Studying the regulation of immune signalling molecules related to immunity during *Avibacterium paragallinarum* infection

By

Poojah Jawallapersand

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Supervisor: **Dr. C.E. Boucher**

Co-supervisors: **Dr. W.J. Janse Van Rensburg**

Dr. W.A. van der Westhuizen

DECLARATION

I, Poojah Jawallapersand, hereby certify that the dissertation submitted by me for the degree Magister Scientiae (M.Sc.) in Biochemistry (Veterinary Biotechnology), is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the University of the Free State. I hereby declare, that this research project has not been previously submitted before to any university or faculty for the attainment of any qualification. I further waive copyright of the dissertation in favour of the University of the Free State.

Poojah Jawallapersand **Date**

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"Great spirits have always encountered violent opposition from mediocre minds."

- Albert Einstein

"*Arise, awake, sleep no more; within each of you there is the power to remove all wants and all miseries. Believe this, and that power will be manifested."*

"All the powers in the universe are already ours. It is we who have put our hands before our eyes and cried out that it is dark."

- Swami Vivekananda

CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

$	Dollar unit of currency
%	Percentage
<	Less than
=	Equal to
>	Greater than
≤	Less than or equal to
®	Registered trademark
°C	Degrees Celsius
µg	Microgram
µg/µl	Microgram per microlitre
µl	Microlitre
µm	Micrometre
µM	Micromolar
ACD	Acid citrate dextrose
ACTH	Adrenocorticotropic hormone
AEC	Animal Ethics Committee
AIDS	Acquired immune deficiency syndrome
AIFM2	Apoptosis-inducing factor-2
AMP	Antimicrobial peptide

APC	Antigen-presenting cell
APP	Acute phase protein
Arg	Arginine
Arp	Actin related protein
ATP	Adenosine triphosphate
BALT	Bronchus associated lymphoid tissue
BCR	B cell receptor
BLAST	Basic Local Alignment Search Tool
bp	Base pair (nucleotide)
BSA	Bovine serum albumin
BTA	Blood tryptose agar
CALT	Conjunctiva-associated lymphoid tissue
CASP6	Caspase-6
CAV	Chicken anaemia virus
CBC	Complete blood count
CC1	Cell conditioning solution
CD	Cluster of differentiation
CFU	Colony forming units
CFU/ml	Colony forming units per millilitre
CH	Cell haemoglobin

CHCM	Cell haemoglobin concentration mean
CHr	Reticulocyte haemoglobin content
cIAP	Cellular inhibitor of apoptosis protein
CO_2	Carbon dioxide
COX	Cytochrome c oxidase
CRF	Corticotropin releasing factor
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CSF	Colony-stimulating factor
CTL	Cytotoxic T lymphocytes
Cys	Cysteine
DAB	Chromagen 3,3′-diaminobenzidine
DAFF	Department of Agriculture, Forestry and Fisheries
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ds	Double-stranded
EASE	Expression Analysis Systematic Explorer

EDTA	Ethylenediaminetetraacetic acid
EDTA-$NA_2.2H_2O$	Ethylenediaminetetraacetic acid, disodium dihydrate
ELISA	Enzyme-linked immunosorbent assays
ER	Endoplasmic reticulum
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
ERK	Extracellular signal-related kinase
FADD	Fas-associated protein with death domain
FB	Fibrinogen
FBC	Full blood count
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FITC	Fluorescein isothiocyanate
FSC	Forward angle scatter
g	Relative centrifugal force
GABRA-1	Gamma-aminobutyric acid type A receptor alpha1 subunit
GALT	Gut- associated lymphoid tissue
GC	Germinal centres
GH	Growth hormone

Gln	Glutamine
GO	Gene Ontology
GPS	Global positioning system
h	Hour
H_2SO_4	Sulphuric acid
HA	Haemagglutinating antigen/hemagglutinin
HCT	Haematocrit
HDW	Haemoglobin distribution width
HG	Harderian glands
HGB	Haemoglobin
HI	Haemagglutination inhibition
HPG	*Haemophilus paragallinarum*
HSP70	Heat shock protein 70 kilodalton
IAV	Influenza A viruses
IBDV	Infectious bursal disease virus
IC	Infectious coryza
ICSH	International Committee of Standardization in Haematology
IEL	Intraepithelial lymphocyte
IFN	Interferon

IFNGR	Interferon-gamma receptor
Ig	Immunoglobulin
IGF-I	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
IL-8L1	Interleukin 8-like 1
IM	Inner membrane
INF	Interferon
IPS1	Induced by phosphate starvation1
IRF3	Interferon regulatory factor-3
JAK	Janus kinase
kb	Kilobase
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LP	Lectin pathway
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M	Molar
Mab	Monoclonal antibody

MALDI-TOF MS	Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
MAP2K3	Mitogen-activated protein kinase kinase 3
MAP3K4	Mitogen-activated protein kinase kinase kinase 4
MAPK	Mitogen-activated protein kinase
MBL	Mannan-binding lectin
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MCVr	Reticulocyte mean corpuscular volume
MD-2	Myeloid differentiation factor-2
MDV	Marek’s disease virus
MHC	Major histocompatibility complex
min	Minute
MIP-1β	Macrophage inflammatory protein-1 beta
MKK3/6	Mitogen-activated protein kinase kinase 3/6
ml	Millilitres
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
mM	Millimolar

MN	Mononuclear
MW	Molecular weight
MyD88	Myeloid differentiation factor-88
N	Normality
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide oxidised form
NADH	Nicotinamide adenine dinucleotide reduced form
NALT	Nasal-associated lymphoid tissue
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear factor kappa B
ng	Nanogram
NGS	Next-generation sequencing
NHLS	National Health Laboratory Service
NK	Natural killer cell
NKT	Natural killer T cell
nm	nanometre
NO	Nitric oxide
NOX2	Nicotinamide adenine dinucleotide reduced form oxidase 2
NS1BP	Non-structural protein-1 binding protein

NUDC	Nuclear distribution gene C
O_2^-	Superoxide anion
OD	Optical density
OM	Outer membrane
p38	p38 mitogen-activated protein kinase
PALS	Peri-arteriolar lymphatic sheath
PAMP	Pathogen-associated molecular pattern
PANTHER	Protein Analysis Through Evolutionary Relationships
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pg/ml	Picogram per millilitre
pH	Power of hydrogen
Phe	Phenylalanine
PI	Post-infection
PI3-K	Phosphatidylinositol 3-kinase
PLG	Plasminogen
PLT	Platelet
PMN	Polymorphonuclear

PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
qPCR	Real-time/quantitative polymerase chain reaction
R	Rand unit of currency
RANTES	Regulated on activation, normal T Cell expressed and secreted
RBC	Red blood cell
rDNA	Ribosomal deoxyribonucleic acid
RDW	Red cell distribution width
REA	Ribotyping and restriction endonuclease analysis
REVIGO	Reduce + Visualize Gene Ontology
RI	Re-infected/re-infection
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNase	Ribonuclease
RNA-Seq	Ribonucleic acid sequencing
ROI	Reactive oxygen intermediate
RPE	R-phycoerythrin
RTX	Repeats-in-toxin
s	Second

SANAS	South African National Accreditation System
SANS	South African National Standards
SDS	Sodium dodecyl sulfate
SE	*Salmonella enteritidis*
Sec61	Channel forming translocon
SHISA5	Shisa Family Member 5
SOP	Standard operating procedures
SPF	Specific pathogen free
ss	Single-stranded
SSC	Side angle scatter
STAT	Signal transducer and activator of transcription
STRING	Search Tool for the Retrieval of Interacting Genes
TAE	Tris(hydroxymethyl)aminomethane -acetate-ethylenediaminetetraacetic acid
TAP	Transporter associated with antigen-processing
Taq	*Thermus aquaticus*
TCR	T cell receptor
TFTC	Too few to count
TGF-β	Transforming growth factor beta 1
Th	T helper

TIR	Translocated intimin receptor
TIRAP	Translocated intimin receptor domain containing adaptor protein
TLR	Toll-like receptors
TLX3	T-Cell leukemia homeobox protein 3
TM/SN	Test medium agar supplemented with chicken serum and NAD^+
TMB	Test medium broth
TMB	3,3',5,5'-Tetramethylbenzidine
TMTC	Too many to count
TNF	Tumour necrosis factor
TNFAIP3	Tumour necrosis factor α-induced protein 3
TNFR1	Tumour necrosis factor receptor 1
TNFSF	Tumour necrosis factor superfamily
TRADD	Tumour necrosis factor receptor type 1-associated death domain
TRAF2	Tumour necrosis factor receptor-associated factor-2
Treg	Regulatory T cells
TRIF	Translocated intimin receptor -domain-containing adapter-inducing interferon-β

Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloric acid
Trp	Tryptophan
TUBB	Tubulin beta class 1
Tyr	Tyrosine
™	Trademark
U	Enzyme activity (micromole per minute)
URT	Upper respiratory tract
US	United States
UV	Ultraviolet
V/cm	Volts per centimetre
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
WASP	Wiskott-Aldrich syndrome protein
WAVE	Wiskott-Aldrich syndrome protein family verproline-homologous protein
WBC Diff	White blood cell differential count
WBC	White blood cell

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ABSTRACT

Infectious coryza is a contagious and acute upper respiratory poultry disease caused by *Avibacterium paragallinarum*, that plagues predominantly layers but also affects broiler breeds. The chicken's immune response to *Av. paragallinarum* serovar C-3 (SA-3) infection (reported to be most virulent in South Africa), and the underlying genetic mechanisms involved, are poorly understood and not well documented. The aim of the study is to understand the complexity of the regulation of immune functions by identifying the molecules that are expressed during *Av. paragallinarum* serovar C-3 (SA-3 strain) infection. In this study, chickens (control versus experimental groups) were directly challenged via infraorbital injection with *Av. paragallinarum* serovar C-3 (SA-3 strain) and the immune response was monitored. The mean disease score and mean daily egg production score were recorded and calculated. Blood and sera were obtained for blood microscopy, leukocyte population profiling by flow cytometry analysis, and antibody/cytokine screening with ELISA assays. Finally, control and experimental chickens were sacrificed based on the clinical scores obtained (0, 1, 2 or 3). Post-mortem examination was conducted, and organs were harvested for immunohistochemistry staining for identification of distinct immune cell populations. The *in vivo* results obtained from the experimental studies in combination with the *in silico* results obtained from bioinformatics tools for the generation of immune signalling pathway maps may provide insight and a birds-eye view into the immune mechanisms between host-pathogen interactions for this disease. Results from our study could potentially assist with diagnostic tests for serovar C-3 and provide insight towards more efficient vaccine development. Hence, if vaccine practices are improved this will limit importations of birds from huge global markets, thus preventing carry-over poultry diseases and zoonosis as well as maintaining a safe and sustainable economy.

Key words: *Avibacterium paragallinarum*; infectious coryza; poultry, immunity; microscopy; flow cytometry, ELISA; post-mortem examination; immunohistochemistry.

CHAPTER 1

LITERATURE REVIEW

Introduction

1.1. Infectious coryza: An overview

In South Africa, the poultry industry comprises of the largest agricultural sector and is a major contributor to the country's economy having a gross income of R37.8 billion per annum (South African Poultry Association, 2012). One of the main aims of the poultry industry is to provide food security and quality. Economic growth and development are correlated with the consumer demand for protein, which implies that as income increases, consumers will enhance their nutritional requirements with protein rich foods such as eggs and chicken meat (Pattison *et al*. 2008). Currently, there is a high consumer demand for poultry meat and egg production, due to dietary preferences, cost of living, processing technology, change in lifestyle and cultural or religious constraints (Taha and Hahn, 2015). Thus, it can be difficult to increase egg and poultry supplies, whereby birds are raised under commercial conditions making them vulnerable to environmental exposure and susceptible to infection by pathogenic microorganisms, eventually leading to disease (Sharma, 1999). One notorious and frequently encountered poultry disease is infectious coryza (Blackall, 1999). The disease has worldwide distribution and economic significance, pertaining to poor growth performance in growing broods and a marked 10-40% decrease in egg production (Yamamoto, 1984; Blackall, 1999; Vargas and Terzolo, 2004; Blackall and Soriano, 2008). An example of an IC outbreak was over a three-year period from 1986 to 1988 in China, whereby IC caused economic losses of about 100 million yuan (approximately US$ 15 million) at the 2018 exchange rate (Chen *et al*. 1993).

Infectious coryza (IC) is characterized as a fast-spreading and acute upper respiratory disease caused by *Avibacterium paragallinarum* (previously called *Haemophillus paragallinarum*) (*Blackall et al.* 2005). During the early phases of infection, IC may be acute to sub-acute, progressing to a chronic respiratory disease state with complications from the presence of other pathogens such as *Mycoplasma synoviae*, *Mycoplasma gallisepticum*, *Salmonella spp*., *Pasteurella spp*., chronic *Escherichia coli* and infectious bronchitis virus (Rimler *et al*. 1978; Reid and Blackall, 1984; Droual *et al*. 1992a; Droual *et al*. 1992b; Sandoval *et al*. 1994; Badouei *et al*. 2014). In 1968, the first IC outbreak was reported on a multi-age farm with approximately 100 000 layers in South Africa (Buys, 1982; Bragg, 2005). Consequently, several vaccinations have been developed since 1975 until today, based on the limited knowledge surrounding virulence factors and antigenicity of affecting serovars (Bragg *et al*. 1996; Mena-Rojas *et al*. 2004). Irrespective, infectious coryza still plagues layer and broiler breeds, especially during the winter season (Bragg *et al*. 1996). Hence, the characteristics and functions of the avian immune response to antigenic and immunogenic proteins from *Av. paragallinarum*, is yet to be studied and elucidated (Mena-Rojas *et al*. 2004; Boucher *et al*. 2015).

1.2. History: Etiological agent

In the 1920s, IC was recognized as a unique and separate clinical entity (Beach, 1920). However, for several years, IC evaded identification and detection, as the disease was masked by other sources of infections, such as fowl pox (Blackall and Soriano, 2008). Early literature clinically describes IC as roup, contagious or infectious catarrh, cold and uncomplicated coryza (Yamamoto, 1972; Blackall and Soriano, 2008; Akter *et al*. 2013). Furthermore, during that era the disease was called *Coryza infectiosa gallinarum* (De Blieck, 1932; Nelson, 1933; Elliot and Lewis, 1934). De Blieck (1932) was the first to isolate and name the etiological agent *Bacillus hemoglobinophilus coryzae gallinarum* (De Blieck, 1932; Hinz

and Kunjara, 1977). Due to the infectious nature of the disease, and that solely the nasal passages were affected, the term “Infectious Coryza” was coined (Beach and Schalm, 1936). However, similar closely resembling microorganisms were also reported (McGaughey, 1932; Nelson, 1933).

The genus *Haemophilus* (derived from Greek nouns *haima*, meaning “blood”, and *philia*, meaning “loving or fondness”), meaning “blood-loving”, was created to classify bacteria growing optimally and specifically in the presence of blood, haemoglobin, serum or protein-containing fluids (Winslow *et al*. 1920). Therefore, the proposed name *Haemophilus gallinarum* as the causative agent of infectious coryza was eagerly accepted (Elliot and Lewis, 1934; Delaplane *et al*. 1934). Knowledge pertaining to the growth essentials of haemophili, and the introduction of the terms X (hemin) and V (nicotinamide adenine dinucleotide (NAD^+) factors was advancing at a fast pace (Thjötta and Avery, 1921; Lwoff, 1937; Lwoff and Lwoff, 1937; Hinz and Kunjara, 1977). McGaughey (1932) reported that the strains isolated only required factor V and not X, whereas Beach and Schalm (1936) and Delaplane *et al*. (1938) instead reported on strains that were both X and V dependant organisms (Beach and Schalm, 1936; Delaplane *et al*. 1938). The work conducted by McGaughey (1932) was largely disregarded and *H. gallinarum* was proposed as an X and V factor dependant strain (Blackall, 1989). Following reports and evidence as of the 1960s onwards, based on avian haemophili requiring solely V factor, Biberstein and White (1969) suggested a new species for those bacteria that were V factor dependent and X factor independent that came to be known as *Haemophilus paragallinarum.* Recently, using methods described by Beach and Schalm (1936), it was shown that X factor independent and V factor dependent strains of *H. paragallinarum* were found to be X and V factor dependent (Blackall and Yamamoto, 1989). Indicating that the authenticity and validity of the work by Beach and Schalm (1936) was under speculation and that there were limitations in their techniques (Blackall, 1989). Moreover, evidence that was once considered as the most credible on X and V factor dependency of *H.*

gallinarum is now an unsettled matter and has been questioned whether it ever existed, as the strains used by Beach and Schalm (1936) were lost (Biberstein and White, 1969). Additionally, McGaughey (1932) recognized that more than one haemophili can exist in chickens, where he found two bacterial groups based on growth characteristics, this work was also largely overlooked, until Page (1962) indicated that the strains he worked with comprised of two avian haemophili groups (Blackall, 1989). These two groups comprised of catalase positive and catalase negative haemophili. The catalase positive group was aerophilic and non-pathogenic to experimental birds, whereas the catalase negative group would not grow in oxygen (capnophilic or microaerophilic) and caused typical infectious coryza symptoms on inoculation of susceptible birds (Blackall, 1989).

H. paragallinarum belongs to a unique group within the bacterial family, *Pasteurellaceae* (Bisgaard, 1993). Blackall *et al*. (2005) investigated the genotypic and phenotypic taxonomy of avian 16S rDNA cluster 18 of the *Pasteurellaceae* family, using DNA sequencing as a tool. The study showed that the avian-associated species *H. paragallinarum*, *Pasteurella gallinarum*, *Pasteurella avium* and *Pasteurella volantium*, forms a monophyletic group, with a sequence similarity of 96.8% (Blackall *et al*. 2005). Based on these results *H. paragallinarum*, *P. gallinarum*, *P. avium* and *P. volantium* were reclassified into a new genus termed *Avibacterium* (Blackall *et al*. 2005). Due to the occurrence of *Avibacterium paragallinarum* (*H. paragallinarum*) and *Avibacterium gallinarum* (*P. gallinarum*) within a single genus, this raised the opportunity for confusion (Blackall *et al*. 2005). Per traditional nomenclature, within the genus *Haemophilus* the prefix ‘para’ was used to highlight a species similar to an existing species, however these two species *Av. paragallinarum* and *Av. gallinarum* differ in growth factor requirements (Blackall and Yamamoto, 1989).

The *Pasteurellaceae* family consists of the genus *Avibacterium*, which further consists of five genuinely named species: *Av. gallinarum*, *Av. paragallinarum*, *Av. avium*, *Av. volantium* and *Av. endocarditidis* and one unnamed taxon, *Avibacterium* species A (Blackall *et al.* 2005; Bisgaard *et al.* 2007). Classification of *Avibacterium* isolates can be challenging when using phenotypic identification due to variable species characteristics (Blackall, 1988a; Blackall and Nørskov-Lauritsen, 2008). A study by Bisgaard *et al*. (2012), using multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST) of *Avibacterium*, successfully identified and confirmed the existence of the species *Av. paragallinarum*. However, the identification of species of other members of *Avibacterium* could not be resolved, even by DNA sequencing (Bisgaard *et al*. 2012). Hence, the validity of the current members of *Avibacterium*, except *Av. paragallinarum*, has been questioned, whereby the discrepancies could have been due to original misclassification of the isolates or the use of a diverse bacterial strain collection during investigations (Bisgaard *et al*. 2012; Alispahic *et al*. 2014). Furthermore, the authors hypothesised that members of *Avibacterium* might be incipient species, due to the close phylogenetic relationship and high similarity observed between some of the *Avibacterium* isolates, with the exception of *Av. paragallinarum* (Bisgaard *et al*. 2012). A study by Alispahic *et al*. (2014) confirmed the findings suggested by Bisgaard *et al*. (2012) and also showed that matrix-assisted laser desorption ionization–time-of flight mass spectrometry (MALDI-TOF MS) can be used as a fast and reliable method for the correct identification and separation of *Av. paragallinarum*. Thus, further taxonomic investigation and re-organization is required for the genus *Avibacterium*, whereby whole genomes will need to be compared among different isolates (Bisgaard *et al*. 2012; Alispahic *et al*. 2014).

1.3. Epidemiology: Host, incidence and transmission

The natural host for *Av. paragallinarum* is the chicken (*Gallus gallus*) (Blackall *et al*. 1997; Blackall and Soriano, 2008). There have been numerous reports of IC in other avian species

such as pheasants, Japanese quails and guinea fowls, however these reports have not been validated by phenotypic or genotypic studies and should be regarded and interpreted with caution (Yamamoto, 1991; Thenmozhi and Malmarugan, 2013). Species like turkey, pigeon, sparrow, duck, crow, rabbit, guinea pig, and mouse are refractory to experimental infection, although, there have been reports on experimentally infected Japanese quail with *Av. paragallinarum* in Australia (Yamamoto, 1972, Yamamoto, 1978; Reece *et al*. 1980; Thenmozhi and Malmarugan, 2013). Upon referral, a flock of peafowl were diagnosed with IC at the University of Ilorin Veterinary Teaching Hospital (Ilorin, Nigeria), this was the first reported case of infectious coryza present in peafowl (Adenkola *et al.* 2016). *Av. paragallinarum* is non-pathogenic to humans, and hence does not have any serious implications on public health (Blackall and Soriano, 2008). A study by Byarugaba *et al*. (2007), showed that turkeys and guinea fowls from Uganda were susceptible to IC and only chickens were infected, however transmission of IC could not be demonstrated from infected chickens or from infected turkeys to turkeys or chickens in close proximity, respectively, even though there was sharing of water and feed in the same containment. Similarly, Yamamoto and Clark (1966) conducted studies on sparrows, and were unsuccessful in showing transmission from infected sparrows to in-contact chickens or transfer of infection from inoculated chickens to the in-contact sparrows under experimental conditions. Thus, it is undoubtedly apparent that *Av. paragallinarum* is the etiological agent of infectious coryza in chickens, and that the role of other avian species in its epidemiology is yet to be revealed.

IC is a cosmopolitan disease and is prevalent wherever chickens are raised and bred (Vargas and Terzolo, 2004). IC predominantly affects layers, however there have been reports in broiler breeds in North and South America, usually found with other bacteria or pathogens (Droual *et al*. 1990a; Droual *et al*. 1990b; Sandoval *et al*. 1994; Conde *et al*. 2011). IC has a global distribution and has been reported in several countries such as Argentina (Linzitto *et al*. 1988), Australia (Arzey, 1987), Bulgaria (Giurov, 1984), Canada (Kerr and Hammarlund,

1982), Egypt (Aly, 2000), United Kingdom (Roberts *et al*. 1964), Guatemala (Matzer, 1974), Holland (De Blieck, 1932), India (Sobti *et al*. 2001), Indonesia (Takagi *et al*. 1991), Iraq (Rashid and Poeiecha, 1984), Switzerland (Baumann, 1982), United States of America (Rooney, 1979; Cutler, 1980; Droual *et al*. 1990a; Droual *et al*. 1990b; Hoerr *et al*. 1994; Matsumoto, 1999), Mexico (Guzman *et al*. 1980; Soriano *et al*. 2001), South Africa (Buys, 1982; Bragg, 2005) and Peru (Mendoza-Espinoza *et al*. 2009). Although, it is a disease mostly associated with the ever growing, expansive and intensive poultry industry, it can also occur in less industrialised situations such as in the kampong (village) chickens in Indonesia and other Asian countries that are just as susceptible to infection as commercial breeds (Zaini and Kanameda, 1991; Zaini *et al*. 1992, Poernomo *et al*. 2000). New Zealand is the only country reported globally that seems to be free of IC (Vargas and Terzolo, 2004).

The main reservoirs of IC are both healthy and chronically infected birds (Blackall and Soriano, 2008). IC has been proposed to be air-borne and can spread through contact with infected chickens, and ingestion of contaminated water and feed (Yamamoto, 1991). However, it has been demonstrated that *Av. paragallinarum* cannot survive too long outside of its host thereby becoming inactivated (Bragg *et al.* 2004). No other vectors- mechanical or biological, have been reported other than healthy and chronic birds (Blackall and Soriano, 2008). Interestingly, IC is non-transmissible through eggs (Blackall and Soriano, 2008).

1.4. Clinical signs and symptoms of disease

Infectious coryza occurs commonly during autumn and winter months in subtropical climatic areas or during the rainy season in tropical regions (Blackall and Soriano, 2008). All age groups are susceptible to IC, which usually occurs within 1-6 weeks after exposure, whereby birds are segregated, moved and quarantined from the brooder house to another cage with

more mature infected birds (Clark and Godfrey, 1961). However, in juvenile birds the disease is less severe (Blackall and Soriano, 2008). A short incubation period, fast and highly contagious spread; high morbidity (20-50%) and low mortality (5-20%) are typical characteristics of IC (Chen *et al*. 1993; Blackall and Soriano, 2008, Pattison *et al*. 2008). The disease develops within 24-48 hours after infection with *Av. paragallinarum* culture or tissue fluid (exudate), followed by a longer progression of the disease in adult birds, especially with active egg-laying hens (Yamamoto, 1984; Blackall and Soriano, 2008, Pattison *et al*. 2008). Susceptible and infected birds will show signs within 1-3 days of infection, whilst flocks will show signs at 7-10 days, depending if the infection is mild or severe, with severe infection taking 3 weeks to display (Pattison *et al*. 2008).

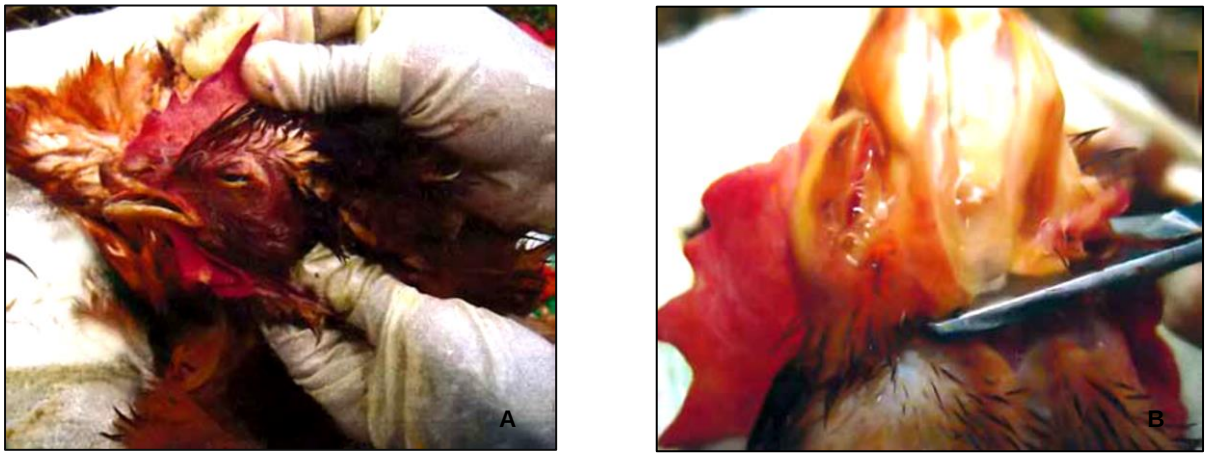

Figure 1.1: Chickens infected with *Av. paragallinarum*. (A). Conjunctivitis with closed eyes and facial swelling. (B). Excessive frothy facial and nasal discharge. (Taken from Akter *et al*. 2013).

Mostly, IC affects the upper respiratory tract, whereas the lower respiratory tract (lungs and air sacs) is affected by chronic complications from other infectious microorganisms (Alder and Page, 1962; Reid and Blackall, 1984; Akter *et al*. 2013). The most typical clinical signs and symptoms include conjunctivitis (Figure 1.1A), nasal and ocular discharge (Figure 1.1B), facial oedema, excessive secretion of tears, swelling of the sinuses, swollen-head syndrome

(especially in males), anorexia, diarrhoea, poor appetite and water consumption, dyspnoea, fetid odour of exudates and poor growth in younger chickens (Blackall, 1999; Pattison *et al*. 2008). Rales may be heard due to infection in the lower air tract, and lesions can be present leading to acute catarrh (Blackall and Soriano, 2008).

1.5. Antigenic structure and virulence factors

There is limited knowledge pertaining to the virulence factors of *Av. paragallinarum*. Gyles and Thoen (1993) showed that the bacterial capsule is linked with virulence, where bacteria with capsules are more virulent than those that are non-encapsulated. *Av. paragallinarum* has a capsule that mediates adhesion to the surface of mucous membranes of its host, leading to colonization and is also considered to be involved in resistance to bactericidal activity of normal chicken serum (Ueda *et al*. 1982; Sawata and Kume, 1983; Sawata *et al*. 1984; Sawata *et al*. 1985a; Sawata *et al*, 1985b, Nakamura *et al*. 1993). Sawata and Kume (1983) demonstrated that a capsular antigen and a haemagglutinating antigen (HA) are responsible for pathogenicity. Kume *et al*. (1984) showed that the hyaluronic-like component has an important function in the capsule since treatment with hyaluronidase leads to degradation of the capsule. A gene encoding for hemagglutinin has been recognized and completely sequenced known as hagA, which functions as an adhesion-binding constituent to respiratory mucin (Hobb *et al*. 2002). Moreover, putative compounds have been identified such as repeats-in-toxin (RTX) proteins (other virulence factors), metalloproteases and haemocin (a toxin secreted by the bacteria that inhibits growth of closely related strains) that may have implications on virulence, moreover it has been reported that these substances may be secreted via membrane vesicles by *Av. paragallinarum* (Terry *et al*. 2003; Rivero-Garcia *et al*. 2005; Ramón Rocha *et al*. 2006; Blackall and Soriano, 2008). In addition, it had been demonstrated that *Av. paragallinarum* can form biofilms due to the presence of HMTp210, a haemagglutinin / adhesion protein (Tokunaga *et al*. 2005; Noro *et al*. 2007; Noro *et al*. 2008;

Wang *et al*. 2014). Biofilms are accretions of bacteria forming a sedimentary layer on biotic or abiotic surfaces which aids the bacteria to develop resistance towards the host immune system (Wang *et al*. 2014). It is still unclear whether the biofilm formation contributes towards virulence in *Av. paragallinarum* (Wang *et al*. 2014). Thus, future studies can be conducted to affirm whether biofilm formation contributes to virulence.

1.6. Vaccines and treatment

Early intervention strategies may be of value in the control and management of IC, which involves the use of biosecurity, medication and vaccination (El-Ghany, 2011). Effective biosecurity measures include minimising exposure of chickens to IC infection. Recovered birds are potential carriers of the disease. Therefore, to eradicate the possibility of IC contamination on farms it is imperative to depopulate infected or recovered birds, especially in cases of an outbreak (Blackall and Soriano, 2008). Moreover, parental stock and starting chicks should not be purchased from unknown sources. On multi-age farms chickens, should be reared and housed in segregation away from older stock (Blackall and Soriano, 2008). Furthermore, to reduce the severity and duration of IC; proper and regular cleaning, fogging and disinfection of equipment, water and premises should be conducted, whereby premises should be left vacant for a few weeks before replenishing with clean or specific-pathogen-free (SPF) chickens (Bragg and Plumestead, 2003; Bragg, 2004).

The treatment of IC involves the use of sulfonamides and antibiotics such as erythromycin, oxytetracycline, streptomycin, sulfodimethoxine, tylosin tartrate, spectinomycin and norfloxacin to reduce the severity of the disease (Blackall *et al*. 1997, Lublin *et al*. 1993). Currently, antibiotic resistance is a major concern due to intensive and widespread utilisation of antibiotics leading to negative consequences that has an impact on both human or animal

health and food safety (Moyane *et al*. 2013). Antibiotic drug resistance to *Av. paragallinarum* is common as such strains have been revealed to carry plasmids, thus conducting tests on antimicrobial sensitivity is strongly advised (Blackall, 1988b). As per, the Food and Drug Administration (FDA) withdrawal periods should be strictly adhered to by individuals in poultry farming and husbandry (Donoghue, 2003). The withdrawal period refers to the amount of time that must pass after the last antibiotic treatment has cleared the animal's system, before the animal is slaughtered (chicken meat) or its product used (eggs) for human consumption (Food and Drug Administration, 1958; Donoghue, 2003). Withdrawal times can vary from a few days to weeks depending on the antibiotic used, whereby the withdrawal times are printed on the antibiotic's label when sold to a farmer or veterinarian (Food and Drug Administration, 1958; Donoghue, 2003). Moreover, farmers are expected to adhere to the specific antibiotic withdrawal times and keep a track record for future reference that verifies compliance. However, compliance to the withdrawal period does not give complete assurance that antibiotic residues are no longer present in the animal tissue (chicken meat) and as such implies that the minimum amount of residue remaining in the organism's system is not considered harmful or a public health concern by governing authorities (Food and Drug Administration, 1958; Donoghue, 2003). In addition, the use of some antibiotics in layers is prohibited in some countries. Some of the drugs could also lead to adverse side and toxic effects in birds; hence dosages need to be administered correctly at appropriate time intervals. However, if treatment is discontinued a relapse can occur, as some birds may still be carriers of the bacteria and harbour the disease (Yamamoto, 1978).

The safest and most effective prevention method against IC is vaccination (Blackall and Reid, 1987; Reid and Blackall, 1987). Infectious coryza vaccination programs do not exempt chickens from getting infected, however the severity of clinical manifestations and progression of the disease is lessened by the reduction of shedding and spreading of the bacteria (El-Ghany, 2011). IC vaccines are manufactured from inactivated and internationally recognised

reference strains of *Av. paragallinarum*; however such vaccines do not provide protection against the local variants of the bacteria (Rimler *et al*. 1977a; Blackall, 1999). Commercialized IC bacterins (a suspension of killed or attenuated bacteria) are commonly available on the market, usually derived from broth-grown cultures (Blackall and Soriano, 2008). The mode of delivery of these bacterins is by injection via subcutaneous or intramuscular routes directly into the leg or breast muscle (Matsumoto and Yamamoto, 1975; Davis *et al*. 1976; Iritani *et al*. 1984; Blackall and Reid, 1987). Whole cell vaccines should consist of 10^8 colony-forming units/ml to be considered effective (Matsumoto and Yamamoto, 1975; Rimler *et al.* 1975; Davis *et al*. 1976; Coetzee *et al.* 1982; Iritani *et al.* 1984; Blackall and Reid, 1987). Some vaccines also contain adjuvants such as aluminium hydroxide gel (Alhydrogel®), mineral oil (Whiterex 307), purified saponin (Quil A®), oil-based emulsion (oil-in-water or water-in-oil) and a combination of aluminium hydroxide and mineral oil (Stone *et al*. 1981; Reid and Blackall, 1987). Adjuvants play a vital role in the enrichment or modulation of immunogenicity of weak antigens by enhancing the speed and duration of immunity, controlling antibody avidity, specificity, isotype or subclass distribution by stimulation of cell-mediated response, as well as improving immune response in immature or senescent individuals (Blackall and Matsumoto, 2003; Lindblad, 2004, Rajput *et al*. 2007). *In ovo* vaccination against IC may become a possible option for farmers, which could allow the birds to develop early protection and immunity. Moreover, *in ovo* vaccination decreases the chances of handling chicks, it reduces stress and anxiety of chicks from manual injection, there is a lesser risk of needle contamination, it is less labour intensive, and the volume dosage delivery is more accurate.

1.7. Avibacterium paragallinarum

1.7.1. Serological classification

Mainly two interconnected classification schemes have been developed to serotype *Av. paragallinarum*, namely the Page and the Kume schemes (Page, 1962; Kume *et al*. 1983). Page (1962) was the first to perform serological classification of *Av. paragallinarum* in the United States, whereby a slide agglutination test was used to identify three serotypes: A, B, and C. Although the plate agglutination test was developed by both Page (1962) and Kato and Tsubahara (1962) there were major disadvantages. Firstly, there was a problem of spontaneous agglutination (Barnard, 2001). Secondly, several field isolates of *Av. paragallinarum* were found to be "untypable" (Blackall *et al*. 1990a).

The Haemagglutination (HA) and Haemagglutination inhibition (HI) tests are currently the only tests available for classification of *Av. paragallinarum*, whereby Kato *et al*. (1965) was the first to demonstrate the haemagglutinating ability of *Av. paragallinarum*. Kume *et al*. (1983) developed a typing system based on the haemagglutinating antigens. The first step is to obtain the haemagglutinating antigens by treating bacterial cells with potassium thiocyanate (KSCN) and sonication of the cells, this is followed by a HA test (Kume *et al*. 1983). The HA test comprises of agglutinating fresh haemagglutinating antigens with glutaraldehyde-fixed chicken erythrocytes (GA-fixed RBC) (Kume *et al*. 1983). Finally, the different isolates were treated using the haemagglutination inhibition (HI) test with fresh rabbit raised antisera against each isolate (Kume *et al*. 1983). Based on the HA/HI tests, the Kume scheme identified three different serogroups, termed I, II and III, and seven different serovars, HA-1 to HA-7 (serovars HA-1 to HA-3 (serogroup I), serovars HA-4 to HA-6 (serogroup II) and serovar HA-7 (serogroup III). Later on, two additional serovars were found; HA-8 belonging to serogroup I by Eaves *et al*. (1989) and HA- 9 allocated to serogroup II by Blackall *et al*. (1990b). Moreover, Blackall *et al*. (1990b) altered the nomenclature on the Kume scheme, since it was demonstrated that the Kume serogroups were linked to the Page serovars A, B and C. Hence, the Kume scheme was modified to nine serovars, which we still use today (Blackall *et al*. 1990b) (Table 1.1).

Table 1.1. Comparison of the classification of *Av. paragallinarum* using the original scheme by Kume *et al*. (1983) and the newly proposed scheme by Blackall *et al*. (1990b). (Taken from Blackall *et al*. 1990b).

Reference isolates	Original scheme (Kume *et al*. 1983)		New scheme (Blackall *et al*. 1990b)	
	Serogroup	Serovar	Serogroup	Serovar
0083/221	I	HA-1	A	A-1
2403	I	HA-2	A	A-2
E-3C	I	HA-3	A	A-3
HP14	I	HA-8	A	A-4
H18	II	HA-4	C	C-1
Modesto	II	HA-5	C	C-2
SA-3	II	HA-6	C	C-3
HP60	II	HA-9	C	C-4
0222	III	HA-7	B	B-1

Currently, several laboratories around the world can perform Page serotyping and this is the most common serotyping scheme in use. The Kume scheme is not used on a routine basis since this scheme is too technically demanding. Serotyping is vital for the production of vaccines, and incorrect typing of a serovar could result in failures in the administration of vaccines for IC (Soriano *et al*. 2004a). However, serological typing is quite time-consuming, cumbersome, expensive and laborious. Moreover, monoclonal antibodies (Mab) are not easily available and difficult to produce. Various haemophilic species exist in chickens as part of the normal microbiota, as a result it is difficult to obtain pure cultures of *Av. paragallinarum* (Mutters *et al*. 1985; Chen *et al*. 1996). Hence, there is a risk of contamination or outgrowth

by other haemophili when isolating and culturing *Av. paragallinarum* (Mutters *et al*. 1985; Chen *et al*. 1996). Therefore, the use of molecular techniques would be most ideal, as it would be possible to type several serovars simultaneously, it would require less time and it would be cost-effective.

1.7.2. Cultivation and growth conditions

Av. paragallinarum is considered to be a fastidious organism, however it is not difficult to isolate (Blackall and Soriano, 2008). Two or three chickens should serve as specimens, for isolation of the bacterium (Blackall and Soriano, 2008). Swabbing can occur in two ways: a small and precise incision is made into the sinus cavity of infected birds using sterile scissors, proceeded by the insertion of a sterile cotton swab into the sinus cavity (invasive technique performed when the bird is dead) or a sterile cotton swab can be used around the nasal areas where nasal discharge is prominent (non-invasive technique performed when bird is still alive) (Blackall and Soriano, 2008). The sinus cavity is rich in *Av. paragallinarum* in its unadulterated form (Blackall and Soriano, 2008). The swab is streaked onto a suitable agar plate or inoculated into media.

Av. paragallinarum grows optimally on blood tryptose agar (BTA) plates or test medium agar supplemented with chicken serum and NAD^+ (TM/SN) or the broth version of TM/SN (test medium broth, TMB) (Yamamoto, 1984; Reid and Blackall, 1987; Eaves *et al.* 1989; Chen *et al*. 1993). BTA plates contain cattle, horse, rabbit, chicken or sheep inactivated serum (Yamamoto, 1984). TM/SN media is a mixture of oleic-albumin complex at 5% (v/v), chicken serum at 1% (v/v) and NADH at 0.0025% (w/v) (Eaves *et al*. 1989). NAD^+-dependent *Av. paragallinarum* strains need NADH (reduced form) or NAD^+ (oxidized form) to grow. Hence, the *Av. paragallinarum* is cross-streaked with a *Staphylococcus* culture and incubated.

Staphylococcus cultures such as *Staphylococcus epidermidis* or *Staphylococcus aureus*, on the agar plate acts as a “feeder” culture, as this bacterial strain has the ability to haemolyse blood resulting in NAD^+ release (Page, 1962; Vargas, 2004).

Media should also contain sodium chloride (NaCl) at 1.0–1.5%, which is necessary for growth of the bacterium (Rimler *et al*. 1977b). The pH of the media should be between 6.9 to 7.6 (Blackall and Soriano, 2008). *Av. paragallinarum* grows ideally in microaerobic or microaerophilic conditions under an atmosphere of 5-10% carbon dioxide (CO_2) (Rimler *et al*. 1976; Vargas, 2004). In the laboratory, a candle is placed and allowed to burn out in a jar with a tightly fitted lid together with growing cultures, to mimic oxygen-deprived conditions (Bragg *et al*. 1997). The minimal and maximal temperatures of growth for *Av. paragallinarum* are 25 and 45°C, with the optimal range being 34-42°C for 16-24 hours after incubation, provided that the correct CO_2 levels, supplements and media is used (Yamamoto, 1984; Vargas, 2004; Blackall and Soriano, 2008).

1.7.3. Morphology and staining

Av. paragallinarum is a Gram-negative and non-motile bacterium. Properly grown bacterial cultures appear as short rods or coccobacilli in shape (Blackall and Soriano, 2008). The dimensions for *Av. paragallinarum* include the length at 1–3 µm and the width at 0.4–0.8 µm, where filament development is possible (Blackall and Soriano, 2008). An outer membrane capsule may be present in virulent strains of *Av. paragallinarum* (Hinz, 1973; Sawata *et al*. 1980). After, 48-60 hours *Av. paragallinarum* degenerates and becomes fragmented, hence it is advisable to work with fresh cultures on a daily basis for laboratory work.

1.7.4. Colony morphology

Depending on optimal conditions and selective media used, colonies belonging to *Av. paragallinarum* appear as tiny dewdrops that are non-haemolytic in nature at 0.3 mm in diameter (Blackall and Soriano, 2008). Some colonies are mucoid (smooth) and iridescent, whereas some colonies are irregular (rough) and do not display iridescence (Hinz, 1973; Rimler, 1979; Sawata and Kume, 1983). Moreover, colonies (NAD^+-dependent strains) show satellitic behaviour in the presence of feeder cultures such as *S. epidermidis* (Blackall *et al*. 1997; Chen *et al*. 1998).

1.7.5. Biochemical properties

Av. paragallinarum though a Gram-negative bacterium, has completely different biochemical properties from its other avian *Avibacterium* counterparts (Table 1.2.) (Vargas, 2004; Kuhnert and Christensen, 2008). Species from the genus *Avibacterium* have the potential to reduce nitrate to nitrite, ferment glucose via specific metabolic pathways without the production of gas and possess oxidase activity, however they are unable to produce indole, hydrolyse urea or gelatine (Blackall *et al*. 1998). *Av. paragallinarum* can produce acid from maltose, D-mannitol and D-sorbitol and lacks catalase activity, unlike other avian haemophili (Blackall and Soriano, 2008). Moreover, *Av. paragallinarum* lacks the ability to ferment galactose or trehalose, as well as produce formazan from 3, 3, 5-triphenyl-tetrazoil chlohydrate (Terzolo *et al*. 1993). There is considerable misinterpretation surrounding carbohydrate fermentation patterns of V-factor dependent species due to variability in results recorded in literature (Blackall and Soriano, 2008). As such, false-negative results obtained are mainly attributed to poor growth (Blackall and Soriano, 2008). For routine identification for the determination of carbohydrate fermentation patterns, a medium consisting of phenol red broth with 1% (w/v) NaCl, 25 μg/ml

NADH, 1% (v/v) chicken serum and 1% (w/v) carbohydrate is used (Blackall, 1983; Terzolo *et al*. 1993).

Table 1.2. Key characteristics and different biochemical tests on the genus *Avibacterium*. (Taken from Kuhnert and Christensen, 2008).

Characteristics	***Av. gallinarum***	***Av. endocarditidis***	***Av. paragallinarum***	***Av. volantium***	***Av. avium***
Haemolysis (ovine blood)	–	–	–	–	–
CO_2 improves growth	+	–	+	–	–
Symbiotic growth (β-NAD requirement)	–	–	V	+	+
Catalase	+	+	–	+	+
Urease	–	–	–	–	–
Indole	–	–	–	–	–
ODC (Ornithine decarboxylase)	–	–	–	V	–
Acid from:					
(+)-$_L$-Arabinose	–	–	–	–	–
(+)-$_D$-Arabinose	V	–	–	+	–
(+)-$_D$-Galactose	+	+	–	+	+
Lactose	V	–	–	V	–
Maltose	+	+	V	+	–
(-)-$_D$-Mannitol	–	+	+	+	–
(+)-$_D$-Mannose	+	+	+	+	+
(-)-$_D$-Sorbitol	–	+	V	V	–
Trehalose	+	+	–	+	+
ONPG (ortho-Nitrophenyl-β-galactoside)	V	+	–	+	–
α- Fucosidase	–	+	–	–	–
α-Galactosidase	–	+	–	–	–
α-Glucosidase	+	+	–	+	+
β-Glucosidase	–	+	–	–	–
β-Xylosidase	–	+	–	–	–
Host	Birds	Chickens	Chickens	Birds	Birds

Data based on: *Avibacterium gallinarum* (Christensen *et al.* 2002; Bisgaard *et al.* 2005); *Avibacterium endocarditidis* (Bisgaard *et al.* 2007); *Avibacterium paragallinarum* (Hinz, 1980; Blackall and Reid, 1982; Blackall *et al.* 2005); *Avibacterium volantium* (Mutters *et al.* 1985); *Avibacterium volantium* (Mutters *et al.* 1985). Characters are scored as: +, ≥ 90% positive; –, ≤ 10% positive; V, 11–89% positive.

1.7.6. Molecular methods of detection

Technology has advanced at a highly fast pace during the past decades that DNA fingerprinting and molecular techniques have become widely available for the identification of *Av. paragallinarum*. Ribotyping and restriction endonuclease analysis (REA) have been beneficial in identifying and linking NAD-independent strains from South Africa, using an rDNA 16S probe (a 16S rDNA operon inserted into a plasmid vector PUC19) (Miflin *et al*. 1995). However, there are only a limited number of molecular typing techniques that have been reported for the differentiation of different serovars of *Av. paragallinarum*, which include Enterobacterial Repetitive Intergenic Consensus (ERIC)-polymerase chain reaction (PCR) (Soriano *et al*. 2004b), multiplex PCR (mPCR) and PCR-Restriction Fragment Length Polymorphism (RFLP) (Sakamoto *et al*. 2012).

ERIC-PCR is a molecular serotyping technique that has been suggested for molecular differentiation of various bacterial species, whereby ERIC sequences are intergenic consensus sequences that are highly conserved, and are located at different loci within a genome for each species or strain (Sharples and Lloyd, 1990; Hulton *et al*. 1991; Chatelut *et al*. 1995; Khan *et al*. 1998; de Souza *et al*. 2015). During amplification using PCR with ERIC sequences as the target sequence, oligonucleotide primers ERIC1R (reverse primer) and ERIC2 (forward primer) are used, which produces different band sizes (Versalovic *et al*. 1991). The different band sizes result in unique banding profiles which can be compared to group isolates, thus subtyping of strains based on the banding patterns obtained is possible (Versalovic *et al*. 1991). Although, Soriano *et al*. (2004b) reported that different *Av. paragallinarum* serovars could be distinguished from one another using ERIC-PCR, the results were difficult to interpret as ERIC-PCR showed several patterns even within each serovar (Sakamoto *et al*. 2012). Additionally, the results obtained from the findings of Soriano *et al*. (2004b) and Khan *et al*. (1998) differ from each other in terms of dissimilar banding patterns

reported. A study by Hellmuth *et al*. (2017) confirmed that ERIC-PCR is unsuitable for the differentiation or for molecular typing of *Av. paragallinarum* serovars, as the banding patterns of field isolates and reference strains cannot be correlated to one another, although isolates of similar origin have unique banding patterns that are shared.

Species-specific PCR or HPG2-PCR is a PCR-based technique that was developed by Chen *et al*. (1996). HPG stands for *H. paragallinarum*. The technique used 4 DNA probes and 2 PCR tests (HPG1-PCR and HPG2-PCR) designed specifically for *Av. paragallinarum*. HPG1-PCR (combination of F1 and R1 primers) yielded a PCR product of 1.6 kb and HPG2-PCR (combination of N1 and R1 primers) yielded a 0.5 kb amplicon (Table 1.3.) (Chen *et al*. 1996). However, the HPG1-PCR gave several false negatives and was unreliable due to the lengthy PCR fragment, whereas HPG2-PCR gave better results even in the presence of normal microbiota (Chen *et al*. 1996). Although, species-specific PCR can detect whether or not *Av. paragallinarum* is present, it cannot be used to distinguish between different serovars A, B and C (Sakamoto *et al*. 2012).

Table 1.3. Three primers (two primer pairs) used for the species-specific PCR by Chen *et al*. (1996).

Primer combinations	Primer Name	Primer sequence	Amplicon size (bp)
F1 and R1	**F1**	5'-CAA TGT CGAT CCT GGT ACA ATG AG-3'	1600
	R1	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'	
N1 and R1	**N1**	5'-TGA GGG TAG TCT TGC ACG CGA AT-3'	500
	R1	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'	

The gene *hmtp210* of *Av. paragallinarum* encodes for a 210-kDa outer-membrane protein that functions as a haemagglutinin (HA), and is a major protective antigen playing a role in pathogenicity (Tokunaga *et al*. 2005; Noro *et al*. 2007, Wang *et al*. 2014). HA is a trimeric autotransporter adhesin functioning not only in hemagglutination but also in cell adherence and biofilm formation (Wang *et al*. 2014). The *hmtp210* gene consists of three regions based on DNA sequence homology (Figure 1.2) (Sakamoto *et al*. 2012). Regions 1 and 3 are highly conserved regions for serovars A and C, whereas the homology of region 2 between serovars A and C was found to be approximately 50% (Figure 1.2) (Wu *et al*. 2011; Sakamoto *et al*. 2012). Region 2 is also known as the hypervariable region of the *hmtp210* gene (Figure 1.2). Therefore, region 2 can be seen as a serovar-specific region, and as such is the best region in the *htmp210* gene to study for protection, against serovar A and C (Figure 1.2) (Sakamoto *et al*. 2012). Sakamoto *et al*. (2012) developed and used multiplex PCR and PCR-RFLP based on region 2 of the *HMTp210* gene. It was found that the DNA sequence homology of this region corresponded to more than 99.8% within each serovar, the strains used in the study were: 221, 083, W, Georgia and Germany (serovar A); Spross and 0222 (serovar B); and 53–47, Modesto and HK-1 (serovar C), respectively (Sakamoto *et al*. 2012). A study by Morales-Erasto *et al*. (2014) showed that mPCR yields poor performance in terms of low sensitivity for recognition of serogroup C isolates and has a relatively high level of inaccuracy pertaining to the results of serogroups A and B. Wang *et al*. (2016) made comparisons between their study and the research conducted by Morales-Erasto *et al*. (2014), whereby both studies found that for certain strains/isolates the mPCR gave correct results across all Page serogroups A, B and C. Furthermore, both studies found that complications arose when the mPCR gave a serogroup B result (Wang *et al*. 2016). Additionally, the previous findings by Morales-Erasto and co-workers indicated that most false serogroup B mPCR results were associated with serogroup C, whereas Wang and co-workers implicated false mPCR serogroup B results to serogroup A (Wang *et al*. 2016). Moreover, it was suggested that different geographic sources of the bacterial isolates/strains of the two studies, was a contributing factor for the difference in results (Wang *et al*. 2016). Both studies suggested that the mPCR and PCR-RFLP

molecular assays are challenging for routine diagnostic use and not suitable for identifying the serogroups of *Av. paragallinarum* isolates (Wang *et al*. 2016).

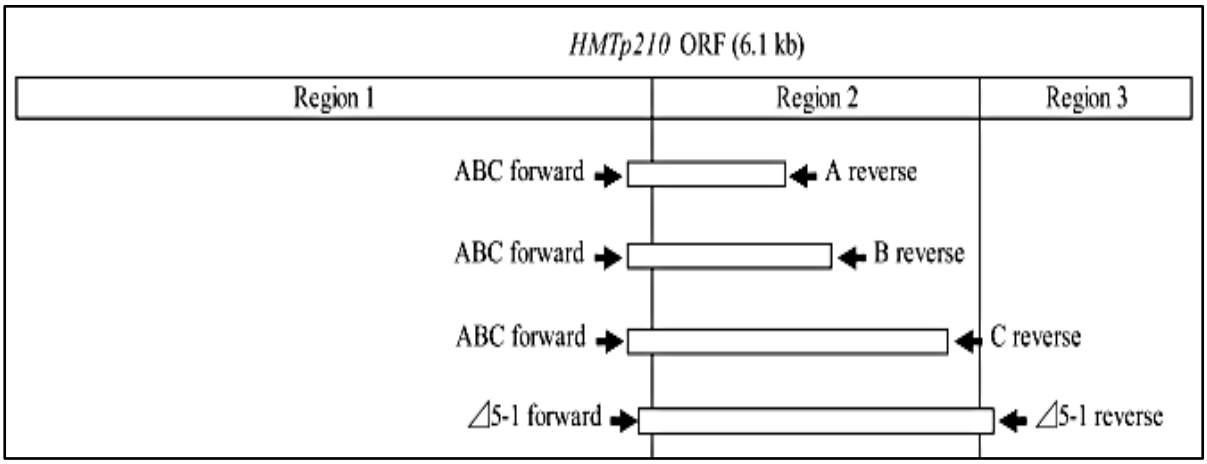

Figure 1.2: Location of primers for amplification of regions within the HMTp210 open reading frame (ORF) region. (Taken from Sakamoto *et al*. 2012).

1.8. Avian immunity

1.8.1. Host-pathogen interactions during Gram-negative bacterial invasion

Chickens like many avian species or other vertebrates are endlessly surrounded by micro-organisms and pathogens (Akira *et al*. 2006; Genovese *et al*. 2013). Prior to the publication of the chicken genome that was first sequenced in 2004, the avian immune system and its unique structural features, such as the absence of lymph nodes and the presence of the bursa of Fabricius for B-lymphocyte development, were still a mystery (International Chicken Genome Sequencing Consortium, 2004; Kaiser, 2010). In order to understand host-pathogen interactions in avian species, we first need to have a comprehensive understanding of the

immunological aspects unique to birds as well as those shared among other species (Kaiser, 2010). However, with the publication of chicken genomic data on genes involved in immune-regulation, comparisons between the chicken, human and murine immune systems have made it possible to understand the evolution, and some of the mechanisms of the avian immune system with its counterparts (Kaiser, 2010). The avian immune system is composed of the innate (non-specific) and adaptive (specific) immune responses (Erf, 1997).

Evolution has highly impacted the development of complex systems used by organisms to sense and respond to an array of stimuli from their surrounding environment (Medzhitov and Janeway, 1997; Keestra *et al*. 2013). The systems primarily consist of sensory receptors that detect the occurrence of specific environmental cues and transduce these signals to intracellular effectors that elicit the applicable cellular response (Keestra *et al.* 2013). A critical feature of the immune system is its capability to recognize pathogens (non-self) while remaining unresponsive to self-antigens (Janeway, 1992). These receptor systems can be found in three distinct forms as endocytic receptors, cell-associated receptors expressed on the cell surface, cytoplasm or intracellularly in immune cells, and are found circulating as humoral proteins in plasma collectively called pattern recognition receptors (PRRs) (Janeway, 1989; Fearon and Locksley, 1996; Janeway and Medzhitov, 2002). These germ line encoded PRRs detect and bind to invading pathogens of microbial but not vertebrate origin based on a series of conserved molecular structural motifs such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycans, flagellin and molecules of viral origin including double and single-stranded RNA and oligonucleotides, known as pathogen associated molecular patterns (PAMPs) (Janeway, 1989; Medzhitov and Janeway, 1997; Ginsburg, 2002; Iqbal *et al*. 2005; Kannaki *et al*. 2010). Thus, the fundamental mechanism pertaining to innate immune recognition is highly conserved from species to species (Akira *et al*. 2006).

Pathogens face an enormous challenge when invading the host's immune system, they need to attach to the host tissue and replicate as fast as they can before the host's system responds and recruits immune cells and molecules against foreign invaders (Gioannini and Weiss, 2007). Our focus will be primarily on PAMPs related to Gram-negative bacteria, as *Av. paragallinarum* belongs to this group of bacteria. Lipopolysaccharide (LPS) is a well-studied PAMP, which is found in Gram-negative bacteria (Miyake, 2004). During bacterial invasion there are primarily two outcomes; damage or no damage to the host (Casadevall and Pirofski, 1999). Gram-negative bacteria are composed of two lipid bilayers and have the following subcellular components; the outer membrane (OM), the peptidoglycan cell wall, the inner membrane (IM) and the periplasm (Silhavy *et al*. 2010). Secreted products in the form of vesicles from the OM, known as OM proteins, that contain virulence factors and other immunomodulatory compounds, contribute to the survival of the pathogen and lead to damage of the host (Kuehn and Kesty, 2005; Galdiero *et al*. 2012)

LPS has long been known for its potent immune stimulatory activity but how this response is induced has remained enigmatic for decades until the discovery of a member of a large family of pathogen sensors or PRRs called Toll-like receptors (TLRs) (Janeway and Medzhitov, 2002; Keestra *et al*. 2013). TLRs function by detecting conserved microbial particles and in turn command eukaryotic cells to respond effectively by producing antimicrobial peptides, cytokines and chemokines (Kawai and Akira, 2007). Therefore, the host is protected from invading pathogens and their corresponding virulence factors are neutralised (Medzhitov and Janeway, 1997). To date at least 10 chicken TLRs have been identified that are orthologous and share gene repertoires with vertebrates (TLR3, 4, 5 and 7), mammals (TLR2A and 2B), fish and amphibians (TLR21) and that are unique to chickens (TLR1LA, 1LB, 15) (Kannaki *et al*. 2010). These TLRs can bind PAMPs, where binding of PAMPS to PRRs triggers the activation of immune cells, which in the case of macrophages leads to the production of inflammatory cytokines but also to activate signals for the adaptive immune system. In order

to identify the broad range of microorganisms additional PRR-families have evolved during evolution and are found in birds as in mammals (Kaiser, 2010). Due to their portend immune system activating activity, PAMPs are now intensively investigated as potential adjuvants by the vaccine industry and have shown promising results under experimental conditions (Powell *et al*. 2015). In a study by Boucher *et al*. 2014, it was indicated that initial pathogen recognition for *Av. paragallinarum* serovar C-3 occurs via TLR 2 and 4 respectively, there was also up-regulation of TLR 7, which the research group indicated and proposed could be as a result of prophages and their remnants, which could contribute to the severe inflammatory immune response observed with IC. In a study by Leveque *et al*. 2003, it was shown that the chicken TLR4 protein shows 46% identity (64% similarity) to human TLR4 and 41% similarity to other TLR family members. Moreover, using linkage studies they showed that TLR4 is associated with resistance to *Salmonella enterica* serovar Typhimurium (Leveque *et al*. 2003). Additionally, they showed using Northern blot techniques that TLR4 is expressed in the same concentrations in the brain, thymus, kidney, intestine, muscle, liver, lung, bursa of Fabricius, heart and spleen (Leveque *et al*. 2003).

1.8.2. Avian innate immune response

The innate immune response in chickens is similar to that in human and the murine system, however there are a few differences in terms of the unique structural features present in the avian immune system. In the field of avian immunology, the chicken is the most researched species however, very little is known about the innate system with regards to other avian species. In order to understand host-pathogen interactions in birds, it is vital to have an in-depth understanding of features that are unique to birds, as well as those that are shared with other species (Kaiser, 2010). Compared to mammals, chickens have different repertoires of Toll-like receptors, cytokines, defensins, antibodies and other immune molecules (Kaiser *et al*. 2005; Boyd *et al.* 2007; Hughes *et al*. 2007; Lynn *et al*. 2007; Kaiser, 2007; Temperley *et*

al. 2008; Cormican *et al*. 2009; Kaiser, 2010). The innate immune system was previously mistaken as a mere scavenger or non-specific system that mainly involved phagocytosis and cell lysis (Playfair and Bancroft, 2013). However, today it is more apparent that there exists a complex interplay between both the innate and the adaptive immune responses involving immune cells, co-stimulatory molecules and cytokines (Schat *et al.* 2014). Mechanisms pertaining to innate immunity is found in a myriad of guises, ranging from the initial non-specific antimicrobial response of bactericidal enzymes, phagocytes and interferon to physical and chemical attack by pathogens to the co-ordinated recruitment of innate immune cells such as macrophages, dendritic cells (DCs), heterophils and natural killer cells (NKs) that are induced via PRRs eventually leading to the release of effector molecules namely cytokines and chemokines that impact inflammatory and acute phase responses, as well as influencing the aftermath effects of the adaptive immune response via the major histocompatibility complex (MHC) through antigen-presenting cells (APCs) (Kogut *et al*. 2005; Kaiser, 2010).

The first-line of defence of the innate system following pathogen invasion consists of a highly effective constitutive physical barrier which includes the epithelial surfaces, mucus, ciliary movement in the airways, fatty acids on skin, peristaltic movement of the intestine, the gastric acidic pH, secretion of mucus and antimicrobial peptides (AMP) (Kaiser, 2010). Following, penetration of these barriers by pathogens this may result in lesions in the skin, the airways and other mucosal surfaces with increased risk of further infection (Schat *et al*. 2011). The normal microbiota, though not of host origin, present on body surfaces also help to prevent colonization by pathogens, and as such prophylaxis with undefined mixtures of normal gastrointestinal microbiota, designated as “competitive exclusion” is fed to day-old chickens to prevent *Salmonella* infections (Van Immerseel *et al*. 2005). Antimicrobial peptides (AMPs) are natural components and have been isolated from most living organisms. AMPs react by forming pores in the membrane of bacteria and fungi leading to cell death (Kagan *et al*. 1990). These peptides have antibacterial, anti-fungal, antiviral or anticancer properties and can

influence inflammation, proliferation, wound healing, release of cytokines, redox homeostasis and chemotaxis (Bals, 2000). Avian AMPs have been shown to be active against a number of microorganisms. In chickens, two classes of AMPs have been identified, cathelicidin-like proteins (Fowlicidin-1 and -2) and defensins (gallinacins (Gal)-1 to -13), showing cytotoxic effects and binding capacity to LPS, resulting in complete blockage of LPS-mediated pro-inflammation (Davison *et al*. 2011). Gal-1 and Gal-1α is reported to be effective in killing *Staphyloccocus aureas, Escherichia coli, Candida albicans, Salmonella enterica,* and *Campylobacter jejuni* but not *Pasteurella multocida* or infectious bronchitis virus (Harwig *et al*. 1994; Evans *et al*. 1995). Gal-11 was found to be primarily active against Salmonella typhimurium and *Listeria monocytogenes* (Higgs *et al*. 2005).

Granulocytes and lymphocytes are immune cells mostly involved in the immune system that are derived from lymphoid stem cells (Playfair and Bancroft, 2013). The most important immune cells in the avian innate immune response are the phagocytes: macrophages and heterophils. NK cells are very poorly phagocytic, however they employ cytotoxic tactics to target and kill pathogens (Playfair and Bancroft, 2013). Macrophages belong to the mononuclear phagocytic system lineage derived from the bone marrow and are the first-line of defence against pathogenic invasion (Skamene and Gros, 1983; Qureshi, 2003). During cell development and differentiation, it takes about 6 days for a monoblast to develop into a pro-monocyte and then into a monocyte under the influence of colony stimulating factors (CSF) (Qureshi, 2003). After entering the bloodstream, it takes a further 3 days for blood monocytes, to seed various tissues and organs, whereby they differentiate into macrophages (Qureshi, 2003). Hence, macrophages are the tissue forms of blood monocytes and are known by various names such as alveolar macrophages (lungs), Kupffer cells (in liver), microglia cells (brain), osteoblasts (bones) and histiocytes (connective tissue) (Qureshi, 2003). Moreover, macrophages participate in a variety of functions, such as phagocytosis (engulfment) of foreign particles, opsonisation, chemotactic targeting of pathogens, annihilation of bacterial

and tumour cells, secretion of prostaglandins and cytokines that regulate activity of lymphocytes and other macrophages, as well as interact with the adaptive aspect of the avian immune response (Bonney and Davies, 1984; Qureshi *et al*. 1986; Kimball, 1990; Qureshi and Miller, 1991;). Phagocytosis is a complex and multi-step process, which starts off with the phagocyte migrating towards the site of infection via chemotactic gradients or chemokines secreted from other cells (Playfair and Bancroft, 2013). Once in the presence of the microbe, the phagocyte attaches to the bacterial cell wall via complement or antibody and engulfs the bacterium into a phagosome (Playfair and Bancroft, 2013). Once the bacterium is in the phagosome, lysosomes are released and come into contact with the phagosome, whereby oxidative and non-oxidative killing mechanisms via reactive oxygen intermediates (ROI) and nitric oxide (NO) pathways, are employed to kill the bacterium (Playfair and Bancroft, 2013). Finally, the act of phagocytosis ends by the internal clearance of the remains by digestion involving enzymes (Playfair and Bancroft, 2013). Additionally, macrophages process antigens and present antigenic fragments via antigen-presenting cells (APCs) to T lymphocytes within the framework of both MHC class I and II cell surface antigens (Unanue and Allen, 1987; Qureshi, 1998).

Polymorphonuclear leukocytes (PMNs) are very important cellular components of innate immunity, and function by killing pathogenic microbes following phagocytosis (Kogut *et al*. 2005). The heterophil is the primary PMN in poultry, the avian equivalent to the mammalian neutrophil (Kogut *et al*. 2005). Like the neutrophil, avian heterophils are involved in the phagocytosis of invading microbes and foreign particles (Genovese *et al*. 2013). Heterophils lack myeloperoxidase, their bactericidal activity has low oxidative burst and their granular constituents seem to differ from those in mammalian neutrophils (Penniall and Spitznagel, 1975; Montali, 1988). Heterophils amass and infiltrate inflamed tissue, whereby tissue damage is caused leading to the formation of heterophil granulomas that are morphologically similar to inflammatory lesions found in reptiles (Montali, 1988; Harmon, 1998). It was found that during

receptor-mediated phagocytosis of opsonized and non-opsonized *Salmonella enteritidis* (SE), avian heterophils differentially expressed transcripts encoding proinflammatory and Th1 cytokines (Kogut *et al*. 2003). Heterophils and monocytes of the innate immune system develop earlier than T and B lymphocytes at hatching and after 2 weeks after hatching the population of PMNs increases in the gut- associated lymphoid tissue (GALT) (Burton and Harrison, 1969; Wells *et al*. 1998; Bar-Shira and Friedman, 2005). It was found that inflammatory stimuli such as LPS, turpentine or various infectious conditions like *E. coli* airsacculitis, staphylococcal tenosyvitis cause a notable influx of heterophils (Harmon, 1998). Moreover, the activation of heterophils by pathogens or by cytokines seem to induce the expression of various pro-inflammatory cytokines such as interleukins (IL) (IL-1, IL-6 and IL-8) (Kogut *et al*. 2005, Kogut *et al*. 2006).

Dendritic cells (DCs) in innate immunity play a unique participating role by activating naïve T cell subsets depending on the availability of high levels of MHCs and co-stimulatory molecules whether of lymphoid or myeloid progenitors (Playfair and Bancroft, 2013). Moreover, DCs determine the type of T cell response, which is dependent on stimulatory cytokines such as interleukin (IL)-10, IL-12 and IL-18 (Davison *et al.* 2011). Immature DCs are kept dormant in tissues and upon pathogen exposure this in turn activates DCs, and mobilise to naïve T-cell rich areas such the lymphoid organs in birds (Davison *et al.* 2011). During this migration phase, the DCs reach maturation differently and have the ability to influence downstream T cell responses, consequently manifested as lowered phagocytic capacity, increase in MHC molecules and accumulation of surface co-stimulatory molecules. However, it should be noted that activation of DCs rely solely on the type of PAMP-PRR interaction (Davison *et al.* 2011).

Natural killer cells (NKs) are morphologically characterised as large lymphocytes with electron dense granula that share many features with cytotoxic T lymphocytes (Göbel *et al*. 1994). In

contrast, to B and T cell development, NKs have thymus-independent development (Bucy *et al*. 1989). Some infections can cause a severe, yet a temporary increase in the NK cell frequency, such a case was reported for *Mycoplasma gallisepticum* infection leading to an accumulation of NK (also $CD8^{+}$) cells in the tracheal mucosa during the first week of infection (Gaunson *et al*. 2006).

The serum complement system is the primary component of the innate response, and as such was observed as a heat-sensitive factor in serum that was complementary to heat-stable antibody during lysis of bacterial and red blood cells (Carroll, 2004).These complement proteins are produced by hepatocytes (liver cells) and macrophages with early synthesis of components such as C1, C2, C4 and C3 (Carroll, 2004). Today, the complement system is known to comprise of approximately 25 serum proteins, 10 or more cell surface complement receptors and regulatory proteins found on numerous host cells (Davison *et al.* 2011). Similar to the mechanism pertaining to PRRs, complement proteins circulate in an inactive form, however upon recognition of molecular motifs from pathogens, they become activated prompting a cascade-like effect, in which the binding of one protein promotes the binding of the next in a sequential cascade (Carroll, 2004). There are three main pathways in the complement system, with the pathways differing in manner of activation, however eventually through proteolytic cleavage reactions they lead to the production of C3 convertase, a key enzyme and factor in the complement system (Carroll, 2004). The classical pathway (CP) is activated by antibody-antigen complexes. The lectin pathway (LP) is activated by microbial carbohydrates in serum and tissue fluids, and the alternative pathway is activated by binding of C3b to microbial surfaces and antibodies (Carroll, 2004). Proteins that cause a change in plasma concentration by 25%, fever, drowsiness, an augmentation in cytokine production and haematological and metabolic alterations, upon inflammatory stimuli are called acute phase proteins (APP) (Schat *et al*. 2014). In addition, APP causing an increase during the response are positive APP while those that cause a decrease in the response are negative APP

(Davison *et al.* 2011). APP play a role in host adaptation or defence, inhibition of serine proteinases, and transport of proteins with antioxidant activity (Davison *et al.* 2011). Other factors such as MBL (mannan-binding lectin), FB (fibrinogen) and CRP (C-reactive protein), including C3 are upregulated via pro-inflammatory cytokines and thus also act as APP (Carroll, 2004; Playfair and Bancroft, 2013). Factor B is the only alternative pathway factor that has been characterised thus far and findings indicate that a factor B dependent alternative pathway is active in chickens (Kjalke *et al*. 1993). The complement system is important in the innate response and adaptive response since it is involved in the induction of inflammatory responses, boosts effects of opsonin activity (phagocytosis) and enhances the direct killing of target cells, as well as B and T cell responses (Playfair and Bancroft, 2013). The end result of complement activation is to trigger inflammation, chemotactically attract phagocytes to the site of infection, promote opsonization, cause lysis of Gram-negative bacteria and cells, expressing of foreign epitopes, participate in B-cell activation and remove harmful immune complexes from the body (Schat *et al.* 2014).

There is a plethora of chemical acute responses during the innate immune response in chickens that serve to protect the host. Serine proteinases regulate extracellular matrix turnover, fibrinolysis and complement activation (Davison *et al*. 2011). However, to down-regulate the activity and effects of these enzymes, serpin inhibitors such as α1-proteinase inhibitor which inhibits neutrophil elastase, α1-antichymotrypsin which inhibits chrymotrypsin-like serine proteinases, and C1 inhibitor which inactivates the blood coagulation factors XIIa and XIIf, protects the integrity of host tissues (Davison *et al*. 2011). APP protects host tissues from toxic oxygen metabolites released from phagocytic activity during inflammation (Davison *et al*. 2011). CRP has both pro- and anti-inflammatory effects *in vitro* and *in vivo* involved in the clearance of damaged tissue, prevention of autoimmunity and regulation of the inflammatory response (Davison *et al*. 2011). In chickens, natural infection with the protozoan parasites such as *Eimeria spp.* and *Histomonas* induce high levels of CRP (Chamanza *et al*.

1999). α1-Acid glycoprotein is a natural anti-inflammatory agent that inhibits neutrophil activation, increases the secretion of an IL-1 receptor antagonist by macrophages and might be involved in the clearance of lipopolysaccharide (LPS) by neutralizing its toxicity (Murata *et al*. 2004). In chickens, elevated levels of α1-acid glycoprotein have been recorded during infections with infectious bronchitis, infectious laryngotracheitis, infectious bursal disease viruses, *E. coli*, and *Salmonella enterica* serovar Enteritidis (Chamanza *et al*. 1999). Fibrinogen (FB) has a primary role in homeostasis, during fibrin formation and in tissue repair, by providing a substrate or matrix for the migration of inflammatory-related cells (Murata *et al*. 2004). Mannan-binding lectin (MBL) is synthesised in the liver and released into the blood stream, however, low expression of MBL has been demonstrated in organs such as the lung, thymus, kidney, small intestine and testis (Wagner *et al*. 2003).

At the onset of pathogen invasion, the innate response is considered the most critical, preventing spread of the pathogen until the adaptive response becomes active with recruitment and mobilization of B and T cells (Juul-Madsen *et al.* 2003). In birds, the activation of complement by the alternate pathway, the action of C reactive protein, and of properdin which contributes to innate resistance in mammals, have yet to be described (Davison *et al.* 2011). The interaction of the lymphoid tissues with the infectious organism is known as specific immunity and is mediated by immune cells and the production of antibodies (Juul-Madsen *et al.* 2003). Thus, innate defence is critical to provide protection during the first weeks of life when the adaptive immune system is still under development.

1.8.3. Avian adaptive immune response

The adaptive immune system is highly specific, it involves the interaction of the lymphoid organs, the lymphocytes, antigen recognition and immunoglobulin production (Playfair and

Bancroft, 2013). Specific immunity encompasses two main immune responses. This entails humoral immunity carried out by B lymphocytes producing antibodies, and the cell-mediated response which refers to T lymphocyte activity in conjunction with the MHCs (Erf, 1997). The B and T lymphocytes develop in the bone marrow of the chicken via haematopoiesis, thereafter they migrate and undergo maturation in unique lymphoid organs, whereby B lymphocytes mature in the bursa of Fabricius whereas T lymphocytes mature in the thymus (Davison *et al*. 1996). The immune system of a newly hatched chick is underdeveloped and therefore is unable to provide complete protection against pathogenic infection upon first encounter with the external environment, and as such, very few lymphocytes are found in the secondary lymphoid organs such as the spleen, caecal tonsil, Harderian gland or the bronchus associated lymphoid tissue (BALT) (Davison *et al*. 2011). Atrophy or prior surgical removal of these organs leads to severe immunosuppression and the birds become more susceptible to disease, as seen in IBDV (infectious bursal disease virus), MDV (Marek's disease virus) or CAV (chicken anaemia virus) infection or in response to (experimental) glucocorticoid treatment (Dohms and Metz, 1991; Davison *et al*. 2011).

T cells mature in the thymus and start to seed the periphery around hatching (Schat *et al*. 2014). Antigen recognition by T cells is a highly complex and remarkable process mediated by the T cell receptors (TCRs) (Chen *et al*. 1989). Unlike other surface receptors that are pre-committed to a specific ligand, T cell antigen recognition via the TCRs are randomly generated, whereby there is recognition of a set of diverse peptides complexed to MHC molecules (Chen *et al*. 1989). The TCR is a disulphide-linked membrane bound heterodimeric protein normally consisting of α and β chains expressed as part of a complex with the invariant CD3 chain molecules (Chen *et al*. 1989). T cells expressing these two chains are referred to as αβ T cells, although a small population of T cells do express an alternate receptor, formed by variable γ and σ chains, referred as γσ T cells (Chen *et al*. 1989; Siu *et al*. 1990). Additionally, to the TCR, the T cell has other accessory molecules or co-receptors in antigen

recognition that were defined using monoclonal antibodies and are helpful as T lymphocyte specific markers (Siu *et al*. 1990). CD4 a molecule, originally referred to as L3T4, is expressed on all T cells that are restricted to MHC class II molecules and is a T helper cell that serves to escalate B and T cell responses through the release of cytokines such as interferon-gamma (INF-γ), IL-4, IL-5, IL-13, IL-17 (Littman, 1987; Davison *et al*. 2011; Playfair and Bancroft, 2013). Cells expressing the CD8 molecule function as cytotoxic T cell, originally known as Lyt 2,3 in mice, which control viral infections and tumour formation via cytokines such as INF-γ (Littman, 1987; Spellberg and Edwards, 2001). CD8 is expressed on all MHC class I molecules (Littman, 1987). T cells must recognize antigen bound to MHC gene products and antigen-presenting cell (APC), before they can partake in any immune-related response (Erf, 2004). Like lymphocytes APCs, develop and mature in the bone marrow and migrate to peripheral lymph organs (Davison *et al*. 2011). Initiation of TCR signalling requires these co-receptors CD4 and CD8 that act as cellular adhesion molecules that bind to their respective MHC molecules and stabilize the interaction of T cells and APCs (Siu *et al*. 1990). T helper cells can be discriminated from each other on basis of function, in particular by cytokine secretion profiles (Spellberg and Edwards, 2001). T helper 1 (Th1) cells produce cytokine interferon-γ (INF-γ), which potently activates macrophages to combat a range of intercellular pathogens such as *Salmonella*, *Mycobacteria* and *Listeria* (Spellberg and Edwards, 2001). Th2 cells secrete cytokines IL-4 and IL-13, helping B-cells to develop into antibody producing cells (Spellberg and Edwards, 2001). These T cells are the classical T helper cells. More recently, additional subsets of CD4 T cells were identified (Chen *et al*. 1994; Fukaura *et al*. 1996; Powrie *et al*. 1996; Hafler *et al*. 1997). Th17 cells release IL-17, which can attract granulocytes into tissues infected by bacteria (Chen *et al*. 1994; Fukaura *et al*. 1996; Powrie *et al*. 1996; Hafler *et al*. 1997). Thus, Th1 and Th17 cells closely interact with innate immune system cells through cytokine secretion to control intra- and extracellular bacteria, respectively (Chen *et al*. 1994; Fukaura *et al*. 1996; Powrie *et al*. 1996; Hafler *et al*. 1997). While these cells of the CD4 lineage induce an immune response and inflammation, CD4 positive regulatory T cells (Tregs)

provide signals (IL-10, transforming growth factor beta 1 (TGF-β)), which control other T cell subsets to prevent immunopathology, auto reactivity, and down-regulation of an immune response after pathogen clearance (Davison *et al*. 2011). However, convincing evidence for the existence of Tregs is still lacking in avian species, due to the difficulties associated with the cultivation and isolation of T lymphocytes (Davison *et al*. 2011).

T cells are tasked to monitor intracellular compartments of the host, whereas B cells are responsible for patrolling extracellular spaces such as blood and tissue fluid (Playfair and Bancroft, 2013). In contrast, B cell maturation shows striking differences to T cells and takes place in a unique organ, the bursa of Fabricius (Schat *et al*. 2014). The molecular mechanisms taking place during avian B cell development are well understood (Davison *et al*. 2011). However, their regulation is still largely unclear. As a net result of B cell maturation millions of B lymphocytes develop, each of them producing a unique antibody that differs from those antibodies generated by other B lymphocytes (Davison *et al*. 2011; Schat *et al*. 2014). Through this mechanism, the entire B cell pool of chicken can generate millions of different antibodies which theoretically should bind any antigen encountered by the bird (Davison *et al*. 2011). However, B cells with a specific antibody are rare and without further activation and enhancement of the immune system, insufficient amounts of antibodies will be generated (Davison *et al*. 2011). During a bacterial invasion, the immunoglobulin (Ig) surface molecule binds antigen, whereby the antigen-Ig complex is endocytosed and digested by enzymes (Playfair and Bancroft, 2013). Following digestion, the constituting peptides are collected and bind to MHC class II molecules (Playfair and Bancroft, 2013). The MHC-peptide complex either binds to the surface of a B cell or T helper cells recognise the MHC-peptide complex presented to them by DCs (Playfair and Bancroft, 2013). Once T helper cells recognise the MHC-peptide complex they activate into T effector cells and finally proliferate into clones (Playfair and Bancroft, 2013). Consequently, the effector T cell secretes cytokines such as IL-2, IL-4, IL-5 and IL-6 to attract a B cell carrying the same MHC-peptide complex, which

activates the B cell (Playfair and Bancroft, 2013). The B cells proliferate into clones. Finally, some B cells differentiate into plasma cells and secrete antibody, while others remain as memory B cells (Playfair and Bancroft, 2013).

A hallmark of antigen-specific B cell responses is the formation of germinal centres (GC) (Davison *et al*. 2011). In birds GCs are found in all secondary lymphoid tissues (Davison *et al*. 2011). Antigen transported to the spleen is presented to T and B cells thereby inducing a complex immune response resulting in the generation of high affinity antibodies (Davison *et al*. 2011). Specialized DCs, T helper cells and B cells physically interact with each other and exchange signals through cell surface receptors and secreted cytokines (Davison *et al*. 2011). During this response GCs form and provide an environment for the selection and promotion of B cells which produce the best antibodies to the presented antigen (Davison *et al*. 2011). Several cytokines involved in B cell maturation and function (antibody production) have recently been identified with the help of genomic data. Some of them have been cloned and shown to have potent B cell activating activities in cell culture systems. These cytokines have enabled extended B cell cultures and provided *in vitro* systems to investigate the interaction of host cells and pathogens with B cell tropism such as MDV or IBDV (Schermuly *et al*. 2015).

Immunoglobulins (Ig) are synthesized by B cells and are globular glycoproteins that have antibody (Ab) activity and are found in the blood, lymph and vascularized tissues of vertebrates (Marchalonis, 1977; Litman *et al.* 1993). Antibodies have the basic unit structure that consists of four polypeptide chains: two heavy (H) and two light (L) that form the monomeric unit (H2L2) (Davison *et al*. 2011). Immunoglobulins are found in two forms, either as membrane-bound antigen receptors or soluble secreted molecules (Davison *et al*. 2011). Some antibodies such as IgG consists of a basic unit, however others like IgM and IgA are more complex and are made up from multiples of the basic unit (Schat *et al*. 2014). Only three main classes of antibodies that have been described for birds: IgM, IgA and IgY (Carlander *et al*. 1999). Both

IgG and IgY antibodies are more systemic antibodies, with IgA being a secretory antibody (Hopkins *et al*. 1987; Carlander, 2002).

The structure and function of chicken IgM is homologous to its mammalian counterpart (Davison *et al*. 2011). IgM is the most prevalent antibody and is the first isotype to be expressed during embryonic development in developing chicks and after initial exposure to a novel antigen (Davison *et al*. 2011). The molecular weight (MW) of chicken IgM is in the range 823–954 kDa (Davison *et al*. 2011). Free M chains have been found in sera of bursectomized chickens and in survivors of infectious bursal disease virus (Choi and Good, 1971; Gauldie *et al.* 1973; Ivanyi, 1975; Higgins, 1976). Like in mammals, the response with IgM is usually transient, although for some chronic bacterial diseases, such as *Bordetella avium* in turkeys, the effect was reported to be active for several weeks (Suresh *et al.* 1994). However, due to the evolutionary conservation of IgM and its transient effect in the immune response, the area of avian IgM research has been rather limited.

Chicken IgY has been found in duodenal contents, tracheal washings and seminal plasma (Carlander, 2002). Chicken IgY in sera is monomeric with MW ranging between 165–206 kDa (Davison *et al*. 2011). IgY is a unique avian maternal antibody transported from the hen to the offspring, involving a two-step process (Carlander, 2002). Firstly, IgY is transferred from the hen's serum into the egg yolk, which is then followed by transmission of IgY from the yolk sac to the developing chick (Carlander, 2002). Phylogenetic studies have shown that the avian IgY is a homologue of mammalian IgG, sharing similarities with both mammalian IgG and IgE (Warr *et al*. 1995). IgY is the predominant and main isotype produced in sera in the secondary antibody response, after IgM is secreted in the primary antibody response (Davison *et al*. 2011). Both IgY and IgG are used interchangeably in literature. Avian immunologists prefer to use the term IgY because this Ig appears to be the evolutionary predecessor of both IgG and

IgE, sharing homology with each of these mammalian isotypes (Warr *et al.* 1995). The major difference between the chicken IgY and the mammalian homologue is the longer H chain in the chicken molecule (Davison *et al*. 2011). Avian IgY consists of five domains (V, C1–C4) and does not have a genetically encoded hinge, in contrast to the four domains that are found in mammalian IgG (Davison *et al*. 2011). Thus, avian IgY has limited flexibility, which may account for some of the unique biochemical properties, such as the inability to precipitate antigens at physiological salt concentrations, seen in chickens and ducks (Davison *et al*. 2011). Although IgY is the major avian systemic antibody active in infections, complete characterization has only been carried out in the chicken and in the duck (Davison *et al*. 2011; Schat *et al*. 2014).

IgA is found in birds, predominantly in bodily secretions such as in the respiratory system, urogenital system and intestine, and play a major role in mucosal immunity (Playfair and Bancroft, 2013). IgA is a dimer that possesses a J chain that binds to a receptor on the surface of epithelial cells (Underdown and Schiff, 1986; Kerr, 1990). This receptor integrates with IgA as a secretory component (SC), whereby the IgA complex gets transported through epithelial cells and is secreted into the lumen of the designated organ (Solari and Kraehenbuhl, 1985). SC provides adhesion of IgA to the epithelial surface and protection from proteolytic degradation within the cells (Solari and Kraehenbuhl, 1985). The phylogenetic origins of IgA are still unknown. A secretory molecule IgX from the African clawed frog (*Xenopus laevis*) has been reported, that has antigenically similar properties to IgM and is secreted into the intestinal tract (Hsu *et al*. 1985). However, IgX is considered an analogue of IgA because of sequence differences and that it does not possess a J chain nor SC molecule (Mussman *et al.* 1996). Birds such as pigeons, penguins and flamingos produce a specific secretion in the crop sac called crop milk, which is regurgitated to feed to the young (Davison *et al*. 2011). Crop milk is rich in nutrients such as fats and proteins, however unlike mammalian milk it does not contain lactose (Davison *et al*. 2011). Moreover, the production of crop milk is under the control of

prolactin (Anderson *et al.* 1984). Crop milk is also rich in IgA with concentrations in the range of 1.5 mg/ml, though it contains little IgY (Davison *et al*. 2011). An increased uptake of IgA from crop milk leads to IgA accumulation in the intestinal tract which cannot pass through the epithelial gut lining into circulation, thus providing local immunity against pathogenic microorganisms within the gut (Davison *et al*. 2011).

1.8.4 Cytokines and chemokines of the avian immune system

Cytokines are peptides having a molecular weight less than 30 kDa that controls the regulation of extracellular signals between cells during the onset and course of immunological responses (Davison *et al*. 2011). Cytokines have various functions and effects on cells as they elicit, modulate, and regulate immune as well as inflammatory responses (Davison *et al*. 2011). Cytokines are generally secreted; however, they also act as cell surface molecules influencing the cells of the immune system and consist of interleukins (IL), interferons (IFN), transforming growth factor-β (TGF-β) family, tumour necrosis factor (TNF) superfamily (TNFSF), colony-stimulating factors (CSF) and chemokines (Davison *et al*. 2011). The IL series have functional roles involving lymphocytes and the IFN series have antiviral effects (Davison *et al*. 2011). TGF-β, TNFSF, CSF and the TGF-β family has a crucial role in regulating inflammatory reactions, whereas TNFSF does not have any anti-tumour activity (Davison *et al*. 2011). Various cytokines and chemokines have been identified in mammals also present in chickens, however there are some exceptions with regards to multigene families, whereby the chicken seems to have fewer members than in mammals, which also explain the unique and fundamental differences in the organs and cells of the avian immune system (Davison *et al*. 2011).

Both IL-1β and IL-18 having functionality in inflammatory responses and have been identified in the chicken genome (Weining *et al*. 1998; Schneider *et al*. 2000). However, no other IL-1 family has been identified in the chicken genome and it is also likely that there are fewer IL-1 family members in chickens compared to mammals (Kaiser *et al*. 2005). Genes encoding IL-2, IL-15 and IL-21, which play a role in T cell proliferation, all lie on chromosome 4 of the chicken, however none of the genes are in synteny (Sundick and Gill-Dixon, 1997; Lillehoj *et al*. 2001; Kaiser *et al*. 2005). IL-12α and IL-12β of IL-12 family, have a biological role in driving inflammatory Th1 responses (Balu and Kaiser, 2003). The chicken has only four members of IL-10 family: IL-10 and IL-19 on chromosome 26, and IL-22 and IL-26 on chromosome 1 (Rothwell *et al*. 2004; Davison *et al*. 2011). IL-10 seems to be conserved in the chicken and acts as an anti-inflammatory cytokine, downregulating the effects of IFN-γ (Rothwell *et al.* 2004). In mammals, IL-19 is a Th2 cytokine, whereas IL-22 and IL-26 are involved in inflammatory responses (Rothwell *et al*. 2004). In the IL-17 family only four genes have been identified in the chicken genome: IL-17A, IL-17B, IL-17D and IL-17F, that seem to play a role in Th17 cell responses, are known to be pro-inflammatory cytokines in both chickens and mammals and are produced early after infection as part of the induced innate immune response (Min and Lillehoj, 2002; Kaiser *et al*. 2005; Chen *et al*. 2006; Harrington *et al*. 2006).

Type I IFN is divided into three subcomponents in chickens: IFN-α, IFN-β and IFN-λ (Sekellick *et al*. 1994; Sick *et al*. 1996). IFN-α and IFN-β both have antiviral activity (Sekellick *et al*. 1994; Sick *et al*. 1996). The chicken IFN-γ gene a Type II IFN was identified and *in vitro* was found to protect chicken fibroblasts from undergoing virus-mediated lysis, was capable of inducing nitrite secretion from macrophages, and showed enhanced MHC class II expression on macrophages (Digby and Lowenthal, 1995; Lowenthal *et al.* 1997). Chicken IFN-γ gene has a crucial role in mediating Th1-controlled responses (Digby and Lowenthal, 1995; Lowenthal *et al.* 1997). The TGF-β family in chickens, consisting of TGF-β1, TGF-β2, TGF-β3 and TGF-β4 are vital players in immune-regulation (Burt and Jakowlew, 1992; Jakowlew *et al*. 1988;

Jakowlew *et al*. 1990; Jakowlew *et al*. 1997; Pan and Halper, 2003). Members of the TNFSF and TNF receptor (TNFR) superfamily (TNFRSF) have central roles in both innate and adaptive immunity, including inflammatory responses, apoptosis, cell proliferation, and stimulation of the immune system (Davison *et al*. 2011). Some TNFSF members should be considered as co-stimulatory molecules, rather than cytokines, however members which can be considered as cytokines are TNF-α, lymphotoxin (LT)-α, LT-β and B cell activating factor of the TNF family (BAFF) (Davison *et al*. 2011). Chicken BAFF has been cloned and was found to mediate B cell survival (Koskela *et al*. 2004; Schneider *et al*. 2004).

Chemokines have a role in the migration of leukocytes (homeostasis) and have a role in the recruitment of cells to sites of inflammation (inflammatory responses) (Davison *et al*. 2011). Chemokines can be divided into four groups – XC, CC, CXC and CX3C –, and are categorised on the basis of the spacing of the first two conserved cysteine residues at the amino termini of these chemotactic proteins, with the exception being XC, which lacks a first cysteine residue (Davison *et al*. 2011). For chemokines, the suffix “L” represents ligands, whereas their receptors are given the suffix “R” (Davison *et al*. 2011). Lymphotactin, the chicken orthologue of mammalian XCL, has been cloned and acts as a chemoattractant for splenic B cells (Rossi *et al*. 1999). The chicken lacks orthologues of CCL11, CCL24 and CCL26 (eotaxin 1–3), which are chemoattractants in mammals for eosinophils and basophils through the receptor, CCR3, which is also absent in the chicken (Davison *et al*. 2011). This clearly indicates that a lack of functional eosinophils in the chicken fits with the lack of eosinophil attracting chemokines (Davison *et al*. 2011).

1.9. Rationale and objectives of the study

There are a plethora of published works on various notable avian diseases related to disease management, vaccine development and gene expression profiling such as Newcastle disease, Marek's disease, chicken anaemia virus, infectious bursal disease (Dohms and Metz, 1991; Davison *et al*. 2011). However, very few challenge studies specifically with regards to the monitoring of the avian immune response have been conducted encompassing disease progression at a genetic, cellular, tissue, and clinical level as a whole. Notable studies conducted on the avian model with regards to immune mechanisms were conducted on *Salmonella* serovars and avian influenza virus (Withanage *et al*. 2004, Withanage *et al*. 2005; Xing *et al*. 2008; Nerren *et al*. 2010).

The aim of this study is to understand the complexity of the regulation of immune functions by identifying immune cells and molecules that are expressed during the chickens' response to *Av. paragallinarum* serovar C-3 (SA-3 strain) infection. This knowledge is important since the exact immune defence mechanisms are poorly understood for this infection. It should be noted that by simply looking at cytokines, immune cells and antibodies, does not give a full understanding of the complexity of the immune mechanisms of *Av. paragallinarum* serovar C-3 (SA-3 strain) infection, however it can provide some insight on how these cells and molecules fit or link as a puzzle into a greater picture involving immunological modulating pathways and immune cell interactions and how the organism is affected by the pathogen causing infection, specifically to serovar *Av. paragallinarum* C-3 (SA-3 strain). As such, this research is a stepping-stone to understanding a disease having dire consequences in the poultry industry and only has a small part to play in a bigger project that has a never-ending scope, which implies that there will always be a drive for future research work in this field, since there is much to discover. Moreover, this project will also further improve diagnostic tests for *Av. paragallinarum* serovar C-3 (SA-3 strain) (reported most virulent serovar in South Africa) in the veterinary field and also provide more perception with regards to vaccine development, since failed vaccination attempts is still a major problem with regards to IC.

Thesis layout with study objectives:

Chapter 2: To perform *in sillico* data analysis, to identify potential immune signalling pathways using existing bioinformatics pathway databases and tools; for understanding future comprehensive experimental data and the systematic workings of the chicken immune system.

Chapter 3: To conduct a pilot study of the immune response of control *versus* experimental chickens to *Av. paragallinarum* serovar C-3 (SA-3 strain); perform bacteriological cultivation, isolation and identification of the infecting serovar C-3 (SA-3 strain); monitor the chickens' immune response for 21 days; conduct full and differential blood counts; to prepare blood smears; conduct haematological analysis of avian blood performing $CD4^+$ (T helper cell population) and $CD8^+$ (cytotoxic T cell population) cell population profiling using flow cytometry; conduct antibody profiling using direct ELISA; and establish research techniques to be used in further chapters.

Chapter 4: To use infectious coryza as an infection model for poultry, monitor disease progression by clinical signs and symptoms, immune cells and molecules; perform bacteriological cultivation, isolation and identification of the infecting serovar *Av. paragallinarum* C-3 (SA-3 strain); conduct cytokine profiling using commercially available ELISA kits; perform microscopy on avian blood smears and conduct morphological classification of immune cells; and conduct haematological analysis of avian blood performing $CD45^+$(pan-leukocyte), $CD4^+$ (T helper cell population) and $CD8^+$ (cytotoxic T cell population) cell population profiling using flow cytometry.

Chapter 5: To conduct necropsy of lymphoid and non-lymphoid organs, as well as perform immunohistochemical staining of tissues obtained.

Chapter 6: Concluding remarks and future studies.

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CHAPTER 2

IMMUNOMICS: *In silico* MAPPING OF IMMUNE SIGNALLING PATHWAYS IN CHICKENS RELATED TO *Avibacterium paragallinarum* SEROVAR C-3 INFECTION

2.1. Introduction

A major problem faced by the poultry industry is that chickens are prone to opportunistic pathogens when reared under intensive conditions (Sharma, 1999). This factor is critical especially for chickens during the first week of life, as their immune system has not yet fully matured and the levels of maternal antibodies on which they are dependent are diminishing (Lowenthal *et al*. 1994). The aftermath, is that there is loss of productivity due to disease and substantial resources are required to treat and maintain the health status of these birds (Lowenthal *et al*. 2000).

Infectious coryza is an important avian disease that has the ability to cause major economic losses in both layer and broiler breeds (Blackall *et al*. 1999). *Av. paragallinarum* forms part of the *Pasteurellaceae* family, however to date factors leading to pathogenicity, immunogenicity and serotyping are not well understood, even among the different serogroups (A, B and C). Over a thirty year period, it was observed that the incidence of serovar C-3 had been notably increasing (Bragg *et al*. 1996). IC, like many other avian infectious diseases, is the

consequence of a complex set of interactions between the pathogen and host responses (discussed in Chapter 1). For more than 100 years, the chicken has been used as a model organism in developmental biology (Stern, 2005). The publication and availability of chicken genomic sequence information has resulted in detailed analysis and characterization of various and numerous genes related to immune signalling and regulation, which can provide insight into the mechanisms that drive the immune system leading towards either elimination of the invading pathogen or reduction in damage to the host caused by bacterial burden (International Chicken Genome Sequencing Consortium, 2004; Medzhitov, 2009; Boucher *et al*. 2014).

There have been studies conducted on immune-related gene expression and the genetic mechanisms that control immunity for the avian model on *Salmonella* serovars, *Eimeria* parasite, Marek's disease virus and avian influenza virus (Xing and Schat, 2000; Withanage *et al*. 2004, Withanage *et al*. 2005; Hong *et al*. 2006; Xing *et a*l. 2008; Adams *et al*. 2009; Nerren *et al*. 2010). Initially, the genetic mechanisms that elicit the immune responses of chickens infected with *Av. paragallinarum* were unknown, until a study by Boucher *et al*. (2015), using high through-put microarray technology screened for genes and regulated biological pathways that correlated to the immunity of birds during disease progression of IC. A study by Boucher and co-workers (2015), highlighted that the regulation of chicken genes to serovar C-3 infection, occurs via Toll-like receptor 4 (TLR4) through the myeloid differentiation factor-88 (MyD88)-dependent pathway, leading to activation of NF-κβ (nuclear factor kappa B), thus resulting in the production of inflammatory cytokines. This finding is similar to studies conducted, whereby C3H/HeJ mice expressing deficient levels of TLR4 showed higher mortality after challenge with live *Salmonella enterica* serovar Typhimurium or *Escherichia coli*, which are Gram-negative bacteria, suggesting that LPS/TLR4 downstream signalling is crucial for host protective immune responses (O'Brien *et al*. 1980; Cross *et al*. 1995; Vazquez-Torres *et al*. 2004).

The chicken's immune response to *Av. paragallinarum* SA-3 serovar C-3 (most virulent in South Africa) infection causing infectious coryza, is still poorly understood and not well documented. A gap in the exact immune defence mechanism involving the regulation of immune signalling molecules against infectious coryza (SA-3 strain) has prompted the need to identify the major antibodies, cytokines, chemokines and cells involved in immunity against this disease, and to study the regulation of immune signalling pathways. The aim of the study is to conduct gene enrichment, functional annotation of differentially regulated genes and identify potential immune signalling pathways using existing bioinformatics pathway databases and tools, for understanding future comprehensive experimental data and the systematic workings of the chicken immune system. The findings obtained *in silico* will enable us to predict potential immune responses *in vivo* during disease progression of chickens affected with IC.

2.2. Materials and methods

2.2.1. Animal ethics, experimental design and data analysis

There was no animal experimental work involved or conducted in this study, rather existing biological data and resources collected from a previous trial that had already been published was used, as described (Boucher *et al*. 2014; Boucher *et al*. 2015). For this study, differentially expressed microarray data that had been processed and validated from Boucher *et al*. (2014, 2015), was obtained, whereby stringent filtering was performed (p-value≤0.02). Stringent filtering targets differentially expressed regulated genes having a higher confidence level. A flow diagram summarizing the bioinformatics pipeline used in the study for the interpretation of up- and down- regulated genes in avian immunity of birds infected with IC is shown (Figure 2.1).

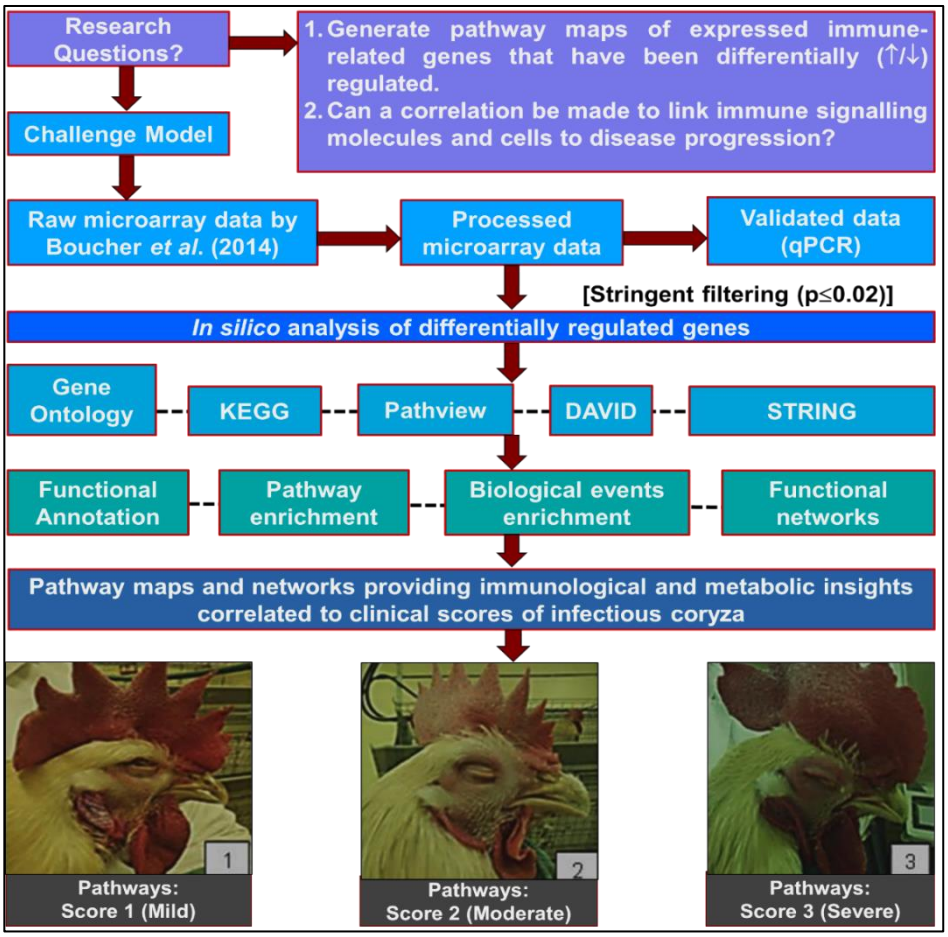

Figure 2.1: Flow diagram of bioinformatics pipeline indicating biological interpretation and outcome of *in silico* data of chickens infected with IC. Chicken images were published with permission from Dr C.E. Boucher.

2.2.2. Bio-statistical analysis

Enrichment analysis of differentially expressed regulated genes was conducted using GO Enrichment Analysis connected to the PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System PANTHER™ GO slim v14.0 (http://geneontology.org/). The enrichment analysis scouts for GO terms that are both over- or under- expressed using annotations for the gene list provided (Ashburner *et al*. 2000; Mi *et al*. 2017; Gene Ontology Consortium *et al*. 2017). Functional annotation of differentially regulated genes in networks

was performed using the web-based tool DAVID v6.8 (Database for Annotation, Visualization and Integrated Discovery) (https://david.ncifcrf.gov/) whereby enriched functional-related gene groups, biological themes and meaning can be obtained from the gene list provided using a set of annotation tools (Huang *et al*. 2008a; Huang *et al*. 2008b). Moreover, DAVID has a larger database for enriched GO terms, which can assist with GO terms not provided using GO Enrichment Analysis.

Results from high-throughput experiments such as microarrays interpreted by statistical testing and generating enriched GO terms may be extensive in size and highly redundant, making it difficult to comprehend (Supek *et al*. 2011). Hence, REVIGO (Reduce + Visualize Gene Ontology) (http://revigo.irb.hr/), a web server was used to reduce redundancy and allow GO terms to be summarised and visualised by searching for a representative subset of the terms via a clustering algorithm based on a measure of semantic similarity (Supek *et al*. 2011). To understand GO term terminology, QuickGO (https://www.ebi.ac.uk/QuickGO/) was used (Binns *et al*. 2009). A KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway identified as significantly differentially expressed, was visualised in a KEGG pathway plot using Pathview (https://pathview.uncc.edu/), whereby fold change was used that involves information on up- or downregulation (fold change >1 for upregulation and <1 for downregulation), an up-regulated gene was shown in red and a down-regulated gene in green (Luo and Brouwer, 2013; Luo *et al*. 2017). Functional association studies were performed using the manually curated STRING (Search Tool for the Retrieval of Interacting Genes) v11.0 database (https://string-db.org/) (Szklarczyk *et al*. 2014).

For the primary study conducted by Boucher *et al*. (2014), birds were sacrificed according to the severity of IC symptoms and at different time periods post challenge. The reason for the 12-24 h intervals between time periods, was to initially allow for adequate time for the

disease to run its course during the infection of chickens with the purpose of properly scoring and assessing IC symptoms, as each chicken would react differently when injected with the *Av. paragallinarum* culture. Moreover, the different time periods are the approximate times when the different clinical symptoms ranging from mild to severe were observed. As per literature, the disease develops in chickens within 24-48 h following infection with *Av. paragallinarum* culture, whereby infected birds will show IC symptoms within 24-72 h of infection (Pattison *et al*. 2008). Group 1 was represented by score 0 chickens, which were also control birds. Group 2 represented score 1 chickens with mild IC symptoms, whereby chickens with mild clinical symptoms were sacrificed at 36-48 h post-infection (PI). Group 3 consisted of chickens with score 2 showing moderate IC symptoms, whereby chickens with moderate clinical symptoms were sacrificed at 72-96 h PI. Group 4 had score 3 chickens with severe symptoms for IC and these chickens were sacrificed at 108-132 h PI. For comparative purposes the differentially expressed and regulated genes from the microarray data of experimental birds (Group 2, 3 and 4) were compared to control birds (Group 1).

2.3. Results and discussion

2.3.1. Gene enrichment analysis

From the GO Enrichment Analysis for the enrichment of differentially expressed regulated genes from the microarray data, pie charts were obtained representing the percentage distribution of the biological process ontology for the comparison of the differentially expressed regulated gene cohorts of Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0). Figure 2.2 shows that as the clinical score progressed during IC infection, from score 1 (mild) to score 3 (severe), there was an increase in the regulation of genes related to the immune response (0.5% $\rightarrow$ 2.2% $\rightarrow$ 2.6%).

A gradual transition from innate to adaptive immunity was also observed from score 1 to score 3.

From the gene enrichment for Group 2 (score 1) vs 1 (score 0), it was found that for 810 differentially expressed genes that was queried, only 422 had been mapped and annotated. The main immune response for Group 2 (score 1) vs 1 (score 0) was found to be the innate immune response. This was in line with the host's response to *Av. paragallinarum*, whereby the innate immune response is the first line of defence to pathogenic infection consisting of physical barriers, the serum complement system, immune cells (natural killer (NK) cells, heterophils, macrophages and dendritic cells) and molecules (cytokines and chemokines) (Kaiser, 2010; Carroll, 2004; Davison *et al*. 2011).

For Group 3 (score 2) vs 1 (score 0), out of 1095 genes that were differentially expressed, only 571 genes were upregulated, mapped and annotated. GO identifiers with percentage distribution associated with the immune response (Figure 2.2) were represented as pie charts (Figure 2.3). Genes representing both innate and adaptive immune responses, as well as the immune effector process were seen to be up-regulated for Group 3 (score 2) vs 1 (score 0), whereas with Group 2 (score 1) vs 1 (score 0) only the innate immune response was found to be up-regulated, showing that there was a transition from innate to adaptive immune responses from score 1 to score 2 (Figure 2.2). Moreover, T cell proliferation and differentiation indicated that the main lymphocytes involved in the immune response were T lymphocytes (Figure 2.3). T lymphocytes are mediators of both the innate and adaptive responses and are expressed as either $CD4^+$ (helper T cells) or $CD8^+$ (cytotoxic T cells) cells, whereby antigen-presentation occurs via MHC class II for CD4 molecules and MHC class I for CD8 molecules on the surfaces of T cells leading to the activation of these cells (Davison *et al*. 2011). During the innate response, $CD4^+$ cells such as Th1 cells produce

INF-γ activating macrophages, Th2 secretes IL-4 and IL-3 which assist in the differentiation of naïve B cells into plasma cells that secrete antibody (Spellberg and Edwards, 2001). $CD8^+$ cells display cytotoxic activity against pathogens during the adaptive immune response (Zhang and Bevan, 2011).

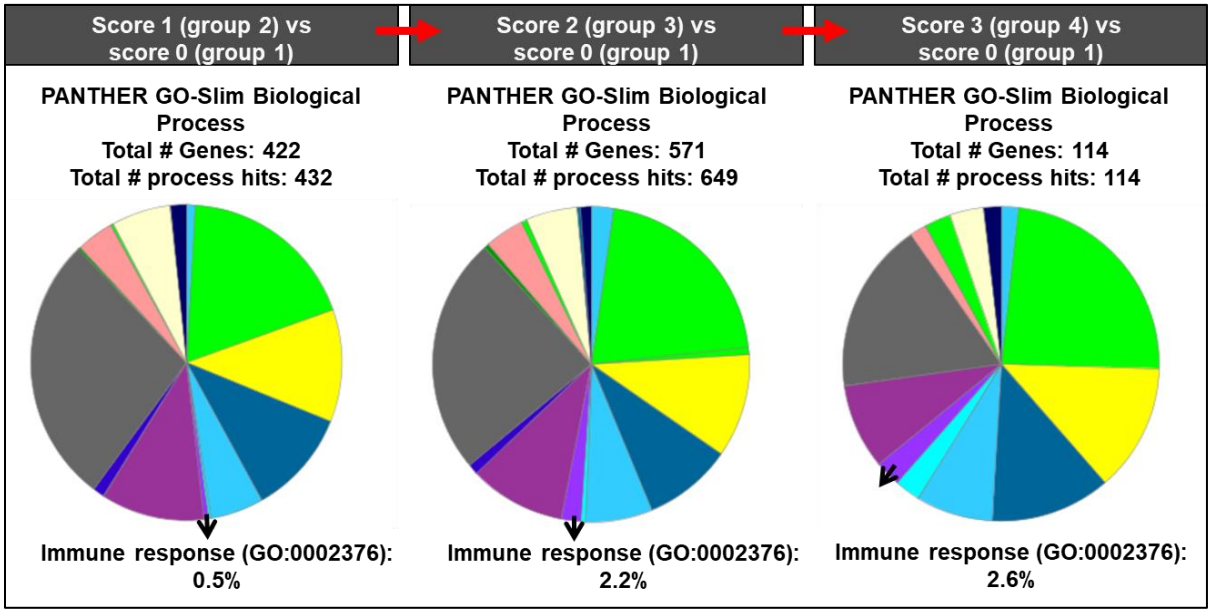

Figure 2.2: Biological process pie charts obtained from GO Enrichment Analysis linked to the PANTHER Classification System representing the percentage distribution of the biological process ontology in the comparison of the differentially expressed regulated gene cohorts of Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0). As the clinical score progressed during IC infection, from score 1 (mild) to score 3 (severe), there was an increase in the regulation of genes related to the immune response (section shown with arrows). This suggests that following the innate and adaptive immune responses there are various mechanisms from different systems (metabolic, endocrine, gastrointestinal, pulmonary, neurological, blood, etc.) involved that prevent the after effects of the immune system from damaging the tissues of the host organism while also deterring infection.

For Group 4 (score 3) vs 1 (score 0), out of 194 genes that were differentially expressed, only 114 genes were upregulated, mapped and annotated. However, for the GO term for immune response (Figure 2.2), further hits for lower level categories could not be obtained.

Thus, further annotations between the immune response of score 3 versus control chickens could not be established.

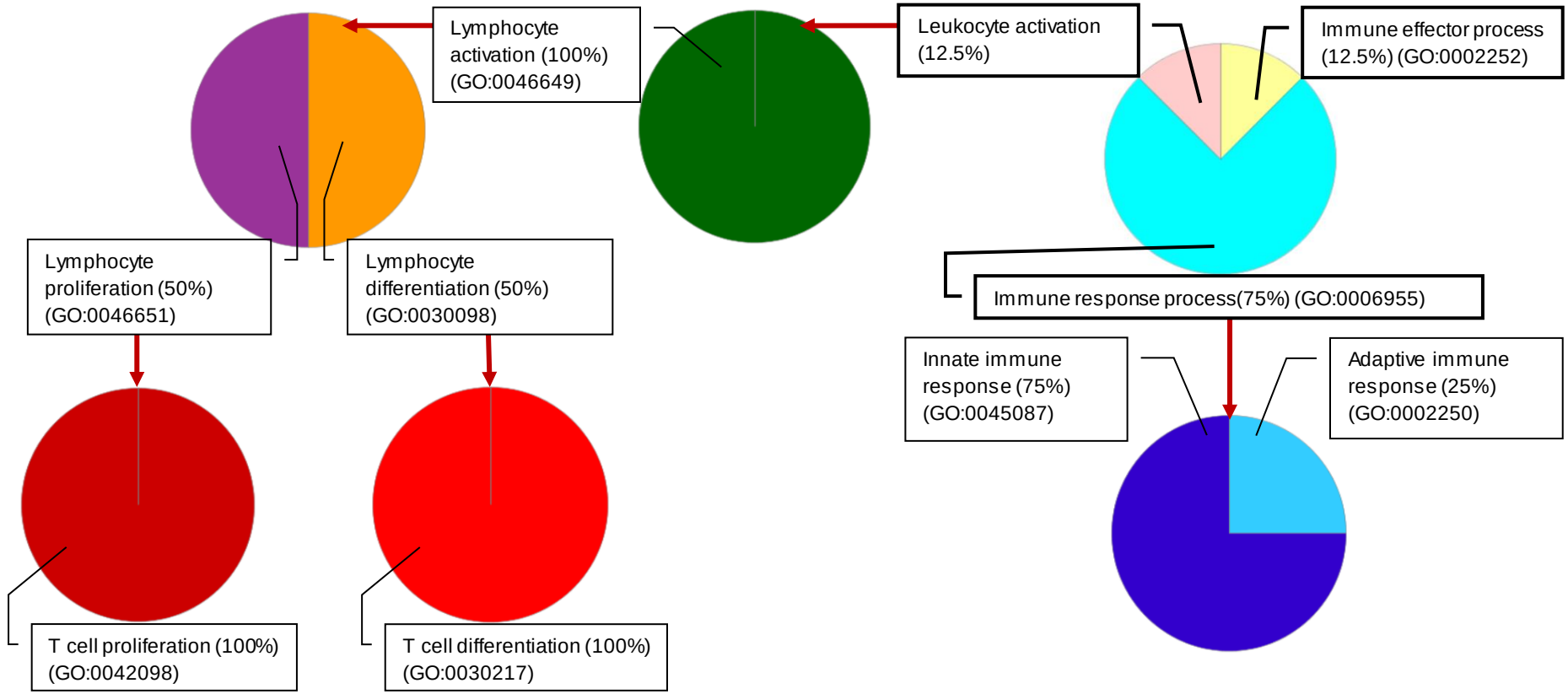

Figure 2.3: Biological process pie charts obtained from GO Enrichment Analysis representing the percentage distribution of the biological process ontology of Group 3 (score 2) vs 1 (score 0). The different GO identifiers for the immune response section shown in Figure 2.2 for Group 3 (score 2) vs 1 (score 0), which was divided into three main activities: immune response process, leukocyte activation and immune effector process (SEE TOP RIGHT PIE CHART). Ultimately, the lymphocyte that had a major role in the immune response was T lymphocytes, seen with T cell proliferation and differentiation.

2.3.2. Functional annotation

Differentially expressed genes between cohorts of Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0) showed highly significant enrichment of genes involved in the regulation of defense and immune responses. A full list of enriched and functionally annotated gene sets of Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0), based on gene ontology (GO) terms using the DAVID bioinformatics tool is provided (Annexure A), whereby the *p*-values observed in the tables represent the EASE (Expression Analysis Systematic Explorer) score or a modification of the Fisher Exact *p*-value, used as a measure of gene enrichment in terms of functional annotation (Huang *et al*. 2008a; Huang *et al*. 2008b). The lesser the *p*-value, the more enriched the genes that have been queried (Huang *et al*. 2008a; Huang *et al*. 2008b). For Group 2 (score 1) vs 1 (score 0) and Group 3 (score 2) vs 1 (score 0), there were potential functional and enriched genes for the immune response, however for Group 4 (score 3) vs 1 (score 0) there were more molecular function and cellular component hits obtained as GO terms.

For visualization of the GO terms obtained from DAVID, REVIGO was used for better interpretation. The results for the gene list of Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0) were shown as illustrations from REVIGO (Figure 2.4 - Figure 2.6).

Each of the GO terms in the scatterplots (Figure 2.4 - Figure 2.6) is represented as a node or bubble (Supek *et al*. 2011). In the scatterplot view of Figure 2.4 - Figure 2.6, the *X*- and *Y*-axes represent the two-dimensional semantic space of the graph, whereby the *x* and *y* coordinates of the bubbles were derived by implementing multidimensional scaling to a

matrix of the GO terms' semantic similarities (Supek *et al*. 2011). The colour of the bubble represents the user-provided *p*-value, where blue and green bubbles are GO terms with more significant *p*-values than orange and red bubbles (Supek *et al*. 2011). Moreover, the size of the bubble indicates the frequency of the GO term, where bubbles of more general terms are larger in size and smaller bubbles have more specific terms (Supek *et al*. 2011). Additionally, the closeness between the bubbles in the *X*- and *Y*- semantic spaces reflects their closeness in semantic similarity, hence similar nodes are found closer together and highly similar GO terms are designated by edges in the graph (Supek *et al*. 2011).

Figure 2.4 shows the REVIGO scatterplot for Group 2 (score 1) vs 1 (score 0), whereby a summarised and visual display of GO terms relating to biological process for mild symptoms to IC infection was observed. Interestingly, some of the immune responses included: response to lipopolysaccharide, xenophagy, cellular response to interferon-gamma, lymphocyte chemotaxis, chemokine-mediated signalling pathway, cellular response to tumour necrosis factor, cellular response to IL-1, reactive oxygen species metabolism and intracellular transport of viral protein in host cell. Most of the immune responses obtained were associated with innate immune responses against IC infection for Group 2 (score 1) vs 1 (score 0) (Figure 2.4). Despite the fact, that chickens with mild symptoms from Group 2 (score 1) vs 1 (score 0) were sacrificed at 36-48 h post challenge, the REVIGO results of Group 2 (score 1) vs 1 (score 0) (Figure 2.4) suggested that during that time period the innate immune responses of chickens was still active, even though innate responses are known to be immediate.

The response to lipopolysaccharide (LPS) refers to the binding of LPS to the TLR4-myeloid differentiation factor-2 (MD2) complex, the impetus required for the activation of pro-inflammatory signalling pathways and leading to a change in the state or activity of the host

such as movement, secretion, enzyme production and gene expression, as well as a cascade of signalling pathways leading to the initial immune responses against Gram-negative bacteria (Figure 2.4) (Bryant *et al*. 2010). Xenophagy is a type of autophagy (self-eating) against antibacterial and antiviral defenses (Figure 2.4) (Mao and Klionsky, 2017). This process is mediated by the autophagosome, whereby organelles in specific regions of the cytosol are sequestered for targeted infected cells and the contents are delivered to lysosomes for degradation (Mizushima, 2007; Xu and Eissa, 2010). Additionally, it was found that autophagy is part of the innate and adaptive immune responses respectively (Xu and Eissa, 2010). It was shown that TLR4 acts as the primary environmental receptor for the process of autophagy during pathogen invasion (Xu *et al*. 2007; Delgado *et al*. 2008; Xu *et al*. 2008). Moreover, studies have shown that LPS induces regulation of TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent, myeloid differentiation factor-88 (MyD88)-independent TLR4 of human and murine macrophages resulting in autophagy (Xu and Eissa, 2010).

The cellular response to interferon-gamma (INF-γ), suggests the mediation of immunity and inflammation by INF-γ a type II interferon that uses the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway to activate STAT1, promote inflammation, limit tissue damage, modulate T helper (Th) and regulatory T (Treg) cells, activate Th1 responses, activate macrophages, facilitate host defense and tumour surveillance (Figure 2.4) (Hu and Ivashkiv, 2009; Green *et al*. 2017). Lymphocyte chemotaxis refers to the directed migration of a lymphocyte towards a specific stimulus and is important for physiological conditions during adaptive immune responses (Figure 2.4) (Cabrero *et al*. 2006). Lymphocyte chemotaxis requires the synchronized activity of adhesion and chemotactic receptors, cytoskeleton and signalling molecules (Vicente-Manzanares and Sánchez-Madrid, 2004). The cellular response to tumour necrosis factor (TNF) relates to the proinflammatory activities of this cytokine through the activation of macrophages or

monocytes (Figure 2.4) (Liu and Han, 2001). TNF also has important cellular functions such as the release/induction of other cytokines, cell proliferation, cell differentiation, modification of the anticoagulant properties of endothelial cells and apoptosis (Liu and Han, 2001). Most TNF-induced responses are via tumour necrosis factor receptor 1 (TNF-R1) mediated pathways (Liu and Han, 2001). Tumour necrosis factor receptor type 1-associated DEATH domain (TRADD) is enlisted to the TNF-R1 complex and consequently effectors such as TNF receptor-associated factor-2 (TRAF2), receptor-interacting protein (RIP), Fas-associated protein with death domain (FADD), cellular inhibitor of apoptosis protein-1 (cIAP1), cellular inhibitor of apoptosis protein-2 (cIAP2) and tumour necrosis factor α-induced protein 3 (TNFAIP3)/(A20), are recruited to the complex, that act as mediators in the activation of proteases, phospholipases, protein kinases and transcription factors via the pathways described (Liu and Han, 2001).

Interleukin (IL)-1 is a highly inflammatory cytokine found in two forms IL-1α and IL-Iβ, whereby they co-function with TNF (Figure 2.4) (Dinarello, 1997). IL-1 has systemic effects and during innate immunity has an effect on all innate immune cells through several functions such as leukocyte recruitment, leukocyte migration, cortisol regulation, humoral innate immunity through the activation of acute phase proteins, lymphoid cell-mediation, as well as survival and effector functions (Garlanda *et al*. 2013). For the regulation and amplification of innate immunity and uncontrolled inflammation, there are four signalling receptors, two decoy receptors (IL-1R2, IL-18BP), two negative regulators (toll interleukin-1 receptor (IL-1R) 8 (TIR8)/ single immunoglobulin interleukin-1 receptor related molecule (SIGIRR), interleukin-1 receptor accessory protein (IL-1RAcPb), seven ligands with agonist activity (IL-1α and IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ), three receptor antagonists (IL-1Ra, IL-36Ra, IL-38) and an anti-inflammatory cytokine (IL-37) in the IL-1 family (Garlanda *et al*. 2013).

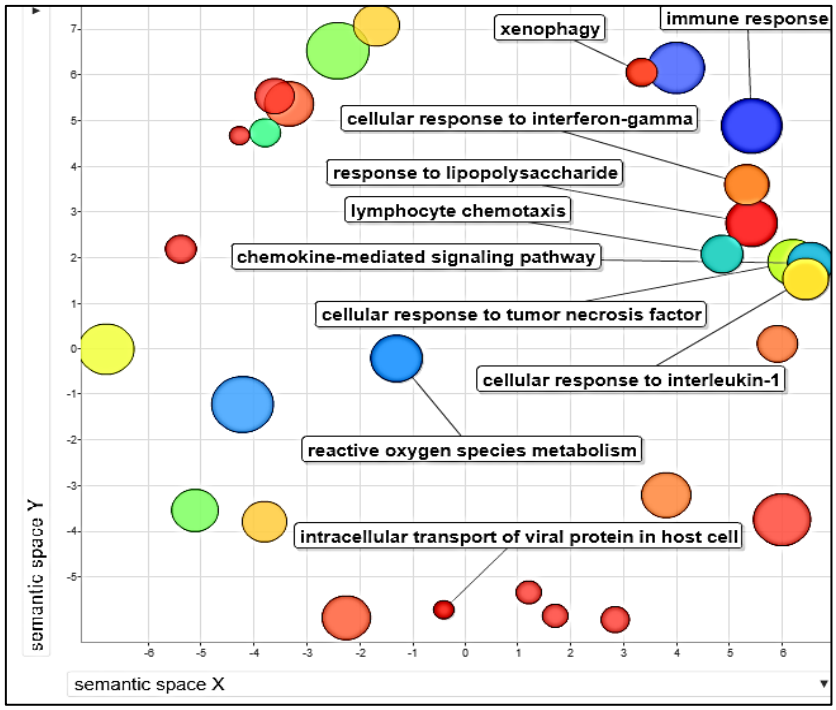

Figure 2.4: Scatterplot of biological process for Group 2 (score 1) vs 1 (score 0) with REVIGO providing a summarised and visual display of GO terms. The scatterplot shows the initial immune responses of chickens with mild IC symptoms. Some of the notable biological processes were as follows: response to lipopolysaccharide, xenophagy, cellular response to interferon-gamma, lymphocyte chemotaxis, chemokine-mediated signalling pathway, cellular response to tumour necrosis factor, cellular response to IL-1, reactive oxygen species metabolism and intracellular transport of viral protein in host cell. In the scatterplot view, each bubble represents a GO term, the *X*- and *Y*-axes represent the two-dimensional semantic space of the graph, the colour of the bubble represents the user-provided *p*-value, the size of the bubble indicates the frequency of the GO term and the closeness between the bubbles in the *X*- and *Y*- semantic spaces reflects their closeness in semantic similarity (Supek *et al*. 2011).

Reactive oxygen species (ROS) metabolism pertains to the chemical reactions and pathways involving a ROS, whereby ROS exist as highly reactive free radicals or minute short-lived oxygen-containing molecules (Figure 2.4) (Chen *et al*. 2016). Moreover, they have a key role in microbicidal activity of phagocytes such as macrophages and serve as second messengers in cell signalling (Bae *et al*. 2009). Upon the transformation of macrophages into phagolysosomes during pathogenic engulfment, NADPH-dependent phagocytic oxidase (NADPH oxidase 2 (NOX2)) is assembled to reduce oxygen to

superoxide anion (O_2^-), involved in the immediate killing and degradation of bacteria inside the phagolysosomes (Slauch, 2011). Phagocytes are the main immune cells involved in innate immunity during pathogenic infection, which would explain ROS metabolism during mild symptoms of chickens during IC infection. The GO term that was interesting to find was that of intracellular transport of viral protein in host cell (Figure 2.4), as similar results were found by Boucher *et al*. (2014). During a study by Boucher *et al*. (2014) there was up-regulation of TLR7 activated by ss-RNA viruses, it was proposed that there was a possibility that the bacterial genome of *Av. paragallinarum* consisted of prophages and prophage remnants that was previously discovered in a study by Roodt *et al*. (2012). Boucher and co-workers suggested that the prophage remnant in *Av. paragallinarum* might contribute to the severe symptoms observed with serovar C-3 (SA-3 strain). All the processes described above might potentially contribute to innate immunity against IC invasion in chickens.

Figure 2.5 shows the REVIGO scatterplot for Group 3 (score 2) vs 1 (score 0), with GO terms relating to biological process. Some of the GO terms obtained from the scatterplot for Group 3 (score 2) vs 1 (score 0) were similar to Group 2 (score 1) vs 1 (score 0), such as response to lipopolysaccharide and cellular response to tumour necrosis factor for the biological process category, which implies that even during moderate symptoms some of the initial innate responses continue to occur (Figure 2.4 and Figure 2.5). In comparison, to Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) generated more GO terms, due to a combination of both innate and adaptive immune responses (Figure 2.4 and Figure 2.5). A new series of biological process GO terms was obtained: chemotaxis, inflammatory response, apoptotic signalling pathway, haemopoiesis, angiogenesis, regulation of catalytic activity, regulation of ERK1 and ERK2 cascade, negative regulation of protein tyrosine kinase activity, positive regulation of peptide hormone secretion, regulation of cell size, positive regulation of cell proliferation, regulation of cell differentiation, negative regulation of phosphatidylinositol 3-kinase signalling and skeletal system development

(Figure 2.5). The chickens with moderate symptoms from Group 3 (score 2) vs 1 (score 0), were sacrificed at 72-96 h, at this time period both late innate and initial adaptive immune responses seemed to occur, as shown with REVIGO results for Group 3 (score 2) vs 1 (score 0) (Figure 2.5). The findings obtained are suggested to be in line with the mechanisms of the avian immune response, since both the innate and adaptive immune responses are interconnected. Following the activation of the avian innate immune response, downstream signalling pathways and molecules trigger adaptive immunity at a later stage during IC infection, which was why moderate symptoms from Group 3 (score 2) vs 1 (score 0) were observed at 72-96 h.

Among the immune responses for Group 3 (score 2) vs 1 (score 0) was inflammatory response, which is a biological response of the immune system that can be activated via nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and JAK-STAT pathways, whereby different organs in the host may be affected (Figure 2.5) (Chen *et al*. 2018). The apoptotic signalling pathway refers to molecular cell signalling pathways controlling apoptosis or programmed cell death, in leukocytes and other cells of the immune system (Figure 2.5) (Siegel and Lenardo, 2002). There are two apoptotic pathways, whereby active apoptosis involves TNF-related receptors known as death receptors which leads to antigen-induced cell death, whereas passive apoptosis occurs when stimulated lymphocytes are denied access to essential growth cytokines and thus death receptors are not needed (Siegel and Lenardo, 2002). Both pathways are carried out by intracellular cysteine proteases referred to as caspases, whereby any defects in either of these apoptotic pathways produce unique pathologies within the host organism (Siegel and Lenardo, 2002).

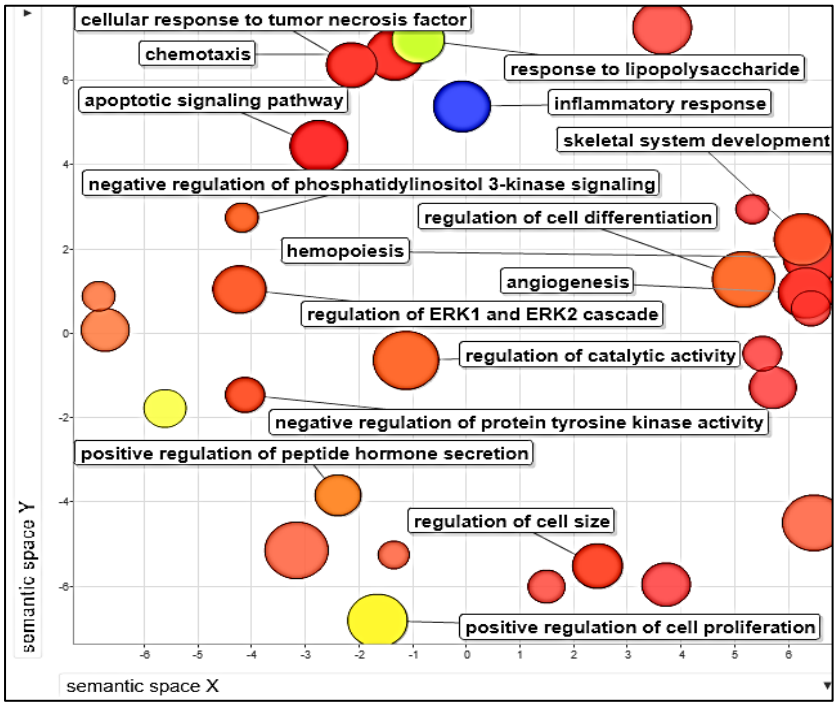

Figure 2.5: Scatterplot of biological process for Group 3 (score 2) vs 1 (score 0) with REVIGO providing a summarised and visual display of GO terms. The scatterplot shows both innate and adaptive responses of chickens with moderate IC symptoms. Some of the notable biological processes were shared with Group 2 (score 1) vs 1 (score 0) such as response to lipopolysaccharide and cellular response to tumour necrosis factor. Distinguished biological process GO terms included: chemotaxis, inflammatory response, apoptotic signalling pathway, haemopoiesis, angiogenesis, regulation of catalytic activity, regulation of ERK1 and ERK2 cascade, negative regulation of protein tyrosine kinase activity, positive regulation of peptide hormone secretion, regulation of cell size, positive regulation of cell proliferation, regulation of cell differentiation, negative regulation of phosphatidylinositol 3-kinase signalling and skeletal system development. In the scatterplot view, each bubble represents a GO term, the *X*- and *Y*-axes represent the two-dimensional semantic space of the graph, the colour of the bubble represents the user-provided *p*-value, the size of the bubble indicates the frequency of the GO term and the closeness between the bubbles in the *X*- and *Y*- semantic spaces reflects their closeness in semantic similarity (Supek *et al*. 2011).

In both innate and adaptive immune responses cells undergo apoptosis primarily with phagocytes, neutrophils and effector T cells for clearance and for elimination of pathogens. Furthermore, the presence of GO terms chemotaxis and angiogenesis suggest the release of neuropeptides during moderate symptoms (Figure 2.5). Neuropeptides from the brain may have similar potent functions to the angiogenic cytokine, vascular endothelial growth factor

(VEGF) and cause a concentration gradient that attracts immune cells (macrophages, monocytes, heterophils) within the avian host (Fischer-Colbrie *et al*. 2005). Neurotransmission implies that the nervous system is involved and one of the symptoms of IC involves lethargy and disorientation in infected chickens. Although, regulation of catalytic activity usually pertains to enzymatic activity, it might also refer to antibody-mediated catalysis by natural antibodies (abzymes) that have the capacity to degrade nucleic acids, protein, and polysaccharide substrates in infection and immunity (Nevinsky *et al*. 2000; Bowen *et al*. 2017).

Haemopoiesis, regulation of cell size, positive regulation of cell proliferation and regulation of cell differentiation showed that there was an influx of cells since the avian immune response consisted of various immune cells to target and eliminate pathogens; and cellular differentiation occurred whereby cells transformed from naïve cells to effector immune cells such as monocytes to macrophages, naïve B cells to plasma cells, naïve T cells to Th or cytotoxic T cells during the innate and adaptive immune phases of chickens with score 2 (Figure 2.5) (Playfair and Bancroft, 2013.). Efferent signals from the brain to the nervous system and finally to the immune system are transmitted by the neuroendocrine and nervous systems, which is possible via shared ligands and receptors, neurotransmitters, neuropeptides, growth factors, neuroendocrine hormones and cytokines (Kelley *et al*. 2007). Therefore, hormones secreted by the neuroendocrine system such as growth hormone (GH) and insulin-like growth factor-1 (IGF-I) play an vital role in the communication and regulation of the cells of the immune system, hence the GO term, positive regulation of peptide hormone secretion was generated from the gene cohort of Group 3 (score 2) vs 1 (score 0) (Kelley *et al*. 2007). The cluster of GO terms: regulation of ERK1 and ERK2 cascade, negative regulation of protein tyrosine kinase activity and negative regulation of phosphatidylinositol 3-kinase signalling, relates to the mechanisms of the TLR2 or TLR4 signalling pathway (Dahle *et al*. 2004). TLR2 signalling commences with the assembly of

large membrane complexes and results in the stimulation and nuclear translocation of NF-κB, whereby pro- and anti-inflammatory cytokines are activated in the immune response (Dahle *et al*. 2004). Moreover, this nonspecific mechanism of cytokine gene induction has been extended on through the recognition of multiple signalling kinases such as p38 mitogen-activated protein kinase (MAPK), extracellular signal-related kinase (ERK1 and ERK2), phosphatidylinositol 3-kinase (PI3-K) and protein tyrosine kinases (Dahle *et al*. 2004). Although, it is known that LPS- mediated signalling is conducted via TLR4, it can also occur via TLR2, which is dependable as both peptidoglycan and LPS are constituents of the Gram-negative cell walls (Arbibe *et al*. 2000; Akira, 2001; Takeuchi and Akira, 2001). This finding, is consistent to that of Boucher *et al*. (2014), whereby initially for mild symptoms, both TLR2 and TLR4 were expressed in expression profiles. Nevertheless, TLR 4 was found to be up-regulated during moderate symptoms. However, there is still a discrepancy related to species-specific differences in the role of the TLRs, since LPS signalling is mediated by TLR4 in mice and by TLR2 in humans, hence it is possible that in avian models LPS signalling is mediated by both TLR2 and TLR4 (Akira, 2001).

Figure 2.6 shows the REVIGO scatterplot for Group 4 (score 3) vs 1 (score 0), with GO terms relating to biological process. In comparison to the scatterplots for Group 2 (score 1) vs 1 (score 0) and Group 3 (score 2) vs 1 (score 0), there are less GO terms for biological process (Figure 2.4 - Figure 2.6). Moreover, since fewer GO terms were obtained for Group 4 (score 3) vs 1 (score 0), this indicated that there were less differentially expressed genes. The results obtained for Group 4 (score 3) vs 1 (score 0) (Figure 2.6) suggested that mechanisms leading to disease tolerance and protection to the host from systemic hyperinflammation of the innate and adaptive immune responses were involved, rather than defence mechanisms against pathogenic infection, as seen with mild and moderate IC symptoms (Figure 2.4 - Figure 2.5). The biological process GO terms for Group 4 (score 3) vs 1 (score 0) were as follows: cellular response to amino acid stimulus, positive regulation

of cortisol secretion, protein heterotrimerization, female sex differentiation, gamete generation, regulation of protein catabolism (Figure 2.6).

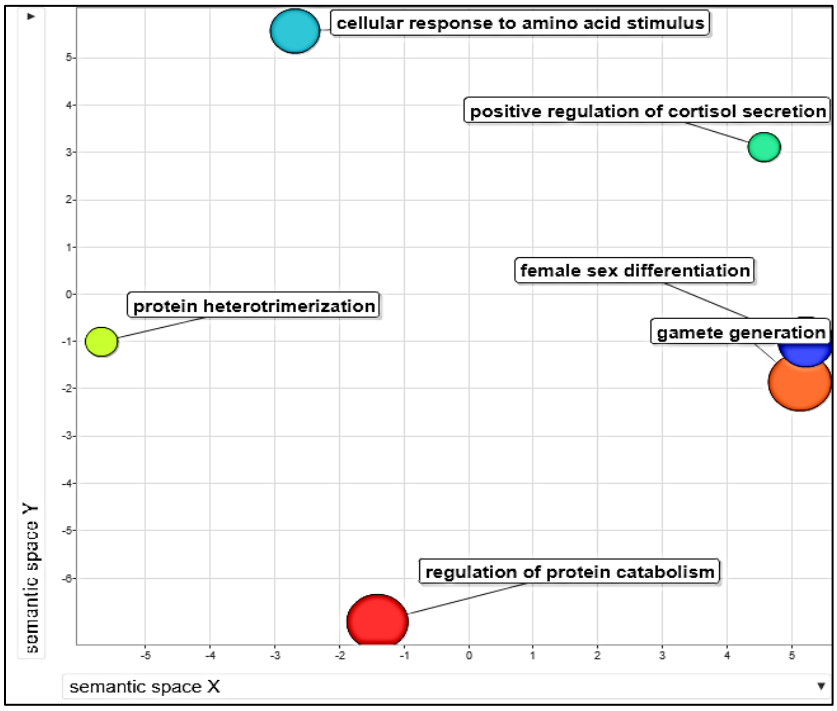

Figure 2.6: Scatterplot of biological process for Group 4 (score 3) vs 1 (score 0) with REVIGO providing a summarised and visual display of GO terms. The scatterplot shows the different biological responses of chickens with severe IC symptoms. The GO terms obtained for Group 4 (score 3) vs 1 (score 0), did not correlate to the immune responses as observed with Group 2 (score 1) vs 1 (score 0) and Group 3 (score 2) vs 1 (score 0). There were fewer GO terms compared to those of mild and moderate IC symptoms (Figure 2.4 - Figure 2.5). Moreover, the GO terms obtained for Group 4 (score 3) vs 1 (score 0) related more to mechanisms leading to disease tolerance and protection to the host from systemic hyperinflammation of the innate and adaptive immune responses, rather than defence against pathogenic infection, as seen with mild and moderate IC symptoms (Figure 2.4 - Figure 2.6). Biological process GO terms included: cellular response to amino acid stimulus, positive regulation of cortisol secretion, protein heterotrimerization, female sex differentiation, gamete generation, regulation of protein catabolism. In the scatterplot view, each bubble represents a GO term, the *X*- and *Y*-axes represent the two-dimensional semantic space of the graph, the colour of the bubble represents the user-provided *p*-value, the size of the bubble indicates the frequency of the GO term and the closeness between the bubbles in the *X*- and *Y*- semantic spaces reflects their closeness in semantic similarity (Supek *et al*. 2011).

The GO term cellular response to amino acid stimulus refers to any alteration in cell morphology or cellular activity due to an amino acid stimulus, however not much could be deduced from this general GO term (shown with a light blue medium sized bubble in Figure 2.6). However, from the scatterplot (Figure 2.6), it was observed that the bubbles for GO terms cellular response to amino acid stimulus, positive regulation of cortisol secretion, female sex differentiation and gamete generation, were closely grouped to the right side of the graph and consisted of a small green bubble (having a GO term that was specific and that had a significant *p*-value), blue and green bubbles (having a GO term that had significant *p*-values) as well as an orange and a dark blue bubble that were in close proximity (indicating high semantic similarity). Therefore, one probable hypothesis how these four GO terms were similarly connected would have been via the neuroendocrine system of the host organism. Additionally, the GO term cellular response to amino acid stimulus could refer to neuropeptides that have a role to play in the adaptive immune response during IC infection. Neuropeptides are neurotransmitters that possess short amino acid chains, involved in neurological transmission and communication, and may affect cell activity due to having potent effects on immunoglobulin synthesis both *in vivo* and *in vitro*, as well as in the proliferation of mast cells and granulocytes (Stanisz *et al*. 1987). Other neurotransmitters such as vasoactive intestinal peptide, substance P, and somatostatin, that are also peptides, have regulatory functions on immune effector cells in gut-associated lymphoid tissue (GALT) and thus are crucial in gastrointestinal physiology (O'Dorisio, 1986). Vasoactive intestinal peptide regulates lymphocyte migration and natural killer (NK) cell activity via a cyclic adenosine monophosphate (cAMP)-dependent mechanism, somatostatin inhibits the effects of both vasoactive intestinal peptide and substance P using a process that appears to include inhibitory guanine nucleotide binding proteins (O'Dorisio, 1986). Another inference to cellular response to amino acid stimulus (Figure 2.6), would be the interplay of catabolic mechanisms of essential amino acids and modulation of the host's robust immune system to prevent hyperinflammation in innate, adaptive, and regulatory responses to infections, as well as prevent infection-driven immunopathology (Grohmann *et al*. 2017). The amino acids

tryptophan (Trp) and arginine (Arg) modulate immune reactivity, whereas phenylalanine (Phe), glutamine (Gln) and cysteine (Cys) balance immune reactivity (McGaha *et al*. 2012).

Ultimately, mammals through evolution have acquired a mechanism to regulate pathogen infection by increasing amino acid catabolism thereby limiting the availability of intracellular nutrients to colonizing pathogens, whereby amino-acid sensing and degradation in immunometabolism occurs via the Trp- (kynurenine (Kyn) pathway) and Arg-catabolic pathways (arginine decarboxylase (ADC), intestinal-renal axis, citrulline-nitric oxide (NO) pathways) (McGaha et al. 2012; Grohmann et al. 2017). Amino acid catabolism described is also in line with the GO term regulation of protein catabolism obtained with REVIGO (Figure 2.6). The GO term positive regulation of cortisol secretion may refer to the activity of corticotropin releasing factor (CRF) or corticotropin-releasing hormone (CRH) from the extrahypothalamic areas in the brain, that stimulates the anterior of the pituitary gland to produce adrenocorticotropic hormone (ACTH) which travels in the bloodstream to the adrenal glands inducing secretion of cortisol, a stress hormone (Figure 2.6) (Ohmura and Yoshioka, 2009). During an immune and inflammatory response, there is stimulation of the neuroendocrine stress system, whereby a Th2 shift is induced which causes stress hormones to electively suppress Th1 responses instead of immunosuppression (Elenkov, 2002). In doing so, the Th2 shift provides protection to the host from systemic hyperinflammatory responses by countering the tissue-damaging effects of macrophages and Th1 cells, as well as Th1/ proinflammatory cytokines (Elenkov, 2002). Nevertheless, the activation of CRH/substance P(SP)-histamine axis, may assist inflammatory responses through initiation of IL-1, IL-6, IL-8, IL-18, TNF-α, and c-reactive protein (CRP) synthesis (Calcagni and Elenkov, 2006). Female sex differentiation and gamete generation, is an invalid GO term, since 10-week old White Leghorn male birds (cocks) with no prior clinical history of IC were obtained for the study and not hens, therefore these biological processes probably occur during embryonic development (Figure 2.6).

2.3.3. Pathway analysis

The Pathview results for differentially regulated genes using fold change for Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0) generated KEGG pathways. The KEGG pathways were selected based on the relevance to some of the GO terms obtained in Section 2.3.2. Genes that were upregulated were shown as red and genes that were downregulated were shown as green. Due to stringent filtering, not many genes were found to be up- or downregulated.

Response to lipopolysaccharide was the shared GO term between Group 2 (score 1) vs 1 (score 0) and Group 3 (score 2) vs 1 (score 0) (Figure 2.4 and Figure 2.5). Hence, the result for toll-like receptor signalling pathway was obtained for both Group 2 (score 1) vs 1 (score 0) and Group 3 (score 2) vs 1 (score 0) (Figure 2.7). It was found that IL-8, regulated on activation, normal T Cell expressed and secreted (RANTES/CCL5) and macrophage inflammatory protein-1 beta (MIP-1β/CCL4) were upregulated for Group 2 (score 1) vs 1 (score 0), which are inflammatory cytokines that function as chemoattractants for mild symptoms of IC. It seems that these molecules mediate the initial responses of innate immunity that eventually culminate into the adaptive immune responses. MIP-1β is a chemoattractant for $CD4^+$ and $CD8^+$ cells which also induces chemotaxis and cell adhesion of T cells, IL-8 attracts T cells and neutrophils, and RANTES has attractant activities for T cells and monocytes of memory phenotype (Schall *et al*. 1990; Schall *et al*. 1993; Tanaka *et al*. 1993). For Group 3 (score 2) vs 1 (score 0) which was for moderate symptoms for IC, TLR2, myeloid differentiation factor 2 (MD-2) complexed to TLR4 and TIR domain containing adaptor protein (TIRAP) were upregulated, showing that TIRAP acts as a bridge between MyD88 to the receptor complex for TLR-2 and TLR4 signalling that mediates NF-κB proinflammatory responses (Figure 2.7) (Verstak *et al*. 2009). Therefore, both TLR2 and TLR4 seem to mediate pathogen recognition in chickens during IC infection for moderate

symptoms (Figure 2.7). TLR4 recognizes LPS and utilises both MyD88 and TRIF upstream, and TIRAP downstream as signalling adaptors to stimulate the activation of NF-κB and MAPK (Figure 2.7) (Takeuchi and Akira, 2010). The TRIF pathway also activates interferon regulatory factor-3 (IRF3), whereby IFN-β and -α are produced that stimulate CD8 T cells as a response to viral infection, antigen-presenting cells (APCs) and other T cells (Figure 2.7) (Welsh *et al*. 2012).

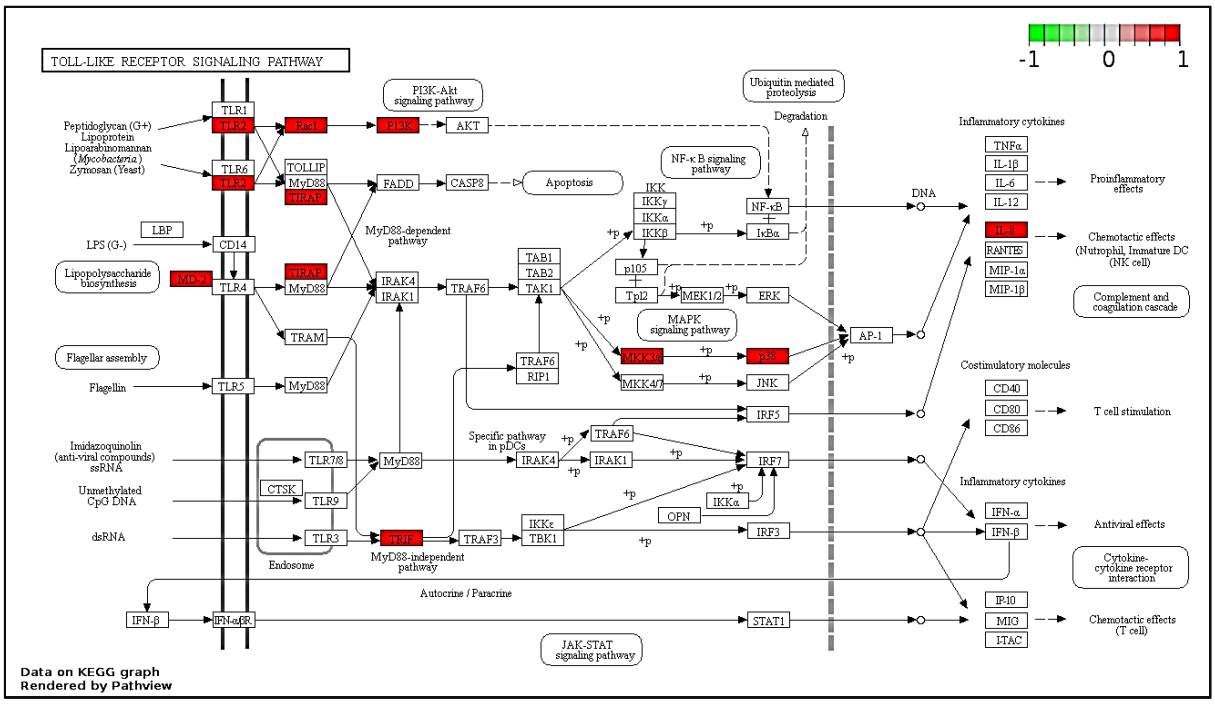

Figure 2.7: Toll-like receptor pathway from KEGG pathways for Group 3 (score 2) vs 1 (score 0). The genes that were upregulated (red) included: TLR2, MD-2, Ras-related C3 botulinum toxin substrate 1 (Rac1), phosphoinositide 3-kinase (PI3K), TIRAP, mitogen-activated protein kinase kinase (MKK) 3/6, p38 mitogen-activated protein kinase (p38) and TRIF. The pathway shown was for moderate symptoms, hence both innate and adaptive responses were involved.

Figure 2.8 shows the signalling pathway of the phagosome for Group 2 (score 1) vs 1 (score 0), an important defence mechanism during innate immunity for the elimination of invasive pathogens. Macrophages are highly motile cells that carry out phagocytosis and chemotaxis

(Rougerie *et al*. 2013.). Genes that were upregulated included F-actin (linear polymer microfilament), TAP (transporter associated with antigen-processing), TUBB (tubulin beta class 1) and Sec61(channel forming translocon), whereas CD36 was downregulated (Figure 2.8).

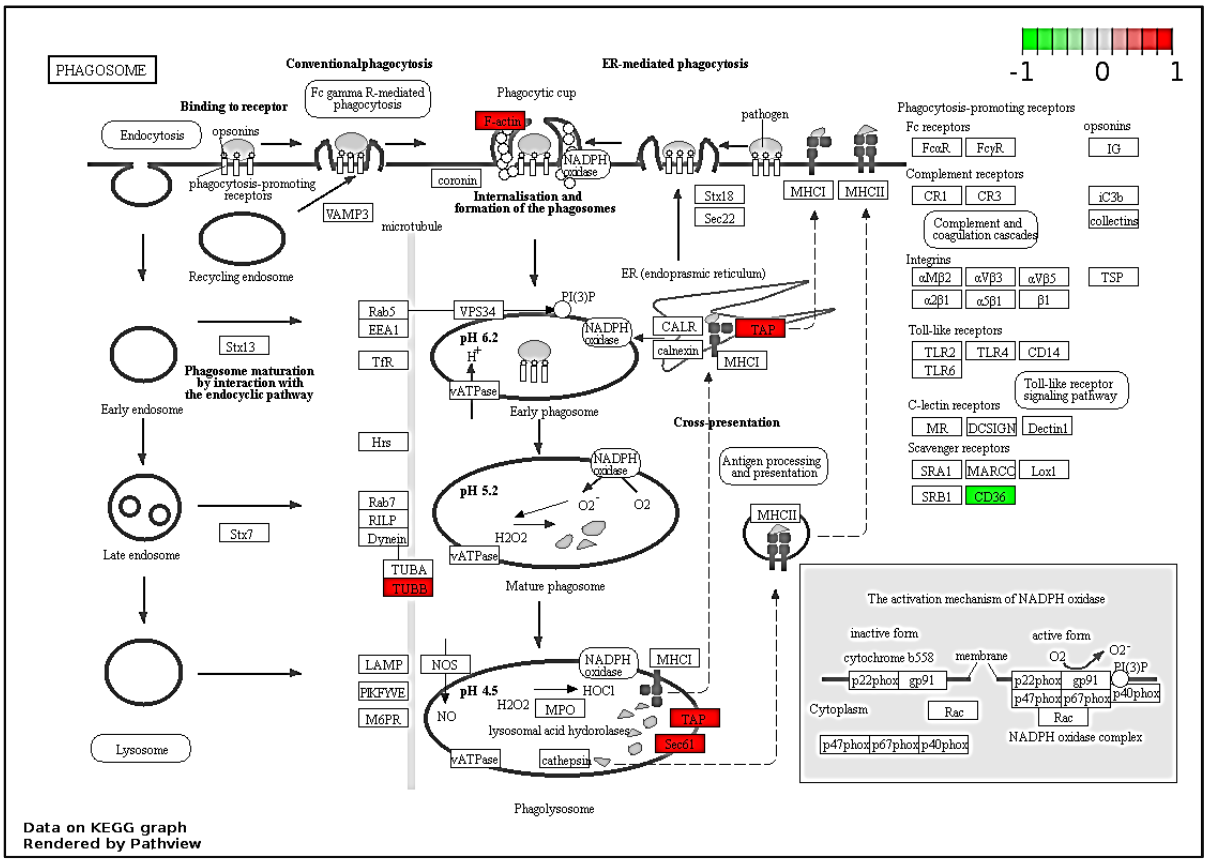

Figure 2.8: Phagosome pathway for the maturation of the phagocyte into a phagolysosome from KEGG pathways for Group 2 (score 1) vs 1 (score 0). The genes that were upregulated (red) included: F-actin, TAP, TUBB and Sec61, whereas CD36 was downregulated (green). The pathway shown was for mild symptoms, during innate immunity.

The differentially regulated genes have a role in the different stages of phagosome maturation (Figure 2.8). During phagocytosis, actin cytoskeletal remodeling is required for the formation of lamellipodia and phagocytic cups that are F-actin rich, requiring regulation of

actin polymerization via actin related protein (Arp) 2/3 activation and nucleation promoting factors like the Wiskott-Aldrich syndrome protein (WASP)/ WASP family Verproline-homologous protein (WAVE) family, as well as remodeling of these actin networks (Rougerie *et al*. 2013). Therefore, the F-actin gene was up-regulated to facilitate the engulfment of the pathogen (Figure 2.8). In addition, TAP transports peptides of foreign origin (from the pathogen), from the cytosol into the endoplasmic reticulum (ER) to MHC class I molecules, whereby the peptide is presented to $CD8^+$ cytotoxic T cells (Ritz and Seliger, 2001).

Interestingly, the pathway for influenza A virus was one of the pathways obtained for Group 3 (score 2) vs 1 (score 0) for moderate symptoms with IC infection, that affects the respiratory system within alveolar and bronchial epithelial cells, alveolar macrophages and type II pneumocytes of both humans and birds alike (Figure 2.9). Genes that were upregulated included 70 kilodalton heat shock proteins (HSP70), MKK3/6, p38, IL-8, TRIF, PI3K, influenza virus non-structural protein-1 binding protein (NS1BP), plasminogen (PLG), interferon-gamma receptor (IFNGR) and tumour necrosis factor receptor 1 (TNFR1), whereas induced by phosphate starvation1 (IPS1) was downregulated (Figure 2.9).

Fibrinolysis is the process of dissolving fibrin into a soluble form conducted by the serine protease plasmin (Berri *et al*. 2013). Plasmin is produced through cleavage of plasminogen generated in the liver and found in the bloodstream, whereby PLG/plasmin has a key role in fibrinolysis-mediated inflammation (Berri *et al*. 2013). Influenza A viruses (IAV) may influence the specific binding and conversion from PLG into plasmin by allowing the virus a substitute protease for cleavage in the form of its hemagglutinin molecule, which is a crucial stage in viral replication leading to disease (Goto and Kawaoka, 1998; Goto et a*l.* 2001; LeBouder *et al*. 2008; LeBouder *et al*. 2010). Additionally, PLG may lead to pathogenesis of IAV infections, by facilitating virus replication or via induction of a fibrinolysis-dependent

damaging inflammatory response in the respiratory tract (Berri *et al*. 2013). PLG was found to be upregulated during IC infection of Group 3 (score 2) vs 1 (score 0) (Figure 2.9).

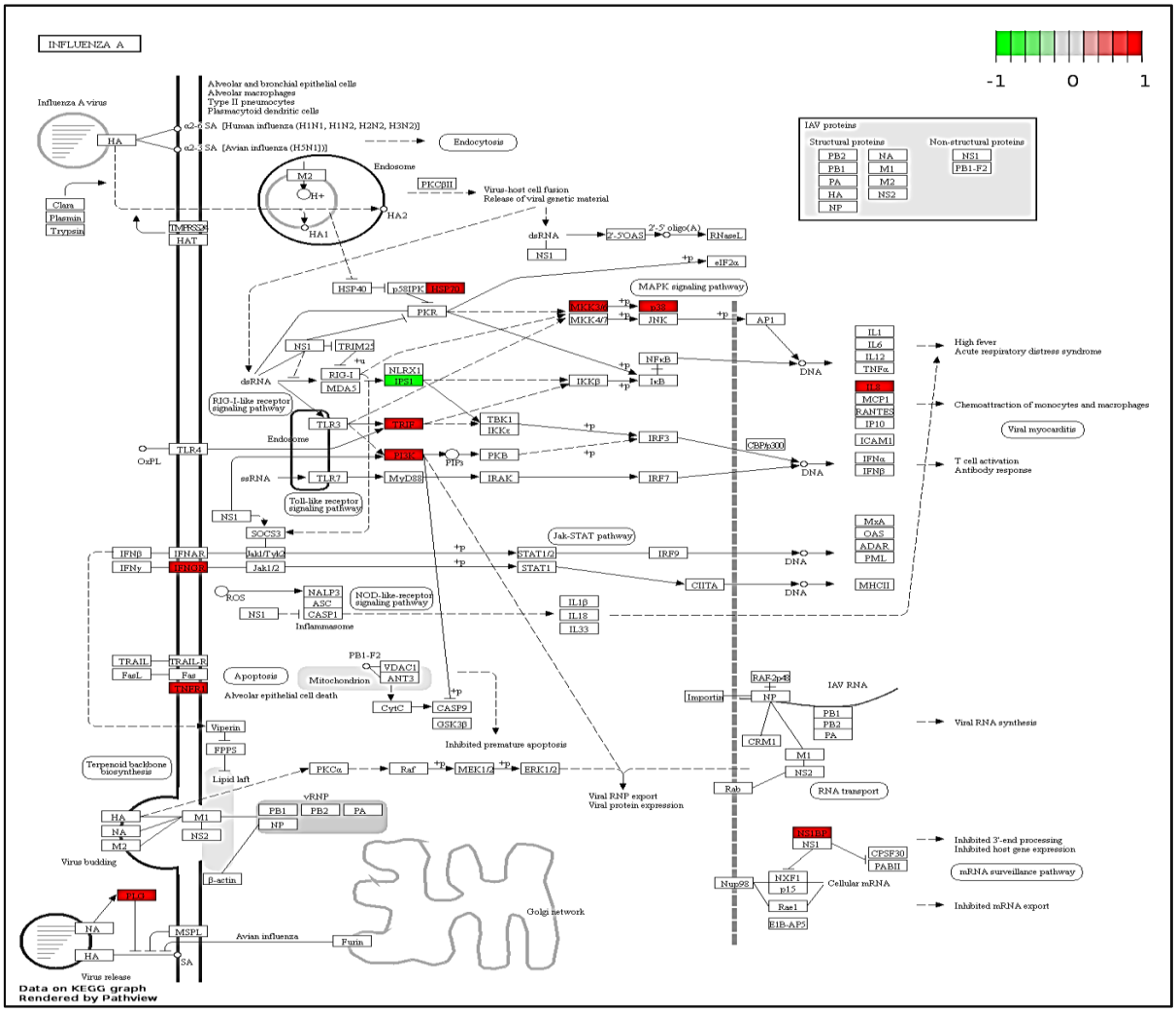

Figure 2.9: Influenza A virus pathway from KEGG pathways for Group 3 (score 2) vs 1 (score 0). The genes that were upregulated (red) included: HSP70, MKK3/6, p38, IL-8, TRIF, PI3K, NS1BP, PLG, IFNGR and TNFR1, whereas IPS1 was downregulated (green). The pathway shown was for moderate symptoms, during innate and adaptive immunity.

Ironically, although *Av. paragallinarum* serovar C-3 is a Gram-negative bacteria, like IAV it also has virulence factors in the form of hemagglutinin (HA) antigens associated with pathogenicity and immunogenicity (Figure 2.9) (Yamaguchi *et al*. 1993; Gamblin and Skehel,

2010). Therefore, it is possible that both *Av. paragallinarum* serovar C-3 and IAV share common mechanisms and genes leading to pathogenesis of the host, in this case the chicken. Since, the mapped pathways of IAV share numerous up-regulated genes (shown in red Figure 2.9) and one down-regulated gene (shown in green Figure 2.9) with IC, this may also explain the severe symptoms observed with IC infection, especially to *Av. paragallinarum* serovar C-3 (SA-3), which is the most virulent strain in South Africa (Figure 2.9). The reason for this shared trait might also be due to the prophage and prophage remnants in the genome of *Av. paragallinarum* discussed in Section 2.3.2. The presence of prophages in *Av. paragallinarum* serogroups has been investigated (Coetsee, 2014). However, since only one of the genes was found to be down-regulated and far more numerous genes were found to be unaffected (Figure 2.9), shared pathogenesis between IC and IAV could only be speculated, whereby this area of research would need further investigation.

Surprisingly, NS1BP was also an up-regulated gene observed with moderate symptoms during IC infection (Figure 2.9). NS1BP that is encoded by the virus genome, blocks IFN signalling (Jia *et al*. 2010). This is achieved by inhibiting the intracellular sensor retinoic acid-inducible gene 1 protein (RIG-I), whereby association with the downstream regulatory proteins IPS-1 (shown as downregulated in Figure 2.9) and IRF3 are disrupted leading to impaired transcriptional activation of IFN-β (Jia *et al*. 2010). Thus, INF-β and other pro-inflammatory cytokines are inhibited and their functions, thereby suggesting a similar mechanism for IC infection (Jia *et al*. 2010).

There was a significant downregulation of numerous genes in the oxidative phosphorylation pathway of differentially regulated genes of Group 4 (score 3) vs 1 (score 0) (Figure 2.10), which might indicate a septic state in the host, which indicates selective inhibition of

downstream kinases and phosphatases that regulate the activity of the oxidative phosphorylation complexes leading to a reduction in adenosine triphosphate (ATP) production, which might cause symptoms observed with IC infection, such as neurological disorientation, lethargy, laboured breathing, weight loss, poor growth and an unthrifty appearance.

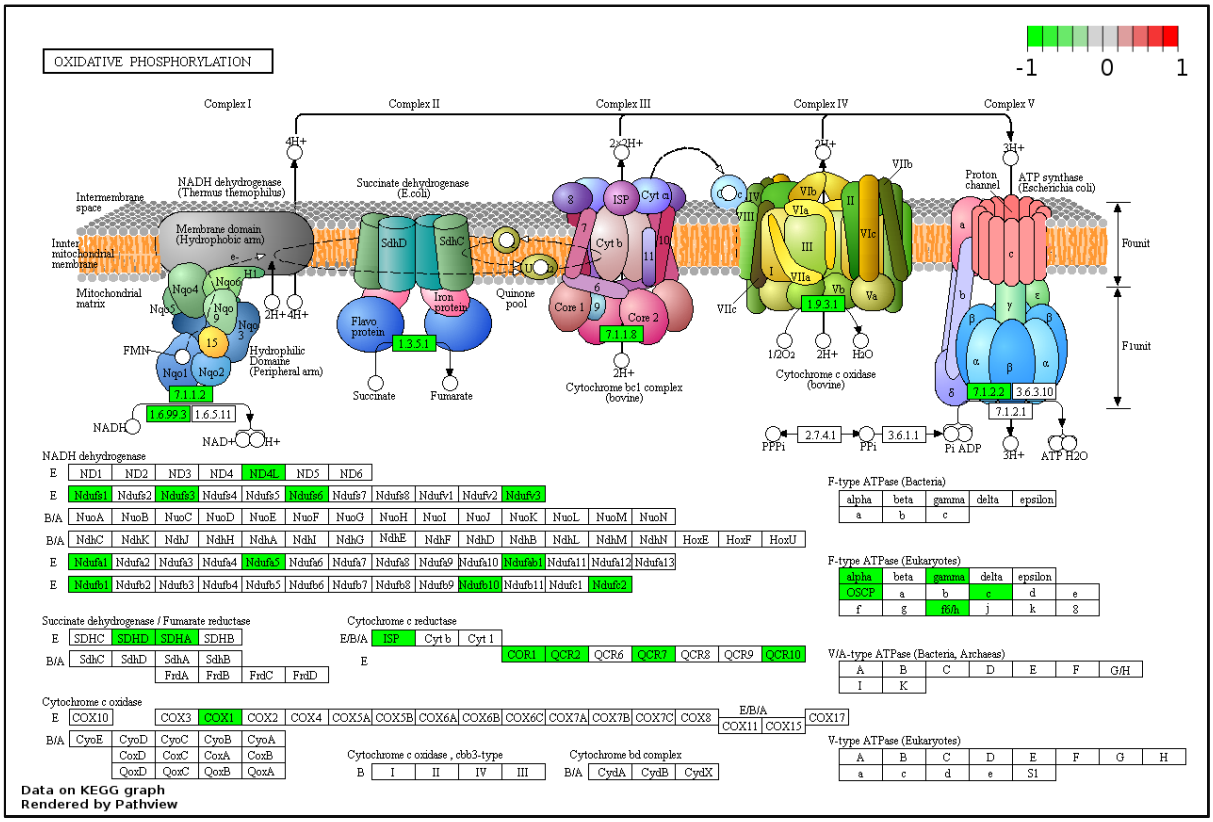

Figure 2.10: Oxidative phosphorylation pathway from KEGG pathways for Group 4 (score 3) vs 1 (score 0). The genes that were downregulated were shown in green). The pathway shown was for severe symptoms, following innate and adaptive immunity. Inhibition of the oxidative phosphorylation process causes a reduction in metabolism leading to energy deficiency causing dire consequences to the host. Score 3 chickens face severe symptoms to IC infection such as hemorrhage and conjunctivitis, which might be indications of upper respiratory sepsis to *Av. paragallinarum* C-3 serovar.

Sepsis is predominantly caused by bacterial infections that can originate from any region of the host's body and may even result in unspecific responses such as tachycardia

(abnormally rapid heart rate) and tachypnoea (abnormally rapid breathing) leading to more drastic consequences such as organ dysfunction and failure (Lee and Hüttemann, 2014). During sepsis and acute inflammation, it was proposed that administration of LPS in animal models causing inflammatory signalling results in alterations in the oxidative phosphorylation state of mitochondrial proteins such as tyrosine (Tyr) 304 phosphorylation of cytochrome c oxidase (COX) catalytic subunit I (Lee and Hüttemann, 2014). Subsequently, there is inhibition of the oxidative phosphorylation process, causing reduced metabolism, a decline in the mitochondrial membrane potential, and an energy deficiency, which can cause organ dysfunction and fatality (Lee and Hüttemann, 2014). The down-regulation of numerous genes (shown in green in Figure 2.10) for the oxidative phosphorylation pathways might lead to the inhibition of various oxidative phosphorylation processes, leading to a dysfunction in the host's metabolism during IC infection.

2.3.4. Functional protein association network analysis

Correlations of differentially expressed gene cohorts of Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0), may reflect differences in the immune response composition between each group. Functional association analysis was conducted to elucidate the functional relevance of this biological variation, and to visualise the networking and interactions between the differentially expressed gene cohorts, using the STRING database (Szklarczyk *et al*. 2014). The STRING functional association network for Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0) are shown (Figure 2.11 A – Figure 2.11 C). The coloured nodes represent the query proteins and the first shell interactors, whereas the white nodes represent secondary shell interactors.

For Group 2 (score 1) vs 1 (score 0), 589 gene products were obtained (Figure 2.11 A). For Group 2 (score 1) vs 1 (score 0) associated with innate immune responses during mild symptoms, the correlating genes included: inflammatory cytokines (IL-4, interleukin 8-like 1 (IL-8L1) and IL-18), acute phase response protein (CRP) and mediators of apoptosis (caspase-6 (CASP6), apoptosis-inducing factor-2 (AIFM2), shisa Family Member 5 (SHISA5), and PLG) (Figure 2.11 A). For Group 3 (score 2) vs 1 (score 0), 796 gene products were obtained (Figure 2.11 B). Some of the correlating genes for Group 3 (score 2) vs 1 included: IL-8L1, TIRAP and MAPK family (mitogen-activated protein kinase kinase kinase 4 (MAP3K4), mitogen-activated protein kinase kinase 3 (MAP2K3) and mitogen-activated protein kinase 11 (MAPK11)) (Figure 2.11 B). Group 3 (score 2) vs 1 correlating genes from the STRING database consists of numerous unknown chicken genes. For Group 4 (score 3) vs 1 (score 0), 151 gene products were obtained (Figure 2.11 C). T-Cell leukemia homeobox protein 3 (TLX3), nuclear distribution gene C (NUDC) and gamma-aminobutyric acid type A receptor alpha1 subunit (GABRA-1) are genes mostly associated with neuronal signalling responses found as correlating genes for Group 4 (score 3) vs 1 (score 0) (Figure 2.11 C).

From the analyses conducted, it was evident that the most genes that were differentially expressed were found in Group 3 (score 2) vs 1 (score 0), which occurred during moderate symptoms of IC (Figure 2.11 B). This clearly showed that both innate and adaptive responses were at play. However, Group 4 (score 3) vs 1 (score 0) had the least number of differentially expressed genes (Figure 2.11 C). Similar results were observed, whereby fewer GO terms correlated to less differentially expressed genes (Figure 2.6). These findings suggest that a lack of up-regulated genes, resulted in more severe disease due to relative suppression of the immune response. Thus, it is possible that in severely ill-birds there may be failure of the immune system as a result of fewer up-regulated genes.

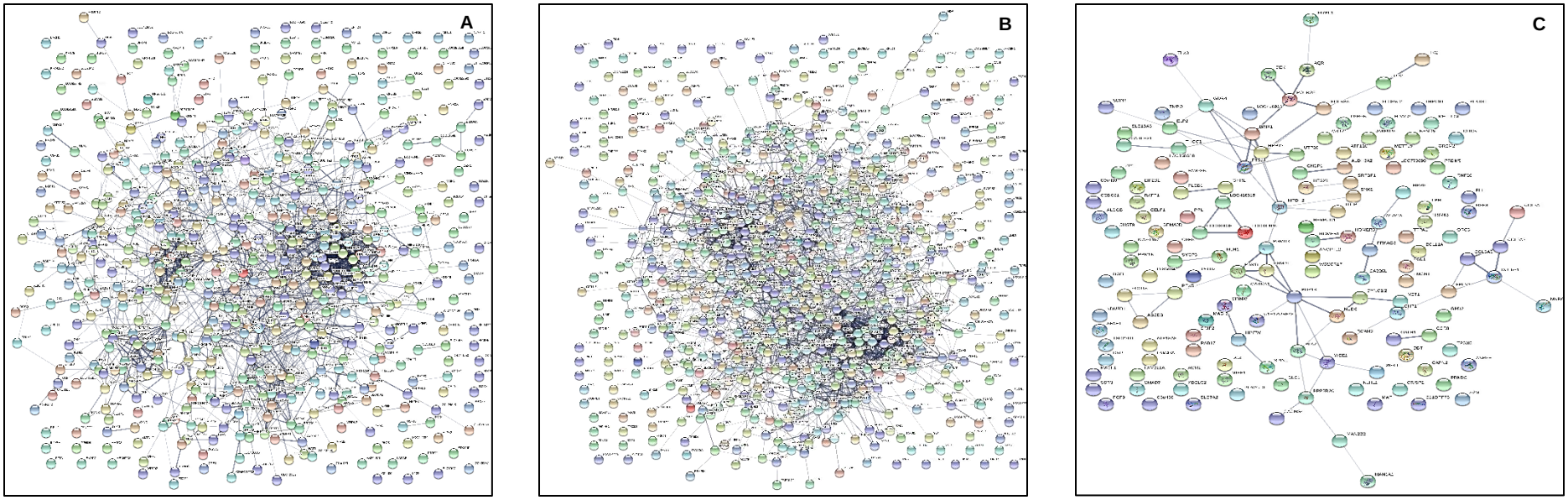

Figure 2.11 (A-C): Functional association network of Group 2 (score 1) vs 1 (score 0), Group 3 (score 2) vs 1 (score 0) and Group 4 (score 3) vs 1 (score 0) correlated genes. The STRING functional association network was generated for differentially expressed genes in the cohorts. Shown here is the confidence view. From the analyses conducted, it was evident that the most genes that were differentially expressed were found in Group 3 (score 2) vs 1 (score 0), which occurred during moderate symptoms of IC, clearly showing that both innate and adaptive responses were at play, whereas Group 4 (score 3) vs 1 (score 0) had the least number of differentially expressed genes.

2.4. Conclusion

In this study, gene enrichment, functional annotation and identification of potential immune signalling pathways of differentially regulated genes was successfully conducted for mild, moderate and severe symptoms obtained from chickens challenged with IC. Focus on functional pathways and interaction networks was conducted to gain comprehensive insights into biological processes relevant to protection and pathogenesis in IC infection which can be harnessed for relevant biomarker signatures. Moreover, pathogenesis of IC infection with serovar C-3, was found to be similar to that of influenza A virus, suggesting that the presence of prophages and prophage remnants may have a crucial role to play in the virulence of the bacterial pathogen, as proposed by Boucher *et al*. (2014). The role of prophages in the virulence of *Av. paragallinarum* still needs further study. IL-8 a pro-inflammatory cytokine and chemoattractant for immune cells was up-regulated during IC infection throughout all symptoms, hence its potential use as a biomarker in IC infection can be considered. It was also found that the metabolism of the host altered during IC infection, whether this was caused by sepsis due to bacterial infection or as a mechanism for protecting the host from hyperinflammatory responses as well as depriving nutrients from the invasive pathogen, remains to be discovered. The *in silico* results obtained from bioinformatics tools for the generation of immune signalling pathway maps may assist in predicting *in vivo* immune responses during IC infection. These findings provide a better understanding of the immune pathology in IC infection, while combined biosignatures may be utilized in future into the development of a pathogenesis-based point-of-care test to monitor and maintain disease in the poultry industry.

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ANNEXURE A

GO terms for biological process, molecular function and cellular component for Group 2 (score 1) vs 1 (score 0)		
GO term	**Description**	***p*-Value**
GO:0006955	immune response	4.32E-03
GO:0006954	inflammatory response	4.63E-03
GO:0072593	reactive oxygen species metabolic process	6.59E-03
GO:0045595	regulation of cell differentiation	6.59E-03
GO:0070098	chemokine-mediated signaling pathway	8.16E-03
GO:0048247	lymphocyte chemotaxis	9.18E-03
GO:0006526	arginine biosynthetic process	1.20E-02
GO:0008637	apoptotic mitochondrial changes	1.57E-02
GO:0055114	oxidation-reduction process	1.67E-02
GO:0071356	cellular response to tumor necrosis factor	2.12E-02
GO:0030097	hemopoiesis	2.62E-02
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	3.06E-02
GO:0002548	monocyte chemotaxis	3.49E-02
GO:0071347	cellular response to interleukin-1	3.81E-02
GO:0009060	aerobic respiration	4.10E-02
GO:0051289	protein homotetramerization	4.26E-02
GO:0071346	cellular response to interferon-gamma	5.46E-02
GO:0030308	negative regulation of cell growth	5.73E-02
GO:0042787	protein ubiquitination involved in ubiquitin-dependent protein catabolic process	6.15E-02
GO:0046627	negative regulation of insulin receptor signaling pathway	6.21E-02
GO:0006099	tricarboxylic acid cycle	6.21E-02
GO:0006633	fatty acid biosynthetic process	7.00E-02
GO:0045454	cell redox homeostasis	7.40E-02
GO:0098792	xenophagy	7.93E-02
GO:0008285	negative regulation of cell proliferation	8.63E-02

GO:0006744	ubiquinone biosynthetic process	8.87E-02
GO:0014054	positive regulation of gamma-aminobutyric acid secretion	9.02E-02
GO:0061732	mitochondrial acetyl-CoA biosynthetic process from pyruvate	9.02E-02
GO:0071421	manganese ion transmembrane transport	9.02E-02
GO:0006121	mitochondrial electron transport, succinate to ubiquinone	9.02E-02
GO:0019060	intracellular transport of viral protein in host cell	9.02E-02
GO:0045901	positive regulation of translational elongation	9.02E-02
GO:1902361	mitochondrial pyruvate transmembrane transport	9.02E-02
GO:0032496	response to lipopolysaccharide	9.63E-02
GO:0016874	ligase activity	2.59E-02
GO:0008137	NADH dehydrogenase (ubiquinone) activity	2.94E-02
GO:0008009	chemokine activity	3.22E-02
GO:0048020	CCR chemokine receptor binding	6.57E-02
GO:0016279	protein-lysine N-methyltransferase activity	6.57E-02
GO:0030955	potassium ion binding	6.57E-02
GO:0046872	metal ion binding	7.06E-02
GO:0016491	oxidoreductase activity	7.85E-02
GO:0009055	electron carrier activity	8.41E-02
GO:0000287	magnesium ion binding	9.28E-02
GO:0015086	cadmium ion transmembrane transporter activity	9.34E-02
GO:0005384	manganese ion transmembrane transporter activity	9.34E-02
GO:0004056	argininosuccinate lyase activity	9.34E-02
GO:0005743	mitochondrial inner membrane	2.38E-07
GO:0043209	myelin sheath	3.43E-06
GO:0005747	mitochondrial respiratory chain complex I	1.11E-05
GO:0005739	mitochondrion	4.39E-03
GO:0031463	Cul3-RING ubiquitin ligase complex	1.07E-02
GO:0005759	mitochondrial matrix	4.07E-02
GO:0045177	apical part of cell	5.64E-02
GO:0005764	lysosome	6.71E-02
GO:0043657	host cell	8.88E-02

GO:0033018	sarcoplasmic reticulum lumen	8.88E-02
GO:0070062	extracellular exosome	9.44E-02

Go terms for biological process, molecular function and cellular component for Group 3 (score 2) vs 1 (score 0)		
GO term	**Description**	***p*-Value**
GO:0006954	inflammatory response	1.09E-06
GO:0032496	response to lipopolysaccharide	4.91E-04
GO:0015986	ATP synthesis coupled proton transport	1.97E-03
GO:0008284	positive regulation of cell proliferation	2.41E-03
GO:0090277	positive regulation of peptide hormone secretion	1.19E-02
GO:0015991	ATP hydrolysis coupled proton transport	1.72E-02
GO:0042127	regulation of cell proliferation	1.81E-02
GO:0006094	gluconeogenesis	2.04E-02
GO:0045595	regulation of cell differentiation	2.12E-02
GO:0005980	glycogen catabolic process	2.31E-02
GO:0014067	negative regulation of phosphatidylinositol 3-kinase signaling	2.31E-02
GO:0050790	regulation of catalytic activity	2.32E-02
GO:0070372	regulation of ERK1 and ERK2 cascade	3.04E-02
GO:0045087	innate immune response	3.22E-02
GO:0051683	establishment of Golgi localization	3.69E-02
GO:0061099	negative regulation of protein tyrosine kinase activity	3.69E-02
GO:0006096	glycolytic process	3.71E-02
GO:0010628	positive regulation of gene expression	3.77E-02
GO:0001501	skeletal system development	3.82E-02
GO:0007155	cell adhesion	3.99E-02
GO:0050727	regulation of inflammatory response	4.19E-02
GO:0008361	regulation of cell size	4.78E-02
GO:0043401	steroid hormone mediated signaling pathway	4.78E-02
GO:0046328	regulation of JNK cascade	7.11E-02

GO:0030836	positive regulation of actin filament depolymerization	7.11E-02
GO:0071356	cellular response to tumor necrosis factor	7.35E-02
GO:0018108	peptidyl-tyrosine phosphorylation	7.35E-02
GO:0005975	carbohydrate metabolic process	7.38E-02
GO:0001525	angiogenesis	7.42E-02
GO:0030097	hemopoiesis	7.52E-02
GO:0006935	chemotaxis	7.52E-02
GO:0030593	neutrophil chemotaxis	7.52E-02
GO:0002548	monocyte chemotaxis	8.11E-02
GO:0035987	endodermal cell differentiation	8.11E-02
GO:0003382	epithelial cell morphogenesis	9.08E-02
GO:0050873	brown fat cell differentiation	9.39E-02
GO:0034446	substrate adhesion-dependent cell spreading	9.44E-02
GO:0097190	apoptotic signaling pathway	9.44E-02
GO:0031532	actin cytoskeleton reorganization	9.72E-02
GO:0070062	extracellular exosome	2.70E-05
GO:0043209	myelin sheath	3.44E-05
GO:0045261	proton-transporting ATP synthase complex, catalytic core F(1)	2.40E-03
GO:0005615	extracellular space	4.45E-03
GO:0005623	cell	5.00E-03
GO:0005829	cytosol	9.81E-03
GO:0009986	cell surface	1.72E-02
GO:0005753	mitochondrial proton-transporting ATP synthase complex	2.27E-02
GO:0005759	mitochondrial matrix	2.59E-02
GO:0005743	mitochondrial inner membrane	3.17E-02
GO:0000139	Golgi membrane	4.66E-02
GO:0005925	focal adhesion	5.40E-02
GO:0005887	integral component of plasma membrane	6.04E-02
GO:0016324	apical plasma membrane	7.41E-02
GO:0032982	myosin filament	8.94E-02
GO:0031012	extracellular matrix	9.50E-02

GO:0046961	proton-transporting ATPase activity, rotational mechanism	1.43E-03
GO:0046933	proton-transporting ATP synthase activity, rotational mechanism	4.79E-03
GO:0005351	sugar:proton symporter activity	1.22E-02
GO:0005003	ephrin receptor activity	1.22E-02
GO:0042802	identical protein binding	1.87E-02
GO:0003779	actin binding	2.08E-02
GO:0000287	magnesium ion binding	2.23E-02
GO:0005031	tumor necrosis factor-activated receptor activity	4.86E-02
GO:0005385	zinc ion transmembrane transporter activity	5.90E-02
GO:0008083	growth factor activity	5.92E-02
GO:0008009	chemokine activity	7.03E-02
GO:0015078	hydrogen ion transmembrane transporter activity	7.03E-02
GO:0009055	electron carrier activity	7.53E-02

Go terms for biological process, molecular function and cellular component for Group 4 (score 3) vs 1 (score 0)		
GO term	**Description**	***p*-Value**
GO:0046660	female sex differentiation	3.53E-02
GO:0071230	cellular response to amino acid stimulus	4.34E-02
GO:0051464	positive regulation of cortisol secretion	4.68E-02
GO:0070208	protein heterotrimerization	5.81E-02
GO:0007276	gamete generation	8.04E-02
GO:0042176	regulation of protein catabolic process	9.13E-02
GO:0005793	endoplasmic reticulum-Golgi intermediate compartment	4.46E-02
GO:0005581	collagen trimer	6.74E-02
GO:0005634	nucleus	6.78E-02
GO:0030686	90S preribosome	7.29E-02
GO:0005782	peroxisomal matrix	7.29E-02
GO:0035256	G-protein coupled glutamate receptor binding	2.40E-02

CHAPTER 3

A PILOT STUDY OF THE IMMUNE RESPONSE TO *Avibacterium paragallinarum* SEROVAR C-3 INFECTION IN *Gallus gallus*

Sections of Chapter 3 have been used for manuscript for submission in a peer-reviewed journal, with the title “Omens and Remnants of Infectious Coryza: A Macabre Tale of Necropsy and Immunohistopathology of Chicken Lymphatic Tissues after Infection with *Av. paragallinarum* serovar C-3” and “The Infectious Coryza Diaries: Disease Monitoring of Immune Cells and Molecules during *Av. paragallinarum* serovar C-3 Infection”.

3.1. Introduction

Infectious coryza caused by the Gram-negative bacterium *Av. paragallinarum*, is a well-recognized and commonly encountered upper respiratory tract disease in chickens (Blackall, 1999). The occurrence of outbreaks has emphasized the significance of the disease in both broiler and layer chickens (Blackall, 1999). In South Africa, IC is regarded as one of the most serious diseases of layers, with C-3 being the most predominant serovar (Bragg *et al*. 1996). The economic impact of IC is primarily observed in the layer industry, with a marked drop in egg production and a significant number of culls due to morbidity in growing broods (Blackall, 1999).

The genetic mechanisms that govern the immune response of birds infected with *Av. paragallinarum* are still unknown and poorly studied. Thus far, the only studies conducted on the avian model with regards to immune mechanisms were conducted on *Salmonella* serovars and avian influenza virus (Withanage *et al*. 2004, Withanage *et al*. 2005; Xing *et al*. 2008; Nerren *et al*. 2010; Matulova *et al*. 2013). Notable studies investigated the role played by candidate genes and genomic regions in disease resistance, through challenge methods with pathogenic *Salmonella* that mimicked the natural routes of exposure to the pathogen, thus allowing the opportunity to study mucosal immunity and the immune response (Lamont, 1994; Lamont, 1998; Kaiser and Lamont, 2002; Lamont *et al*. 2002; Kramer *et al*. 2003). Studies by Boucher *et al.* (2014, 2015), investigated the up- and down- regulation of genes using microarray technology and RNA isolated from chicken sinuses presented with clinical symptoms when infected with serovar C-3, thus opening the way to the immune mechanisms pertaining to *Av. paragallinarum* host-pathogen interactions. Moreover, with regards to innate and adaptive immunity in birds challenged with *Av. paragallinarum*, it is still uncertain which immune cells and molecules are involved that serve as immune mechanisms against pathogenic infection. This prompted us to investigate and study the regulation of immune signalling molecules related to immunity during *Av. paragallinarum* infection. However, before we could commence, a pilot study was conducted to validate whether the study would be feasible. The pilot study would also provide a robust and logical workflow and experimental design, that will enable us to troubleshoot any discrepancies or errors encountered and take further necessary measures for the actual experiment to be conducted.

For this research project, a pilot study was conducted in accordance with clear study aims and objectives, as an exploratory tool to enable us to determine a suitable experimental design, master and understand techniques, implement correct laboratory procedures and practices, involving chickens challenged with a highly virulent *Av. paragallinarum* serovar C-3 (SA-3 strain). This was done to elicit an immune response and monitor disease progression. A pilot

study is essential since it would encourage the necessity of the study, establish a framework within which to work and therefore guarantee that it is scientifically valid (Lancaster *et al*. 2004).

3.2. Materials and methods

3.2.1. Ethics statement, animal husbandry and study design

The research conducted and handling of animals in the study was performed in accordance with current South African legislation, The South African National Standard for the Care and Use of Animals for Scientific Purpose (SANS 10386:2008), and the Animals Protection Act, 1962 (Act No. 71 of 1962). Prior to experiments, permission to perform animal research was applied for and clearance was obtained under Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) from the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa. The study and specific experiments were conducted and monitored according to an approved ethics protocol by the Interfaculty Animal Ethics Committee (AEC) of the University of the Free State, Bloemfontein, South Africa (Project number: UFS-AED2016/0105).

IC affects predominantly layer breeds. Hence, layer chickens were chosen for the study (Droual *et al*. 1990). A total of 40 specific pathogen free (SPF)/unvaccinated White Leghorn chickens at 20 weeks of age, were obtained from Deltamune (Lyttleton, Centurion, Pretoria, South Africa). The chickens were transported in carton crates, with each carton containing 10 chickens. Special care was taken during transportation of the chickens such as proper air ventilation throughout the vehicle, limited exposure to the environment and disinfection of surface areas with a pressure sprayer (2% dilution Virukill®, ICA Laboratories, Stellenbosch, South Africa) to prevent any contamination or carry-over diseases.

The chickens were separated into two cohorts namely; the experimental group consisting of 30 birds and the control group consisting of 10 birds. The chickens were housed and maintained by the University of the Free State Animal Unit (University of the Free State, Bloemfontein) (GPS Coordination: 29°06'49.143"S, 26°11'2.169"E). Cages and isolators were properly cleaned and thoroughly disinfected using a pressure sprayer (2% dilution Virukill®, ICA Laboratories, Stellenbosch, South Africa) prior to the arrival of chickens. Care was taken to prevent over-crowding in cages; hence, the experimental chickens were placed into individual cages, whereas the control chickens were placed into isolators of 5 chickens each. Isolators had filtered air that supplied ventilation and air to the chickens, thus implying that the chickens were not in direct contact with the experimental chickens nor the outside environment. The experimental set-up was similar to that described by Bragg (2002). Cages were in rows of five cages in the facility. The drinking water system consisted of an adjoining water supply that travelled through each cage sourced from a header tank; within each cage a nipple drinker was provided. There was also a communal feed trough that passed each row of the cage. The birds were given animal feed (Layer mash, Senwes) and water daily *ad libitum*. Cages were cleaned on a regular basis and kept clean through disinfection of cages with a pressure sprayer (2% dilution Virukill®, ICA Laboratories, Stellenbosch, South Africa). Regular check-ups were conducted to ensure the well-being of the chickens. Additionally, a qualified laboratory animal technician was on standby for any emergencies or assistance needed. The chickens were kept under observation and were monitored over a week before the experimental phase commenced.

3.2.2. Bacterial isolate used for challenge

Av. paragallinarum SA-3 strain is indigenous to South Africa, however to ensure that a mixed culture or the wrong strain was not used which could interfere with the disease progression of the study, a reference isolate and not a field isolate was needed. Import permits and all

necessary documentation required for the shipment of the strain was conducted in accordance with the Department of Agriculture, Forestry and Fisheries (DAFF). The SA-3 (or C-3 under Blackall classification) reference isolate was obtained from Prof. Patrick Joseph Blackall at the University of Queensland, Australia. The reason for choosing or working with SA-3, was primarily because it was reported as the most virulent strain of *Av. paragallinarum* in South Africa (Bragg *et al.* 1996).

3.2.3. Microbial cultivation and identification

3.2.3.1. Cultivation of *Av. paragallinarum* serovar C-3

Av. paragallinarum is a fastidious organism and is typically grown in test media with supplements such as chicken serum and nicotinamide adenine dinucleotide (NAD^+) to simulate the environment of the host (Miflin *et al*. 1995). *Av. paragallinarum* serovar C-3 (SA-3 strain) was cultured on blood tryptose agar (BTA) plates containing cattle blood (Onderstepoort Biological Products, Pretoria) and cross-streaked with *S. epidermidis* obtained from the culture collection of Prof. Celia Hugo at the University of the Free State, South Africa. This serves as a feeder culture and nourishes the organism with a supply of NAD^+ (Page, 1962). The culture was passaged every 2 days on BTA plates to keep the bacterial culture viable. Tryptic soy broth (TSB) (Merck) supplemented with 0.2% (v/v) NAD^+ (Merck) was the growth media used to culture *Av. paragallinarum* serovar C-3 (SA-3 strain). TSB media was autoclaved at 121°C for 15-20 min and the supplements were added, only after the media was cooled down, by filter sterilisation using a syringe filter with a pore-size of 0.20 μm (GVS ABLUO).

A pre-inoculum was prepared that contained a bacterial culture of less than 24 h of age. This was then inoculated into a flask containing 250 ml of TSB supplemented with 0.2% NAD^+ (v/v)

for a further 10-14 h with continuous shaking of the flask at 120 rpm at 37°C (Labwit Scientific), grown to an optical density (OD_{600}) of 1.0. The bacterial culture (<24 h) obtained was then centrifuged at 3000 x *g* for 10 min, to obtain a pellet which was re-suspended in 10 ml of 1X phosphate-buffered saline (PBS) (pH 7.4, Merck) which was kept at 4°C overnight. A volume of 1-3 ml of the bacterial suspension was kept for bacterial identification and the rest was kept for the infection of chickens. Thus, a 48 h old bacterial culture previously re-suspended in 1X PBS, was used to infect the chickens for the first and second injections respectively, whereby for the second injection, fresh bacterial culture was prepared again and re-injected on Day 9.

3.2.3.2. Genomic DNA extraction

To identify the bacterial species that would be used for infecting the chickens, genomic DNA needed to be extracted from the 1-3 ml of the bacterial culture that was reserved, using a modified version of the phenol-chloroform DNA extraction technique by Labuschagne and Albertyn (2007). Bacterial cells were harvested by centrifugation at 3000 x *g* at 4°C for 15 min. Once a pellet was obtained, the cells were re-suspended in 500 µl of cell lysis buffer [100 mM Tris-HCl, Roche Diagnostics, Merck; 50 mM ethylenediaminetetraacetic acid (EDTA), Merck; 1% SDS, BDH Laboratory Supplies, pH 8.0]. The cell suspension was then incubated at 37°C for 20 min. After incubation, the mixture was vortexed for 30 s, followed by 30 s on ice and repeated for a period of 4 min. A volume of 275 µl of ammonium acetate (7 M; pH 7.0, Merck) was added to precipitate any proteins bound, followed by a quick vortex or slow inversion of the Eppendorf tube with contents. The mixture was incubated for a further 5 min at 65°C on a heating block (FMH instruments, Labotec), followed by 5 min on ice. A volume of 500 µl of chloroform (Sigma-Aldrich) was added to dissolve proteins in solution, followed by a quick vortex and centrifugation at 20 000 x *g* for 2 min at 4°C. The mixture forms three distinct layers: the aqueous phase (supernatant) containing DNA, the interphase containing proteins and the organic phase containing RNA and lipids (Köchl *et al*. 2005). The upper aqueous phase was

carefully transferred to a clean 1.5 ml tube without touching other phases of the mixture. An equal volume of isopropanol (Merck) was added to the supernatant to precipitate the DNA out of solution, and the mixture was centrifuged at 20 000 x *g* for 2 min at 4°C. The supernatant was discarded, and the pellet obtained was washed with 70% ethanol (ice-cold) and centrifuged at 20 000 x *g* for 2 min at 4°C, this procedure was repeated. Once, the supernatant was discarded, the pellet was either air dried or placed in the miVac centrifugal vacuum concentrator (SP Scientific, Genevac Ltd., UK). The air-dried pellet was then re-dissolved in 50 µl of TE buffer [10 mM Tris-HCl, Roche Diagnostics, Merck; 1 mM EDTA, Merck, pH 8.0], to which 2 µl of RNase (10 mg/ml, Qiagen®) was added. The solution was incubated for 1 hour at 37°C in a water bath (Labotec®), to digest any residual RNA that might be present. The NanoDrop 1000 (Thermo Scientific) was used to measure the concentration and purity of DNA extracted from bacterial samples. The extracted DNA was then stored at -20°C for long-term storage or until further use.

3.2.3.3. Identification of bacterial strain

The isolate was first phenotypically identified based on the shape and appearance of the colonies formed on BTA plates, as well as the satellitic behaviour observed when crossed-streaked with “feeder” culture *Staphylococcus* species (De Blieck, 1932; Page, 1962; Vargas and Terzolo, 2004). Molecular identification involved carrying-out a species-specific PCR also known as the HPG2-PCR described by Chen *et al*. (1996). The species-specific PCR consisted of 50 µl reaction volumes. The reaction mixture consisted of: 1µl of each of the primer set as described by Chen *et al*. (1996) (0.2 µM, Table 3.1) (Integrated DNA Technologies Inc.), 5 µl of 1x ThermoPol® reaction buffer (New England BioLabs® Inc.), 1 µl of dNTPs (100 µM, Thermo Scientific™), 0.4 µl of Taq DNA polymerase (2U/ 50 µl PCR, New England BioLabs® Inc.), 36.6 µl of nuclease-free water and 5 µl of the appropriate amount of template DNA (4-1000 ng/µl) were added. The PCR reaction was performed in the 2720

Thermal Cycler (Applied Biosystems, Life Technologies) with an initial 1 min denaturation step at 95°C. The PCR amplification and cycling conditions had 35 cycles that consisted of the following steps: denaturation at 95°C for 25 s, annealing at 55°C for 30 s, and an elongation step at 68°C for 45 s, followed by a final elongation/holding step at the end of the cycle at 68°C for 7 min. The samples were then stored at -20°C until further use.

To confirm that the bacterial species that were used during experimentation was indeed *Av. paragallinarum* serovar C-3, a 16S rDNA PCR was conducted. The 16S rDNA PCR consisted of 50 μl reaction volumes. The reaction mixture consisted of: 1μl of each of the primer sets as described by Edwards *et al*. (1989) and Mendoza-Espinoza *et al*. 2008 (0.2 μM, Table 3.1) (Integrated DNA Technologies Inc.), 5 μl of 1x ThermoPol® reaction buffer (New England BioLabs® Inc.), 1 μl of dNTPs (100 μM, Thermo Scientific™), 0.4 μl of Taq DNA polymerase (2U/ 50 μl PCR, New England BioLabs® Inc.), 36.6 μl of nuclease-free water and 5 μl of the appropriate amount of template DNA (4-1000 ng/μl) were added. The PCR reaction was performed in the G-Storm GS482 (Gene Technologies Ltd.) with an initial 5 min denaturation step at 94°C. The PCR amplification and cycling conditions had 30 cycles that consisted of the following steps: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and an elongation step at 72°C for 1 min 45 s, followed by a final elongation/holding step at the end of the cycle at 72°C for 5 min. The samples were then stored at -20°C until further use.

Both the species-specific and 16S rDNA PCR reactions consisted of a negative control (no template control) and two positive controls from *Av. paragallinarum* (SA-3 strain serovar C-3 ($C3^+$) and Modesto strain serovar C-2 ($C2^+$)). The negative control is used primarily to check for contaminants, as it contains all PCR reagents except the template. A positive control is a

sample that had previously been amplified using the same PCR reagents and conditions but is used to test for inhibitors.

Table 3.1: Oligonucleotide primers for species-specific and 16S rDNA PCR amplification of target region.

Molecular technique	Primers	Sequence	Expected amplicon size (bp)	Authors
Species-specific PCR	HPG2 forward	5'-TGA GGG TAG TCT TGC ACG CGA AT-3'	500	Chen *et al*. 1996
	HPG2 reverse	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'		
16S rDNA PCR	8F	5'-AGA GTT TGA TCA TGG CTC AG-3'	1500	Edwards *et al*. 1989; Mendoza-Espinoza *et al*. 2008
	1525R	5'-AAG GAG GTG ATC CAG CCG CA-3'		

3.2.3.4. Agarose gel electrophoresis and visualisation of correct DNA fragment size

The agarose gel was prepared with SeaKem® LE Agarose (Lonza), 1X TAE buffer [50X TAE stock: 0.1 M Tris, 0.05 M $Na_2EDTA.2H_2O$, pH 8.0, 0.1 mM glacial acetic acid; Merck] and stained with ethidium bromide (0.3 µg/µl). PCR products from the species-specific PCR and 16S rDNA PCR were resolved on a 1% (w/v) agarose gel, with each well containing 1 µl of 6X Orange Loading Dye (Thermo Scientific™) and 4 µl of PCR product. The O'GeneRuler™ DNA Ladder Mix (100-10,000 bp, Thermo Scientific™) served as a molecular marker and was run in parallel with samples on agarose gels respectively, this was done to determine the relative

sizes of the DNA fragments by comparing their electrophoresis site of mobility with that of the molecular marker. Gel electrophoresis was performed in 1X TAE buffer for 30 min at 9 V/cm. The gel and visualisation of correct DNA fragment size was conducted under ultraviolet (UV) light using the Gel Doc™ EZ Imager and Image Lab™ Software (Bio-Rad).

3.2.3.5. Sequencing of 16S rDNA PCR products

To confirm the identity of the bacterial strain and that the nucleotide sequence indeed corresponded to *Av. paragallinarum*, sequencing needed to be performed. Following the visual confirmation of the correct fragment size of the 16S rDNA PCR product, the gel band was cut out using the UV transilluminator (Spectroline®).

The agarose gel slice containing the 16S rDNA PCR product was purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. The gel slice was weighed-off and a volume of 1 μl of Membrane Binding Solution was added per 1 mg of gel slice. The mixture was vortexed thoroughly and incubated at 65°C until the excised agarose gel had been completely dissolved. An SV column was placed into a collection tube per purification to be performed. The dissolved gel mixture was transferred to the column assembly and incubated at room temperature for 1 min. The mixture was then centrifuged at 16 000 x *g* for 1 min, the flow-through was discarded and the column was placed back into the collection tube. A volume of 700 μl of Membrane Wash Solution was added to the column, to remove excess dNTPs, enzymes and primers not used during amplification and the column was centrifuged at 16 000 x *g* for 1 min. The flow-through was discarded and the column was re-inserted back to the collection tube. The wash step was repeated with a volume of 500 μl of Membrane Wash Solution and the contents centrifuged at 16 000 x *g* for 5 min. The collection tube was emptied, and the column assembly was re-centrifuged for 1 min with

the lid open to allow evaporation of any residual ethanol. The column was re-inserted to a clean 1.5 ml tube. A volume of 50 μl of nuclease-free water was then added to the column. This was followed by incubation of the column assembly at room temperature for 1 min and centrifugation at 16 000 x *g* for 1 min, to elute and recover the purified PCR product. Purified DNA samples were stored at -20°C until needed.

PCR sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™). Since the size of our PCR product is approximately 1500 bp, a final reaction quantity of 10-40 ng of the PCR product was needed. The sequencing PCR consisted of 10 μl reaction volumes. The reaction mixture consisted of: 1μl of the 8F and 1525R primers in separate reactions as described by Edwards *et al*. (1989) and Mendoza-Espinoza *et al*. 2008 (0.32 μM, Table 3.1) (Integrated DNA Technologies Inc.), 1 μl of BigDye® Terminator v3.1 Ready Reaction Mix (premix), 2 μl of 5X Sequencing Buffer, 4 μl of nuclease-free water and 2 μl of the appropriate amount of template DNA (10-40 ng) were added. The sequencing PCR was performed in the G-Storm GS482 (Gene Technologies Ltd.) with an initial 1 min denaturation step at 94°C. The sequencing PCR amplification and cycling conditions had 30 cycles that consisted of the following steps: 96°C for 10 s, 50°C for 5 s and 60°C for 4 min, followed by a final holding step at the end of the cycle at 4°C for 5 s.

The BigDye® EDTA/ethanol precipitation post-sequencing reaction clean-up protocol was carried-out according to manufacturer's instructions for a clean and consistent signal during sequencing analysis, as well as to remove unused primers and unincorporated dye-labelled terminators. The sequencing reaction volume was adjusted to 20 μl by adding a volume of 10 μl of sterile nuclease-free water to the 10 μl PCR products obtained from the sequencing reaction. The mixture was transferred to a clean Eppendorf tube. A volume of 60 μl of 100%

(v/v) ethanol (final concentration: 70.6% (v/v)) and 5 µl of 125 mM EDTA at pH 8.0 (final concentration 7.35 mM) was added to precipitate DNA, remove unincorporated BigDye® terminators and to chelate divalent ions for inactivation of DNases. The mixture was vortexed for 5 sec and left to precipitate at room temperature for 15 min. DNA was pelleted by centrifugation at 4°C at 20 000 x *g* for 15 min and the supernatant was completely aspirated. The pellet was washed with a volume of 60 µl of ice-cold 70% (v/v) ethanol. The suspension was centrifuged at 20 000 x *g* for 5 min. The supernatant was discarded, and the samples were dried using the miVac centrifugal vacuum concentrator (SP Scientific, Genevac Ltd., UK). Once dried, the samples were stored in the dark at 4°C.

Sequencing was performed with the Applied Biosystems 3130xl Genetic Analyzer at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State. Sequence analysis was performed using Geneious® 9.8.1 (Biomatters Ltd.) (Kearse *et al*. 2012). The forward and reverse sequences were aligned, and the consensus sequence was compared with known sequences in GenBank, using a nucleotide BLAST analysis (Altschul *et al*. 1990).

3.2.4. Challenge methods and clinical scoring

Handling of the chickens, by scientists in training and a qualified animal technician, was done to prevent any injury to the chickens, by holding the chicken's wings and feet securely under the arm, thereby restricting movement (Figure 3.1). Infection of the chickens was conducted via infra-orbital injection directly into the sinus cavity on the left side of the facial area as described by Boucher *et al*. (2014), as IC is an upper respiratory disease and the sinus cavity is an ideal location for infection. For the first trial, the control group was injected using 100 µl of 1X PBS (pH 7.4, Merck) only and the experimental group was injected with 100 µl of 1X

PBS (pH 7.4, Merck) solution containing a suspension of *Av. paragallinarum* serovar C-3 (SA-3 strain) bacterial cells at an optical density (OD) of 1.0. The bacterial culture used for the first injection was 48 h old (Section 3.2.3.1). After infection, the signs and symptoms were monitored, to ensure that the chickens had been exposed and infected. On Day 9, all experimental birds except control birds were re-infected with a 48 h old bacterial culture as described above (Section 3.2.3.1), the disease was allowed to progress, and signs and symptoms were monitored.

Following infection of the chickens, we allowed the signs and symptoms of IC to progress to score 1, 2 and 3, the data was recorded on a daily basis on a monitoring sheet for both groups (Appendix A). The adapted criteria and clinical manifestations of IC by Bragg (2002) was used, based on a scoring system (Matsumoto and Yamamoto, 1971). A score of 0 is used to indicate “No clinical signs”. A score of 1 indicates “Mild clinical signs with nasal discharge with or without mild facial oedema”. A score of 2 indicates “Moderate signs with nasal discharge on both left and right sides and slight facial oedema” and a score of 3 describes “Severe signs with severe bilateral oedema with or without haemorrhage and conjunctivitis”. Blood was collected from 5-7 chickens after every 2-4 days (48-96 h), based on the disease score and symptoms observed in chickens. For each scoring system, chickens were unbiasedly/randomly selected from the total population of experimental subjects, where 2-4 chickens showing the same score were sacrificed for histopathology, for observation of lymphoid organs and the rest of the chickens were left for the disease to progress further. This process was repeated until a score of 3 was observed. The total number of eggs produced, and the total number of eggs laid per bird per day from each group were carefully recorded.

Figure 3.1: Handling of a bird by qualified personnel. Utmost care was taken to ensure that the well-being of the birds was considered and that the chickens were treated as humanely as possible during injection procedures, as well as during bleeding times without causing stress, pain, suffering, anxiety or physical injury.

3.2.5. Blood collection and processing

Blood samples (2-3 ml) were collected via the branchial vein (wing vein) into commercial 4 ml EDTA coated SGVac PET Blood Collection Tubes (The Scientific Group). An additional volume of EDTA (0.5 M, Merck) of 800 μl- 1 ml was supplemented to the EDTA tubes, to prevent quick coagulation of the blood collected, as chicken blood coagulates quickly due to high levels of calcium present (Lewis and Stoddart, 1971; Mikaelsson, 1991; Preda *et al*. 2014). Following blood collection, the EDTA tubes were quickly inverted 3-4 times to thoroughly mix the blood and EDTA together to prevent coagulation and to minimise clots. The collected blood was then separated into three parts into separate 1 ml EDTA coated SGVac PET Blood Collection Tubes (The Scientific Group) with a maximum volume of approximately 1 ml per tube for flow cytometry, blood microscopy and to obtain plasma to perform direct enzyme-linked immunosorbent assays (ELISAs). The blood samples were

processed within 6-8 h. To obtain plasma, one of the tubes containing whole blood, was centrifuged at 3000 x *g* for 15 min, to separate the blood components from plasma. The plasma obtained was then stored at -80°C for long-term storage and until further use.

3.2.6. Avian full blood counts, differential blood counts and microscopy

EDTA tubes containing a volume of 1 ml of the blood sample collected from each experimental subject, were sent to the National Health Laboratory Service (NHLS, Universitas, Bloemfontein, South Africa) and to Pathcare Vetlab (Westdene, Bloemfontein, South Africa), respectively for preliminary complete/full blood counts (CBC/FBC) and white blood cell differential counts (WBC Diff). The NHLS mainly conducts diagnostic testing for human patients and the Pathcare Vetlab caters for animal diagnostics. These laboratories were chosen as we did not have the equipment, expertise or facilities in our laboratory to carry out these tests. We also needed to conduct blood tests and the smears as soon as blood was drawn according to the experimental design, whereby a laboratory in close proximity was ideal to prevent a delay in the blood processing which could lead to erroneous and inaccurate results. Moreover, these laboratories are South African National Accreditation System (SANAS) accredited laboratories, whereby calibration, quality assurance and controls and standard operating procedures (SOPs) are followed and used on a routine basis.

Blood smears of the avian specimens at the NHLS (Universitas, Bloemfontein, South Africa) were performed according to the SOP described by the NHLS below. The EDTA tube containing whole blood was gently inverted 8 times. An applicator stick was used to check for blood clots. Using a glass capillary tube or applicator stick, a small drop of blood was placed on the glass slide at 1 cm from one end in the midline. A spreader was placed on a flat surface

and held down firmly with the thumb and forefinger at opposite ends. The spreader was then held at an angle of 45°, and was pulled backwards until it touched the drop of blood, which allowed the blood to spread across the breadth of the slide on both ends. The spreader was dragged forward smoothly and rapidly, maintaining contact between the two slides. The blood film formed is 3-4 cm long, evenly spread with no ragged tails. Moreover, the thickness should be such as to allow the erythrocytes to be separated from each other in the last quarter of the film. The film was left to air-dry before staining the slide. Staining was conducted using the slide stainer Hematek® 3000 System (Siemens Healthineers) with Hematek Modified Wright's stain and Hematek Wright-Giemsa stain to stain peripheral blood and identify different blood cells. Visualisation of the slides was conducted with the Eclipse 50i microscope, DS-Fi1 digital microscope camera and NIS-Elements F 4.00.06 Build 786 microscope imaging software (Nikon), whereby images were taken at a 100X magnification.

Complete/Full blood counts (CBC/FBC) and white blood cell differential counts (WBC Diff) were conducted as described by the SOPs followed by the NHLS (Universitas, Bloemfontein, South Africa) below. The ADVIA® 2120i (Siemans Healthineers) was used according to manufacturer's instructions and operator's manual, which is an automated flow-cytometry based analyser that provides complete blood counts, complete differential count, reticulocyte absolute and reticulocyte percentage in a single run with a single blood sample provided (Harris *et al*. 2005a; Harris *et al*. 2005b). There are several processes involved in the analyser such as cytochemical reactions, cytometric measurements of specific cell properties and algorithms converting data into familiar results for cell classification, cell count, cell size and haemoglobin. Firstly, a volume of approximately 175 µl of the EDTA anti-coagulated whole blood was aspirated by an automatic/manual sampler, this is followed by the anti-coagulated whole blood sample being passed and separated into different reaction chambers comprising of different reagents and reaction settings (HGB, BASO, RBC/PLT, PEROX and RETIC). For the haemoglobin concentration (HGB), the haemoglobin method was used which is a

modification of the manual cyanmethaemoglobin method developed by the International Committee of Standardization in Haematology (ICSH) (ICSH Standard EP6/2, 1977; ICSH Standard EP6/3, 1977; Dacie and Lewis, 2006). For a white blood cell (WBC) count, ADVIA® 2120i BASO reagent containing acid and surfactant was mixed with the whole blood (Cremins and Orlik, 1996). ADVIA® 2120i BASO reagent causes haemolysis of RBCs, whereby the white blood cells were able to be analysed using 2 angle laser light scatter signals (Cremins and Orlik, 1996). For red blood cell (RBC) and platelet (PLT) counts, whole blood was mixed with ADVIA® 2120i RBC/PLT reagent. The red blood cells (RBCs) were lightly fixed with glutaraldehyde present in the reagent to preserve their isovolumetrically spherical shape (Kim and Ornstein, 1983). Both RBCs and PLTs were detected from a single optical cytometer/detector with 2 gain settings, whereby platelet signals are amplified considerably more than the RBCs. A coincidence correction was performed to each of the counts so that the accurate counts are conducted over a wide range of each cell type. For sizing RBCs and platelets, simultaneous measurement of laser light scattered at 2 different angular intervals was performed, which eliminates the effect of variation in the cellular haemoglobin concentration when determining cell volume (Kerker, 1969; Groner and Tycko, 1980). Moreover, red cell indices like the mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), were derived from mathematical calculations based on the RBC count, the total haemoglobin and the mean corpuscular volume (MCV) determination (Wintrobe, 1932). The haematocrit (HCT) values were calculated from the RBC count and the MCV (Wintrobe, 1932). The red cell distribution width (RDW) and haemoglobin distribution width (HDW) values were calculated from the cell-by-cell measurement of the cell volume and haemoglobin concentration (Bessman, 1981). The CH represents the cell haemoglobin content histogram from internal complexity of intact RBC by side scattered of a low angle laser light and cell haemoglobin concentration mean (CHCM) is calculated as the mean of the RBC haemoglobin concentration histogram (Mohandas *et al*. 1986).

The WBC Diff was performed based on the peroxidase method (Cremins *et al*. 1990). Leukocytes possess the enzyme peroxidase that is active in cells. In the presence of hydrogen peroxide and an appropriate electron acceptor chromogen, peroxidase develops a darkly coloured precipitate in the cells. The peroxidase cytochemical reaction involves two steps and is conducted simultaneously with the CBC. In the first step, ADVIA® 2120i PEROX 1 reagent is mixed with whole blood, whereby surfactants and thermal stress cause lysis of the red blood cells. Moreover, ADVIA® 2120i PEROX 1 reagent contains formaldehyde that fixes the WBCs. In the second step, ADVIA® 2120i PEROX 2 reagent and ADVIA 120 PEROX 3 reagent were added to the peroxidase reaction chamber. ADVIA 120 PEROX 2 reagent contains 4-chloro-1-naphthol and ADVIA 120 PEROX 3 reagent contains hydrogen peroxide, which stain the sites of peroxidase activity in the granules of neutrophils, eosinophils, and monocytes. There are no granules with peroxidase enzyme activity in lymphocytes, basophils, and large unstained cells. The cell suspension from the Perox reaction chamber passes through the flowcell, whereby the two fluids flow independently and without mixing, with the ADVIA 120 PEROX SHEATH stream encasing the sample stream. The absorbance and the forward light-scattering measurements of each blood cell are captured, and the optical signals are converted to electrical pulses by photodiodes. The raw data is processed, and the information is presented in two histograms namely, Perox Y histogram containing the forward-scattering data (cell size) and Perox X histogram containing the absorption data (peroxidase staining). These two histograms are merged to form the Perox cytogram, from which cells are identified and counted. The basophil/lobularity method was performed and was based on Cremins and Orlik (1996), whereby basophils are unaffected by lysis using a combination of acid and surfactant. This method provides rapid recognition of basophils, accurate basophil counts and a measure of cellular lobularity. During this reaction, EDTA anticoagulated whole blood was mixed with ADVIA 120 BASO reagent in the BASO reaction chamber, where RBCs and leukocytes were lysed or have their cytoplasm stripped from them, except basophils. Thereafter, a two-angle laser light scattering detection method was performed to analyse the cell suspension using a laser diode. Finally, the leukocytes could be classified into three

categories: basophils, mononuclear (MN) cells, and polymorphonuclear (PMN) cells respectively.

The reticulocyte cell count was conducted using a nucleic acid dye (oxazine 750). EDTA anticoagulated whole-blood sample was mixed with the ADVIA 120 autoRETIC reagent. The ADVIA 120 autoRETIC reagent preserves the isovolumetrically sphere shape of erythroid cells and stains cellular RNA. This was followed by a low-angle laser light scatter, and high-angle laser light scatter, whereby the absorption characteristics of all cells were counted and measured. The absorption data was used to categorize each cell as a reticulocyte or mature red blood cell depending on its RNA content. The sizing of reticulocytes was conducted via the simultaneous measurement of laser light scattered at two (2) different angular intervals, whereby variation in cellular haemoglobin concentration on the determination of the MCVr parameter is eliminated. The last parameter analyzed was the CHr, which was the mean of cellular haemoglobin content (CH) histogram for the reticulocyte population (Brugnara *et al*. 1994; Fishbane *et al*. 1997). The CHr is an indicator of functional iron deficiency in human patients (Brugnara *et al*. 1994; Fishbane *et al*. 1997). Blood smears, CBC and WBC Diff were also requested at Pathcare Vetlab (Westdene, Bloemfontein, South Africa). Unfortunately, protocols and standard operating procedures could not be obtained from Pathcare Vetlab (Westdene, Bloemfontein, South Africa).

3.2.7. Staining and flow cytometry analysis

Fresh chicken blood was obtained that were anticoagulated from venesection as described above (Section 3.2.6) and processed no later than 8 h. Flow cytometric analysis was performed using the following mouse anti-chicken antibodies: CD4-FITC (2-35 clone, MCA2164F) and CD8-RPE (11-39 clone, MCA2166PE) (Bio-Rad). A modified version of the

method described by Macey *et al*. (1999) was used. A volume of 5 µl of each antibody was added to 25 µl of whole blood in a round bottom, snap cap tube (Greiner Bio-One International). The labelled cells were incubated for 10 min in the dark at room temperature. A volume of 2 ml of working strength 1X FACS Lysing Solution (Becton Dickinson, Immunocytometry Systems, San Jose, CA) was added and the cells were lysed for 10 min. The cells were centrifuged for 3 min at 2000 x *g* and the supernatant discarded. The cells were then washed once with 2 ml of 1X PBS (pH 7.4), vortexed and centrifuged for 3 min at 2000 x *g* and the supernatant discarded. Finally, the cells were resuspended in 1 ml of 1X PBS (pH 7.4) and vortexed thoroughly. Sample analysis were performed on the BD FACSCanto II and BD FACSDiva 8.0.1 software (Becton Dickinson), whereby calibration and set-up were performed on a routine basis using BD FACS 7-color setup beads (Becton Dickinson).

To separate RBCs from lymphocytes in whole blood for flow cytometry: either lysis of RBCs was conducted, whereby 2 ml of 1X FACS Lysing Solution (Becton Dickinson, Immunocytometry Systems, San Jose, CA) was added to 25 µl of whole blood and the cells were lysed for 10 min, or Ficoll-Paque™ PLUS (GE Healthcare) according to the manufacturer's instructions was used. A volume of 4 ml of Ficoll-Paque™ PLUS (GE Healthcare) was layered to a diluted blood sample consisting of 2 ml of whole blood and 2 ml of balanced salt solution. The blood sample was centrifuged at 400 x g for 30-40 min at room temperature. Following centrifugation, the formation of different blood cell types was obtained. The upper layer consisting of plasma was drawn off, followed by the extraction of the lymphocyte/ peripheral blood mononuclear cell (PBMC) layer using aseptic techniques.

3.2.8. Direct Enzyme-linked immunosorbent assay (ELISA)

A volume of 500 ml of broth, containing strain SA3 (C-3) was cultivated as described above (Section 3.2.3.1) and centrifuged at 3000 x *g* for 10 min to obtain a pellet. The pellet was re-suspended in 10 ml of PBS. The bacterial culture was standardised to an OD of 1.0 at a wavelength of 600 nm and kept in the fridge at 4°C until further use, for a period of not more than 1 week due to gradual degradation of the antigens with time. Enzyme-linked immunosorbent assays (ELISAs) were performed in 96-well Costar® high-binding polystyrene plates (Corning Inc.). The method described had been previously optimised. The Costar® plates were coated with 100 µl of antigen with the bacterial cells prepared. The tests were conducted in quadruplicate and the controls were performed in duplicate. Two wells per batch tested had only PBS added (no antigen controls) and 2 wells per batch tested were coated with antigen with no plasma added (no plasma controls). The coated plates were incubated overnight at 37°C or for 1 h, before the removal of excess liquid by shaking-off excess liquid by decanting in one motion or through complete aspiration of the well contents. The wells were washed 6 times with 200 µl of PBS-Tween® 20 0.1% (v/v) (Sigma-Aldrich), to remove any unbound antigen and to wash the cells. Blocking was performed using 200 µl of 3% (w/v) Bovine Serum Albumin Fraction V (BSA) (Roche) in PBS-Tween® 20 0.1% (v/v) (Sigma-Aldrich) to prevent non-specific binding of antibodies and antigens to the microtiter well (Xiao, 2012), followed by incubation for 1 h at 37°C. Following blocking, another washing step was performed, whereby wells were washed 6 times with 200 µl of PBS-Tween® 20 0.1% (v/v) (Sigma-Aldrich) and shaking-off excess liquid by decanting in one motion. Chicken plasma was diluted at 1:100 with PBS-Tween® 20 0.1% (v/v) (Sigma-Aldrich) and a volume of 100 µl was added to appropriate wells except the control wells, this was followed by incubation at room temperature for 1 h with gentle mixing on the Mini BioMixer (Benchmark Scientific). The wells were washed 8 times with 200 µl of PBS-Tween® 20 0.1% (v/v), followed by shaking-off excess liquid by decanting in one motion. Anti-chicken IgY (IgG) (whole molecule) peroxidase antibody produced in rabbit (Sigma-Aldrich) was diluted at 1:10 000 with PBS-Tween® 20 0.1% (v/v) and a volume of 50 µl was added to appropriate wells and incubated for 1 h at room

temperature with gentle mixing on the Mini BioMixer. A final washing step was conducted, whereby the wells were washed thoroughly 8 times with 200 µl of PBS-Tween® 20 0.1% (v/v), followed by 5 min intervals on the Mini BioMixer and shaking-off excess liquid by decanting in one motion after every washing step. A volume of 50 µl of 3,3',5,5'- Tetramethylbenzidine (TMB) (Roche) was added as a substrate to appropriate wells and incubated at room temperature for 15 min, until a gradient colour change was observed, this final incubation time should not exceed 30 min. A volume of 50 µl of 2N (1M) sulphuric acid (H_2SO_4) (Merck) was added to all the wells to stop the reaction. The absorbance values of the wells were measured at a wavelength of 450 nm using the ELx800™ plate reader with Gen5™ software (BioTek). Statistical analysis was performed to determine the statistical significance of the data using Microsoft Excel. The mean and standard deviation of the data were calculated, and a $p<0.05$ was found to be statistically significant, from which a graph was plotted.

This was a preliminary screening for antibodies present in plasma of control and IC infected chickens, hence only 16 samples were used for the ELISA from Day 0, 3, 7 and 14. There were 4 samples for Day 0, 4 samples for Day 3, 6 samples for Day 7 (consisting of 4 post-infected (PI) and 2 control chickens) and 2 samples (consisting of 1 control and 1 re-infected (RI) chicken) for Day 14. There were no known positive or negative control serums available for the ELISA. However, no antigen and no plasma controls were included for quality control.

3.3. Results and Discussion

3.3.1. Microbial cultivation and identification

3.3.1.1. Identification of bacterial strain

Tiny dewdrop colonies and satellitic behaviour adjacent the *S. epidermidis* "feeder" cultures on the BTA plates were observed, typical of *Av. paragallinarum* (Figure 3.2) as described in literature (Blackall *et al*. 1997). As bacterial cultures were grown in 2-5 flasks, only flasks with an OD_{600} closest to 1.0 was selected for the first (3IC: $OD_{600\ (flask)}$= 0.938; $OD_{600\ (1X\ PBS)}$= 0.834) and second injection (5IC: $OD_{600\ (flask)}$= 0.804; $OD_{600\ (1X\ PBS)}$= 0.972). Turbidity in cultures was checked for any bacterial contamination.

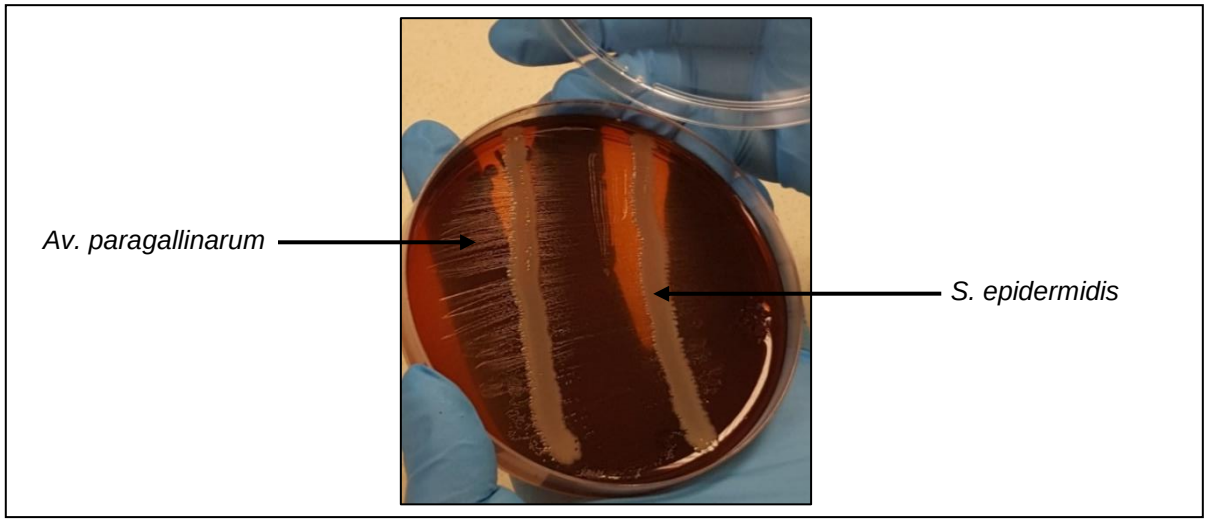

Figure 3.2: BTA plate with *Av. paragallinarum*. Tiny dewdrop colonies are observed near the cross-streaked "feeder" cultures thus displaying satellitic behaviour.

A species-specific PCR was performed, using the DNA extracted from bacterial cultures from the flasks containing broth (TSB) (Merck) supplemented with 0.2% (v/v) NAD^+ (Merck) and the bacterial suspension of C-3 in 1X PBS (pH 7.4, Merck) which was kept at 4°C, for the first and second injection respectively. This was done as a precautionary measure from the flask stage to the injection stage, to ensure that the bacterial cultures used were sterile and contamination-free, as in the past we had experienced an outbreak of spore-forming *Bacillus* species. The species-specific PCR/ HPG2-PCR was performed with the DNA extracted from the reference

isolate SA-3 (C-3) cultivated and an expected amplicon size of 500 bp was obtained (Figure 3.3).

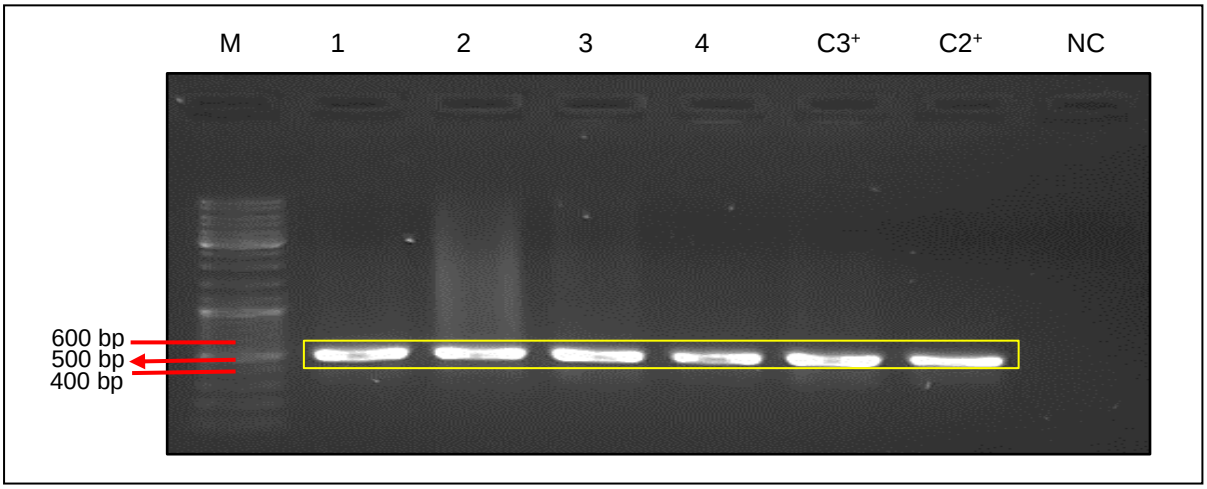

Figure 3.3: HPG2-PCR for the SA-3 (C-3) reference isolate cultivated for experimental procedure, whereby amplification was observed for all samples, with an expected band size of 500 bp. Lane M- molecular marker O'GeneRuler™ DNA Ladder; lane 1: SA-3 strain in 1X PBS used for first injection; lane 2: SA-3 strain in 1X PBS used for second injection; lane 3: SA-3 strain in supplemented TSB for first injection; SA-3 strain in supplemented TSB for second injection; lane $C3^+$: SA-3 strain serovar C-3 positive control; lane $C2^+$: Modesto strain serovar C-2 positive control; lane NC: negative control.

3.3.1.2. Sequencing of 16S rDNA PCR products

The identity of the *Av. paragallinarum* reference isolate was confirmed using 16S rDNA amplification based on the 8F and 1525R region, which is a highly conserved evolutionary region amongst bacterial species used to study bacterial phylogeny and taxonomy. PCR products of an amplicon size of 1500 bp were obtained for all samples (Figure 3.4).

Following amplification of samples with 16S rDNA PCR, Sanger sequencing was conducted. The sequences obtained, were analysed using Geneious® 9.8.1 (Biomatters Ltd.) (Kearse *et*

al. 2012). Both the forward and reverse sequences of each sample were aligned, and the consensus sequence was compared with known sequences in GenBank, using a nucleotide BLAST (Basic Local Alignment Search Tool) analysis program (Altschul *et al*. 1990). A sequence identity of >97%, indicated DNA-DNA relatedness/homology and as such we were able to identify the bacterial strain to a species level, which matched the identity of our bacterial strain of interest. The sequencing results for the samples were recorded in Table 3.2 Using the results from both the species-specific PCR and 16S rDNA sequencing results from the National Centre for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/), we were successful in identifying the bacterial strain used for the study, which coincides with the *Av. paragallinarum* SA-3 strain, which was a crucial step for the entire experimental study.

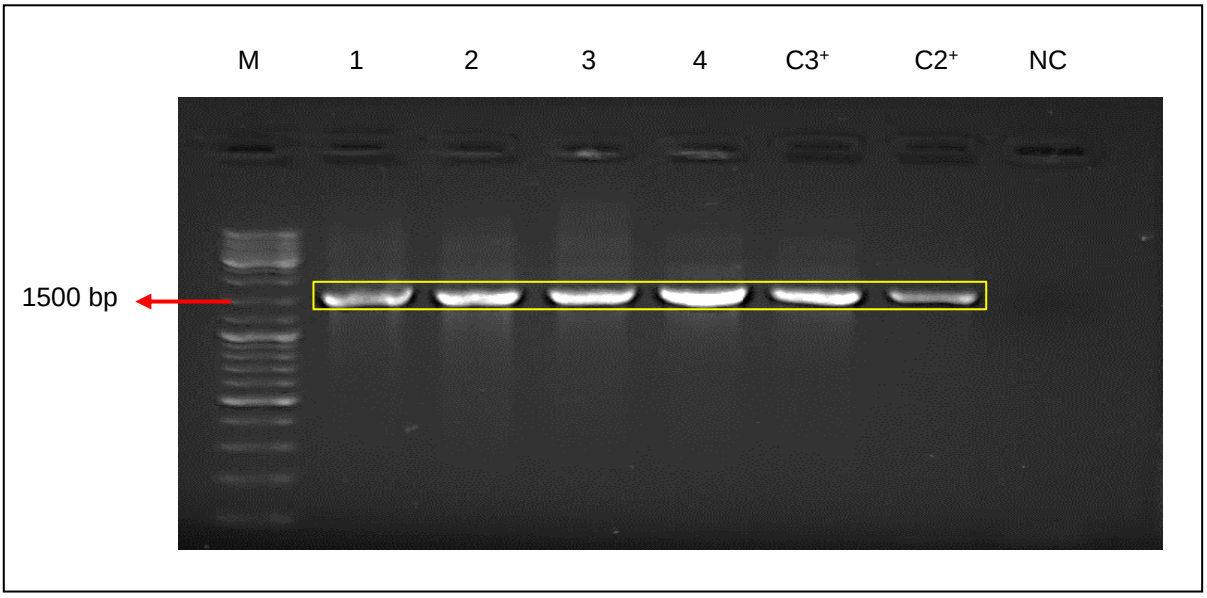

Figure 3.4: 16S rDNA PCR for the SA-3 (C-3) reference isolate cultivated for experimental procedure, whereby amplification was observed for all samples, with an expected band size of 1500 bp. Lane M-molecular marker O'GeneRuler™ DNA Ladder; lane 1: SA-3 strain in 1X PBS used for first injection; lane 2: SA-3 strain in 1X PBS used for second injection; lane 3: SA-3 strain in supplemented TSB for first injection; SA-3 strain in supplemented TSB for second injection; lane $C3^+$: SA-3 strain serovar C-3 positive control; lane $C2^+$: Modesto strain serovar C-2 positive control; lane NC: negative control.

Table 3.2: Nucleotide BLAST results for all 16S rDNA PCR products with species identification, GenBank® accession numbers, query length, query coverage, E-value and high sequence identities.

Sample	Isolate/Species	Accession number	Query length (bp)	Query coverage	E-value	Identity
3IC	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	653	100%	0.0	100%
5IC	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	529	100%	0.0	100%
1SD	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	697	100%	0.0	100%
2SD	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	664	100%	0.0	100%
C3^{+}	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	644	100%	0.0	100%
C2^{+}	*Haemophilus paragallinarum* strain Modesto 16S ribosomal RNA gene, partial sequence	AY498870.1	546	100%	0.0	100%

3.3.2. Challenge methods and clinical scoring

Once all chickens were injected accordingly, they were closely monitored for a period of 21 days for any visible IC related symptoms, the daily mean score was recorded during disease progression for both the experimental and control groups (Figure 3.5). After 24 h, very slight swelling at the site of injection was observed, which is a typical inflammatory response due to the protective barrier of the skin being damaged by a foreign object (needle) or introduction of the pathogen. However, following 24-48 h, we still could not observe any IC related symptoms and the slight swelling subsided around the site of infra-orbital injection near the sinus cavity. Only on 72 h, did we see the mean disease score change to 0.1 (Figure 3.5), which indicated that 3 in 30 chickens showed symptoms which was still a very low number. Furthermore, on 72 h the chickens were on a score 1 with facial swelling and slight nasal discharge on the left side of the nasal area, diarrhoea and lethargy (Figure 3.6). On Day 4-7, the mean disease score increased until a mean disease score of 0.5 was reached on Day 7, which could be because of the innate immune response being at its peak, with supplementary symptoms in addition to the symptoms mentioned on Day 3 such as sneezing as well as swollen wattles and combs (Figure 3.6). In addition, on Day 7 only 1 chicken reached a score 2, with nasal discharge on both sides of the nasal region. However, after Day 7, there was a gradual drop in the mean disease score, indicating that the chickens were recovering. Unfortunately, for a duration of 1 week a mean disease score of 1 could not be reached in the experimental group, the chickens started to recover, and the disease did not progress any further. Hence, we had to reinject the experimental chickens on Day 9, to “boost” the disease progression and to see whether this strategy had any effect. The scoring for the control group during the first week (Day 0-7), was stable at a constant mean disease score 0, which indicated that no clinical signs and symptoms of IC were observed nor did any cross-contamination occur between the two cohorts.

For the second injection, after 24 h (Day 10), most chickens in the experimental group gave facial/inter-mandibular swelling, but no nasal discharge and only experimental chickens

labelled E10 and E17 (E10 and E17: E stands for experimental chicken and the number 10 and 17 indicate the cage number) showed prominent nasal discharge. E10 was at a score 1 and had very slight nasal discharge on the left side only with mild facial swelling (Figure 3.7). E17 was on a score 2 and had nasal discharge on both sides with mild facial swelling (Figure 3.7). The disease did gradually progress after the second injection and on 72 h (Day 12), the mean disease score was 0.9 (Figure 3.5), showing that the second injection did stimulate the adaptive phase of the immune response. However, after 72 h the chickens started to recover from the infection and inter-mandibular swelling was diminished. The scoring for the control group during the duration of the study after the second injection (Day 9-21), was once again stable at a mean disease score 0, no clinical signs and symptoms of IC were observed, and this was a good indication that no cross-contamination between the two cohorts occurred. The disease did not progress for most of the experimental group and a score 3 was not reached once again with a second injection, which was quite puzzling given that the SA-3 strain (serovar C-3) is a virulent, indigenous strain to South Africa.

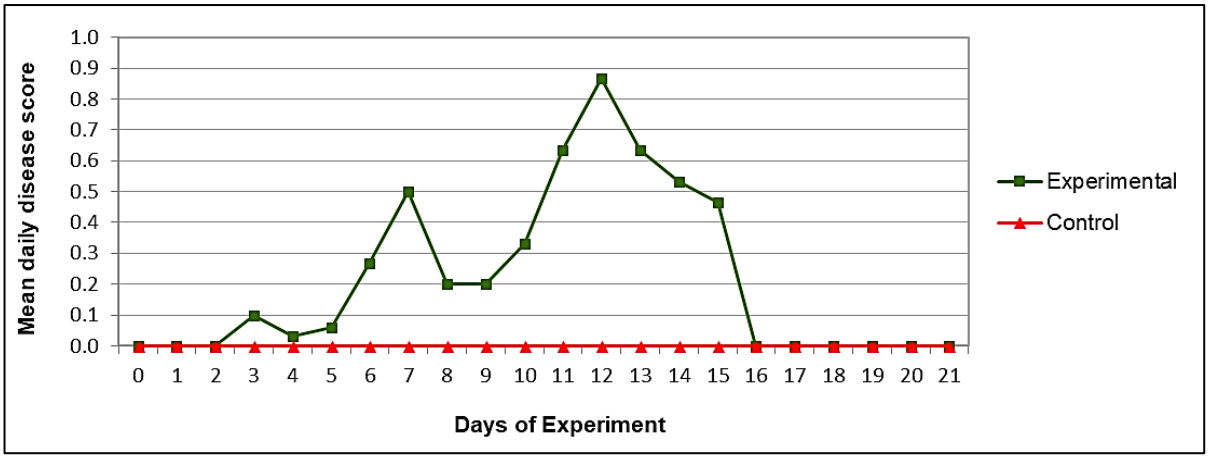

Figure 3.5: The disease profile showing the daily mean disease score of the control (red) and experimental (green) cohorts. The trend of the control group was consistent at a score 0 throughout the duration of 21 days, as they were not challenged or exposed to C-3 isolate. The experimental group had a different trend whereby an initial innate immune response was observed on Day 7 with the first injection and an adaptive immune response due to a secondary exposure occurred on Day 12. In between Day 7 and Day 12, there is a gradual drop in the daily mean disease score, which was mainly due to recovering chickens given that IC does not cause mortality.

According to literature, IC symptoms usually develop in chickens between 24-72 h after exposure to the bacteria and symptoms last over a period of days (Blackall and Soriano, 2008). However, the lack of clinical signs and symptoms indicated either there was a good initial immune response or there was an issue with the bacterial inoculum with the first and second injections respectively. Our results were contrary to the study conducted by Bragg *et al*. (2004), whereby in a chicken population of untreated and unvaccinated birds challenged with C-3 isolate yielded a rapid disease progression whereby on Day 5 based on a slightly different scoring reached the status of “moderately affected” similar to a score 2 in this study. As the disease progressed by Day 9 the chickens had “severely affected” clinical signs and symptoms equivalent to score 3. This indicated that there were issues in the current study and that immediate troubleshooting needed to be performed to resolve the problem.

Figure 3.6: Clinical signs and symptoms related to IC after the first injection. (A) Male presented with swollen wattles and combs. (B) Diarrhoea frequently occurring in sick birds. (C) Nasal discharge observed on left side of facial region and slight facial swelling can be seen. (D) IC leads to lethargy in birds.

We suspected that various reasons could have led to the experiment not being successful. Firstly, the culturing conditions needed improvement. Although, the SA-3 strain bacterial culture was grown to an OD_{600} of 1.0, we did not establish whether the culture had enough viable bacterial cells to cause infection. In other words we did not ensure that in the 100 µl that was injected into the experimental chickens, there were 10^8 colony forming units (CFU) (Byarugaba *et al*. 2007), implying that there might not have been enough viable bacterial cells to breech the infection threshold that would cause and prolong disease progression. Moreover, after we re-suspended the bacterial pellet in 1X PBS (pH 7.4, Merck), we kept the suspension at 4°C, which could have shocked the bacterial cells and caused the bacterial cells to either die or become dormant. *Av. paragallinarum,* being a poultry pathogen, thrives best at a temperature similar or close to the body temperature of a chicken, which is 41.8°C (Bolzani, 1979).

Secondly, the sinuses of the chickens at 20 weeks of age, were not yet well developed, being the site of infection, which is why we could not observe immediate signs and symptoms of IC and the disease could not spread or progress. Lastly, it could be that the chickens were not SPF and had been exposed to *Av paragallinarum* or other pathogens prior to using them in the experiment, although they were obtained from a reliable source. Thus, implying previous exposure to other organisms indicates that the immune system was already established to pathogenic infection and any other exposure would cause a fast-immune response to occur, which is why the birds recovered faster, as opposed to if they were SPF whereby an infection would take longer to progress and heal. If the chickens, were indeed not SPF or were previously exposed to other micro-organisms, chicken plasma before infection would contain antibodies. As such, we decided to conduct a direct ELISA screening as described (Section 3.2.8) and the results were recorded (Section 3.3.5).

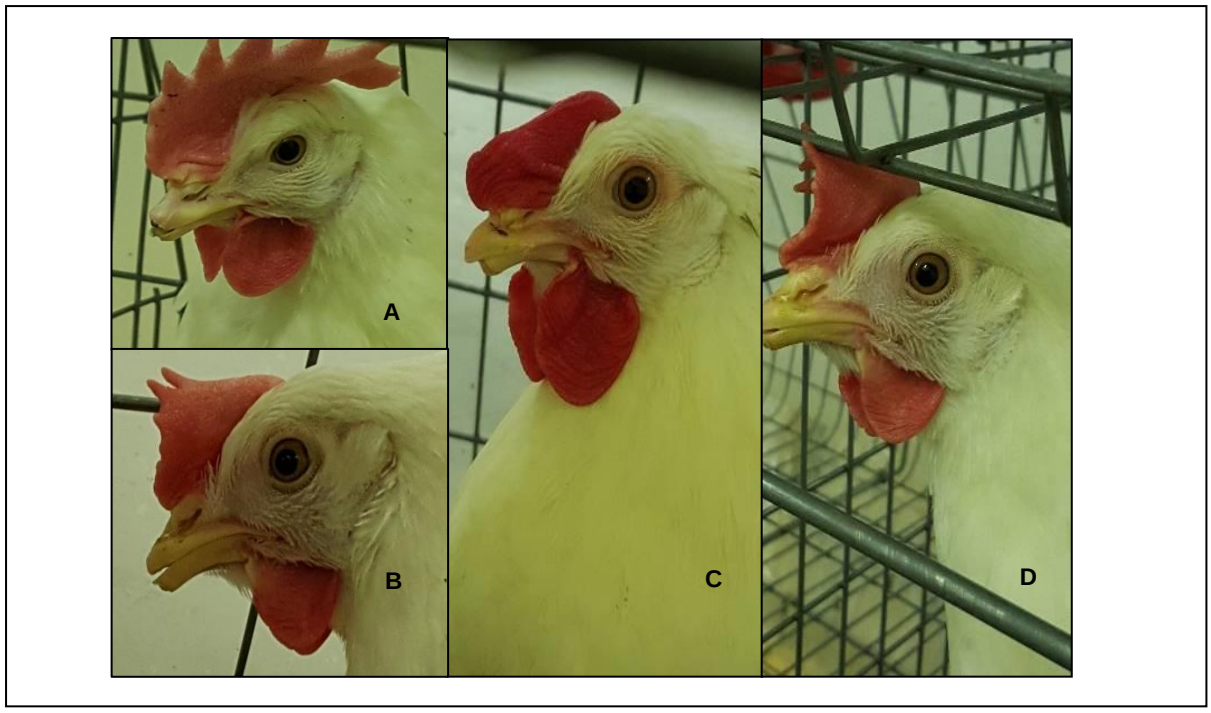

Figure 3.7: Clinical signs and symptoms related to IC after the second injection. (A) and (B) Birds with inter-mandibular swelling, with no nasal discharge. (C) E10 with a score 1 showing very slight nasal discharge on the left side only with mild facial swelling and swollen wattles and comb. (D) E17 with a score 2 presented with nasal discharge on both sides of the nasal cavity with mild facial swelling.

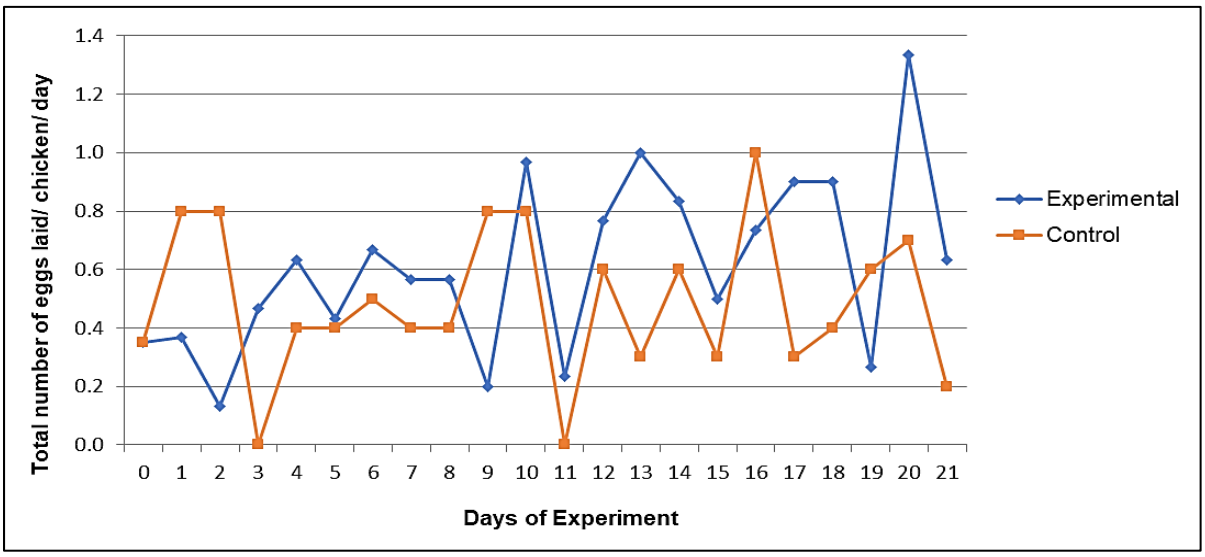

Figure 3.8: Egg production indicated as the total number of eggs laid/chicken/day in the experimental and control groups. Both cohorts showed highly variable egg-laying trends, making it difficult to deduce any significant weekly or daily egg-laying patterns.

The total number of eggs laid/ chicken/ day was recorded for both experimental and control groups. Both experimental and control groups had highly variable egg-laying trends (Figure 3.8). As such, we could not observe any stable or consistent trend with regards to the control group or experimental group infected with IC. Moreover, we did not observe any significant decline in egg production.

3.3.3. Avian full blood counts, differential blood counts and microscopy

We had a major setback during blood collection with chicken blood, it coagulated very rapidly once we collected the blood, which was ideal for serum, but not whole blood collection. During collection of blood samples of 2-3 ml collected via the branchial vein into commercial 4 ml EDTA coated SGVac PET Blood Collection Tubes (The Scientific Group). Supplemented was 800 µl- 1 ml of additional EDTA (0.5 M, Merck) into the EDTA coated SGVac PET Blood Collection Tubes followed by quick inversions of the tubes 3-4 times. This was conducted to prevent quick coagulation of the blood collected, as chicken blood coagulates quickly due to high levels of calcium present in chicken blood. EDTA chelates free calcium ions present in the blood, hindering coagulation. Previously, we had also tried acid citrate dextrose (ACD), heparin and Alsever's solution as anticoagulants for supplementing into the EDTA tubes however, none of these solutions worked as effectively as EDTA. The high levels of calcium were due to the rich layer feed provided for egg-laying chickens intended for egg development. Calcium plays a role in the blood coagulation cascade, however high levels of calcium could lead to conformational changes in proteins such as factor V and factor VIII, which has an increased effect on pro-coagulation, leading to clotting occurring at a faster rate (Michaelsson, 1991). Chickens could not be bled daily, and blood was drawn every 2-4 days. This was due to hematoma development, which is a collection of clotted blood outside of a blood vessel as a result of a collapsed vein at the sight of bleeding. This takes from a few days up to a week

to heal. Thus, bleeding was done on one side of the wing and the other wing was left available for bleeding upon healing. If both wings were bled from no blood was drawn until one of the wings had healed.

We had obtained the CBC and WBC Diff results from NHLS (Universitas, Bloemfontein, South Africa) and Pathcare Vetlab (Westdene, Bloemfontein, South Africa) respectively (Annexure B). However, we learned that the analysers from both NHLS and Vetlab were not ideal for avian blood but only human blood. The reason was that avian blood have nucleated RBCs and human RBCs do not. Thus, when human whole blood is used, the analysers can distinguish RBCs from WBCs as well as other immune cells. Regrettably, with the avian whole blood that was provided, the analysers mistook the nucleated RBCs with WBCs leading to erroneous CBC and WBC Diff counts. Hence, on the result statements there was an escalated number of WBCs in comparison to RBCs, which was not the case.

As such, the results were inaccurate. Pathcare Vetlab (Westdene, Bloemfontein, South Africa), sent blood smears to be evaluated manually by a veterinary pathologist, since the analyser counts were incorrect. However, this could still not provide any insight as to what the true counts were. Moreover, after querying the results for clarity, the veterinary pathologist at Vetlab indicated that they do not offer avian CBC and WBC Diff, even though these options were available on the request form (Appendix B) with prior enquiry and consultation about whether these haematology tests could be performed before commencing the project. Furthermore, blood smears prepared at Vetlab (Westdene, Bloemfontein, South Africa) for our study, were discarded without any notification and we could not obtain results. The NHLS (Universitas, Bloemfontein, South Africa) told us that they could only use human samples and they also could not help us further, however we did obtain blood smears (Figure 3.9) which showed us the cell morphology in whole blood, which could provide insight during IC infection.

After a week, we had to cease and abandon the CBC and WBC Diff from the NHLS and Vetlab, for the pilot study due to high cost with inaccurate results being obtained.

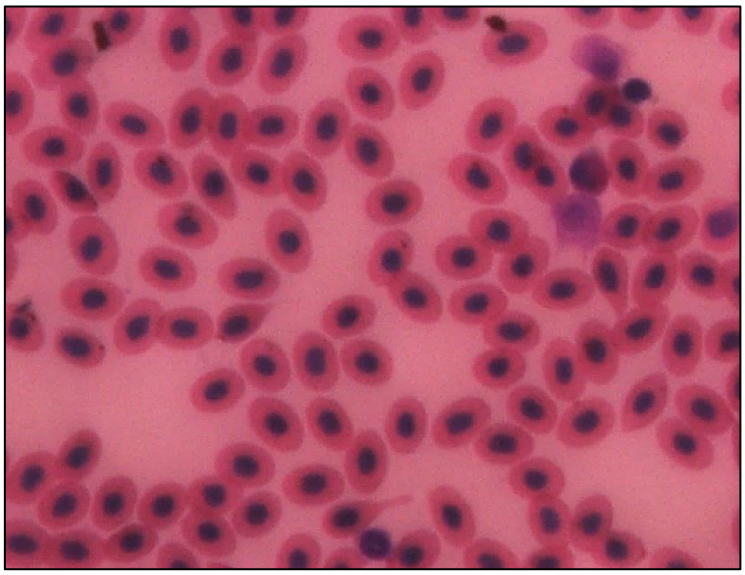

Figure 3.9: Avian blood smear from a Day 0 chicken. Peripheral blood film showing nucleated RBCs and some leukocytes (100X Magnification).

According to literature, the correct way for counting erythrocytes of chickens is by using the erythrocyte Unopette 5850 system (Becton Dickinson) with Neubauer-ruled haemocytometer or using Natt and Herrick's method. To count leukocytes of chickens the eosinophil Unopette brand 5877 system (Becton Dickinson) with Neubauer-ruled haemocytometer or the direct leukocyte count using Natt and Herrick's method can be performed (Natt and Herrick, 1952; Campbell, 1995). These manual techniques are the best alternatives to the automated analysers. However, the disadvantage is that the process is laborious, time-consuming and has a high cost, especially with a large sample size. For the study, the RBC count was not important for us, however the WBC count was. Hence, the combination of blood smears with flow cytometry results were looked at (Chapter 4), as an alternative to obtain different cells counts (excluding RBCs), as an indication of disease progression.

3.3.4. Flow cytometry and antibodies

Cell population profiles of the T-cell population mainly the CD4 T cells (T helper cells) and CD8 T cells (cytotoxic T cell) using flow cytometry were studied and generated. Flow cytometry profiles were generated when fluorescently labelled cells passing through the interrogation point interact with a laser, whereby light scattering is produced that could be measured and correlated with relative cell size and structures inside the cell. The measurements were termed forward angle scatter (FSC) which is based on the size of the cell and side angle scatter (SSC) which is based on the granularity/complexity of the cell.

During the runs on the BD FACSCanto II (Becton Dickinson) while simultaneously performing the analysis on the BD FACSDiva 8.0.1 software (Becton Dickinson), it was difficult to locate and gate the precise location of the entire leukocyte population and lymphocyte population. This was due to the nucleated RBCs that overlapped with the leukocyte and lymphocyte population, thus making it difficult to gate the leukocyte population, which was also where the lymphocyte population was, containing both CD4 and CD8 cells (Figure 3.10). Hence, the cell counts of the cell populations of interest could not obtain, as gating could not be performed.

To resolve this problem, the RBCs were separated from the lymphocytes either by lysing the RBCs or using the Ficoll-Paque™ PLUS (GE Healthcare) according to the manufacturer's instructions (Figure 3.10), however this was to no avail the same plots as in Figure 3.10 were obtained. Ficoll-Paque™ PLUS (GE Healthcare) separates whole blood into different components present in blood and plasma where platelets are also found and the lymphocyte/ peripheral blood mononuclear cell (PBMC) layer, Ficoll-Paque™ PLUS layer, granulocyte layer and erythrocyte layer (Figure 3.11). Finally, an anti-CD45 pan-leukocyte marker was used upon repeat of experiments (Chapter 4), which would be the best solution to tackle the

avian nucleated RBC dilemma, CD45 would only be present on leukocytes (granulocytes and lymphocytes) but not erythrocytes (RBCs). Moreover, avian nucleated RBCs are much smaller in size in comparison to leukocytes, hence when the anti-CD45 pan-leukocyte marker would be used they would be easily separated and distinguished from the leukocytes, making it easy for gating purposes.

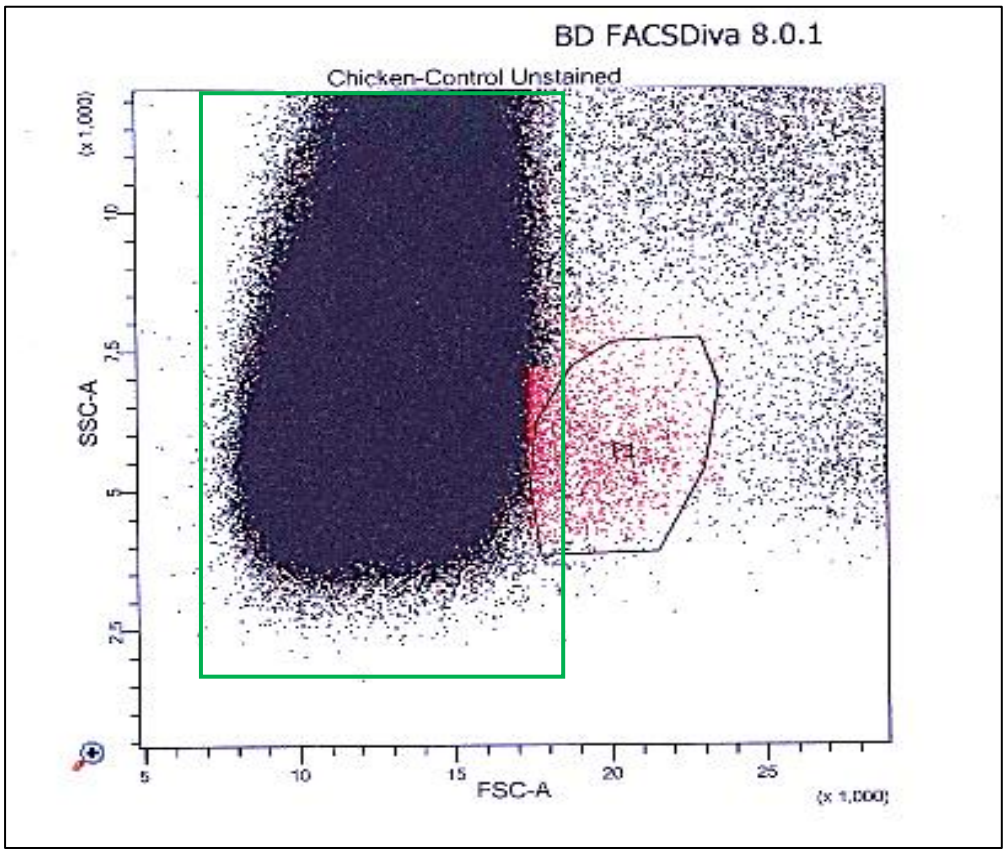

Figure 3.10: Flow cytometry profile of one of the control chickens showing the forward scatter (FSC-A) and side scatter (SSC-A) plot. The sequestered region outlined in green shows the RBC population, this area overlaps with the gated region P1 (shown in red) which is the approximate location of the lymphocyte population. It was very difficult to gate and perform further analysis, since we could not differentiate between the different cells and gate the precise location of the lymphocytes where the overlapping occurred.

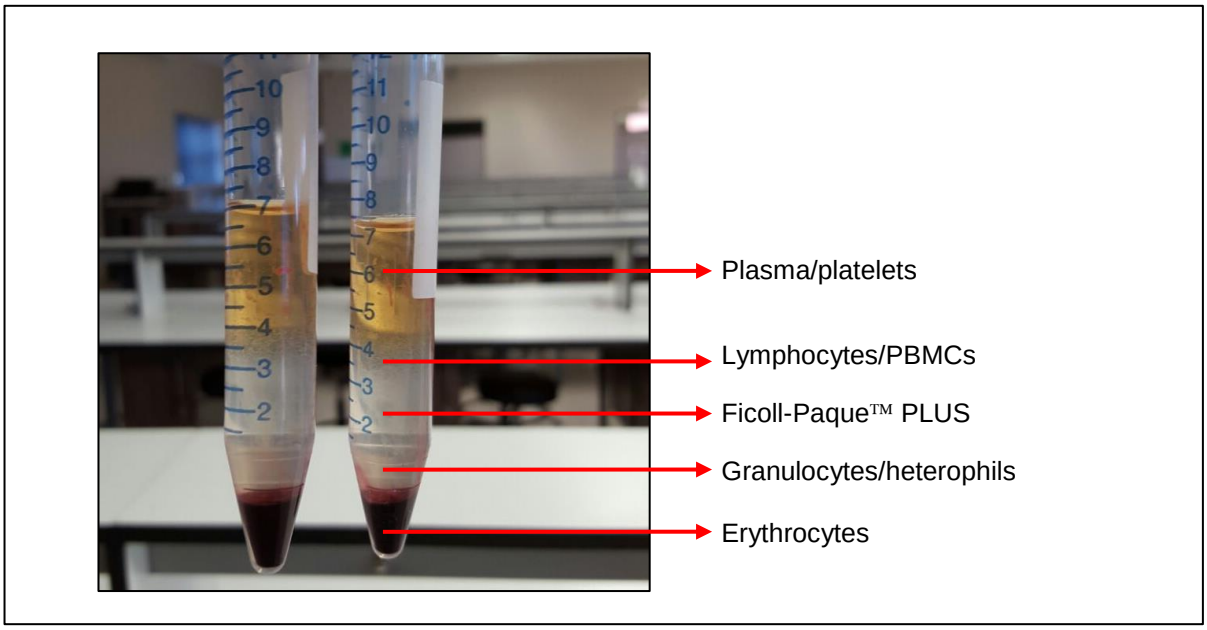

Figure 3.11: Whole blood separated by Ficoll-Paque™ PLUS (GE Healthcare) into different components after using a swing-bucket centrifuge at 400 x *g*. Despite using this technique, we still could not gate the lymphocyte population and there was still overlapping occurring.

3.3.5. Direct enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was carried out ($p<0.05$) and the results are shown in (Figure 3.12 and Figure 3.13). The results showed that although the chickens were supposedly SPF, they had a well-established immune system, since they were able to produce antibodies even before exposure to SA-3. Day 0 chickens were not yet infected, yet we could still see high absorbance values in chickens 1, 2, 3 and 5. Although, the antibody production was different for each chicken that was randomly bled, we could see that post-infection (PI) with the first injection the antibody titres against the anti-chicken IgY (IgG) (whole molecule) peroxidase antibody produced in rabbit (Sigma-Aldrich), did increase for some of the chickens PI such as E12 and E19 on Day 3 and E28 on Day 7.

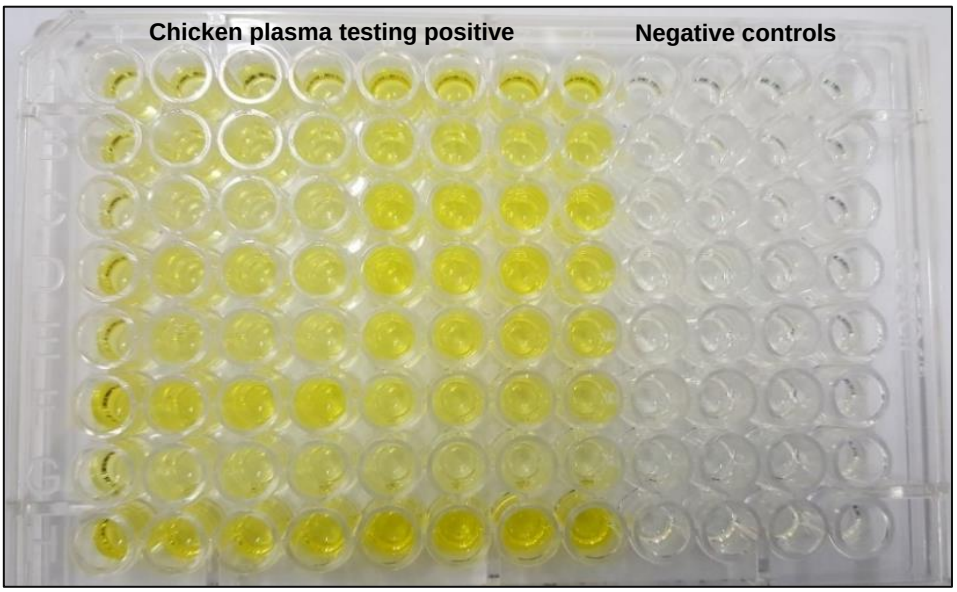

Figure 3.12: Screening for antibodies using chicken plasma from both experimental and control chickens. A coloured yellow product indicates presence of antibodies in the chicken plasma sample used, whereas the clear wells are the negative controls showing that no antibodies are present.

It was surprising that despite there being no cross-contamination or IC related symptoms in the control group we still obtained antibody production on Day 7 for the chickens control 1 and 2, and a very high antibody titre for bird control 3 on Day 14. One of the chickens (E7) that was re-infected (RI) with the second injection, also showed a high antibody titre on Day 14. From the results, it was apparent that the chickens might have been previously exposed to microorganisms. The only improvements in our ELISA assay would be to include plasma positive and negative controls to validate results, and to perform the experiment for each sample for each score obtained in duplicate, triplicate or quadruplicate.

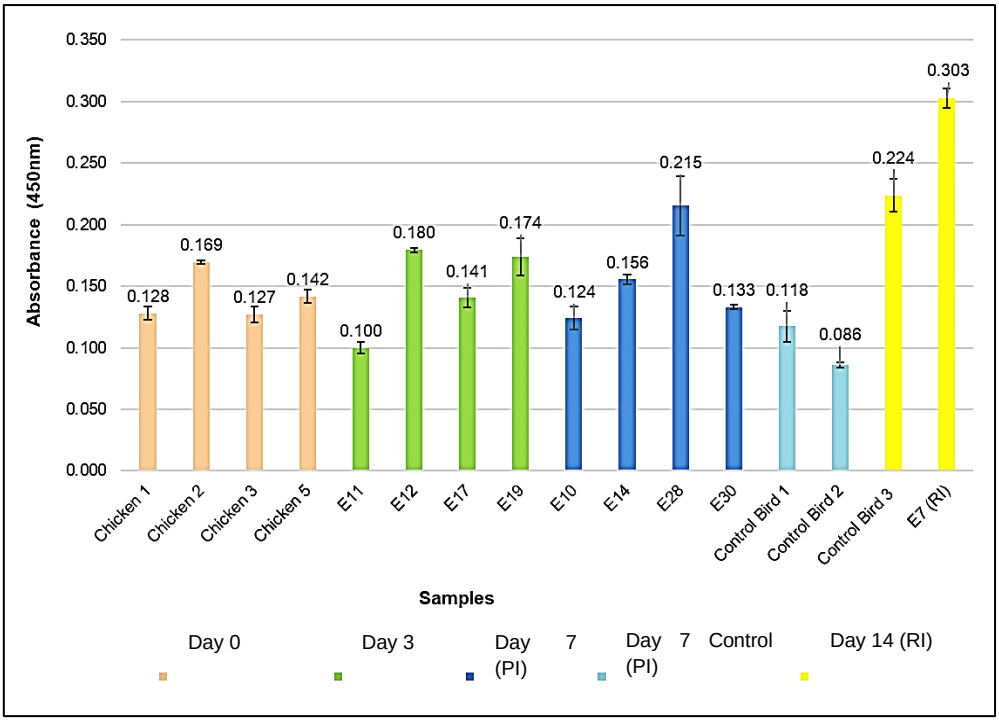

Figure 3.13: Graphical representation and statistical analysis of ELISA assay conducted on chicken plasma samples from the experiment ($p<0.05$). Day 0 (beige), chickens were not infected, yet antibody titres were still observed. On Day 3 (light green), high antibody titres were observed for chickens E12 and E19, as symptoms for these chickens aggravated with SA-3. On Day 7 (blue), chicken E28 at a score 1 showed high antibody titres. Antibody production could be seen in Day 7 (light blue) and 14 (yellow) control chickens as well. Overall, we saw an increase in antibody production in experimental chickens as they were infected with SA-3. On Day 14 (yellow), with the re-infected chicken E7 we saw a high antibody titre due to a second injection with SA-3.

3.4. Conclusion

The pilot study was not as successful as planned. This was because disease progression with regards to IC infection was not observed in chickens infected with SA-3 (serovar C-3). As such, no IC related signs and symptoms of injected chickens were seen. On the contrary, the injection of serovar C-3 in the chickens, yielded a fast and effective (good) immune response leading to quick recovery, which we suspect was due to prior immunity established in the chickens before the study, from previous exposure, whereby memory cells had been formed. Therefore, the clinical signs and symptoms that was anticipated and expected of an IC

infection was not fully observed during the course of the disease progression, as the chickens recovered swiftly following infection with serovar C-3, which is the reason why a score 3 was not attained. These hypotheses, were proven, based on existing antibodies present in chicken blood of Day 0 chickens (which were supposedly SPF), of Day 7 chickens control 1 and 2, as well as a very high antibody titre for the chicken control 3 on Day 14 (Section 3.3.5). It should be emphasized that the control chickens were not infected or exposed to serovar C-3.

However, despite the negative outcome of the study, we were still able to find valuable insight that could further improve the outcome of the project for future studies (Chapter 4 and 5). We tried to troubleshoot all the shortcomings of the methodology and have made a few core findings. Firstly, there was a problem with the culturing of the bacterial cells, whereby we must ensure that the final bacterial culture has 10^8 CFU to ensure that there are enough bacteria to cross the disease threshold to cause disease. Moreover, the temperature should be kept at 37°C before injecting the chickens in the next study, to simulate the environment of the chicken host to ensure that *Av. paragallinarum* SA-3 remains viable. Moreover, we were not certain if the SA-3 serovar C-3 strain might have lost its virulence during multiple passaging in our laboratory, hence we should cultivate a fresh bacterial culture from a new vial of the freeze-dried bacterium that had not been passaged. Furthermore, in younger birds, the sinuses are not well developed, which could have contributed to the poor immune response obtained in the study, hence in the next experiment (Chapter 4 and 5) older SPF birds would be used, as their sinuses would be more developed. Lastly, from our ELISA results it was apparent that the alleged SPF chickens were previously exposed to microorganisms or even vaccinated, as their immune system was already well established observed from the antibody titres on Day 0 and for control chickens on Day 7 and 14 respectively, leading to no IC related symptoms. However, due to the lack of positive and negative control serums, it is difficult to have any confidence in the serology and the interpretation of the detected absorbances, hence the ELISA's should be repeated in future studies with known positive and negative control serums.

From these core findings, we hoped that the next trial would be successful. The main reason for conducting a pilot study is chiefly to determine initial data for the primary outcome measure, as well as to perform a sample size calculation and to further improve the techniques for the next round of experimentation. However, in our case, we had to establish the necessary techniques and keep track of the experimental time frame for the main experiment *in vivo* study to be conducted for the project.

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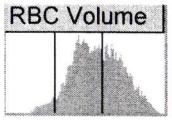
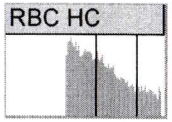
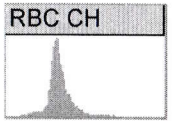
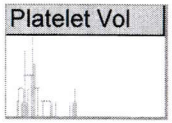
ANNEXURE B

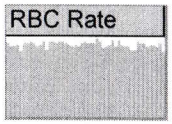
NHLS Universitas ADVIA 2120i B	
SID	HU00398543
Patient Name	CHICKEN 1 , NO NAM
Aspiration Date/Time	03-May-17 12:21:42 P
Sample Type	PATIENT
Rack & Position	0 0
Instrument Number	IR35111412
Sample Selectivity	CBC\DIFF
Age & Sex	U
FOR LABORATORY USE ONLY	

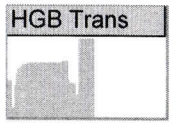


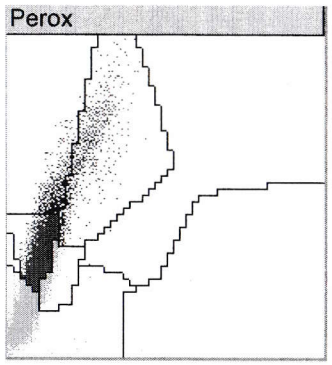


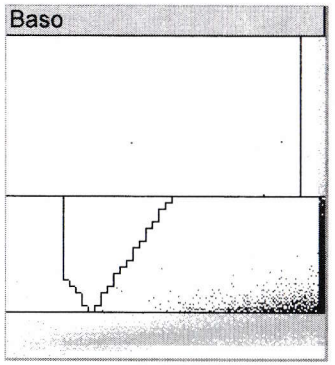


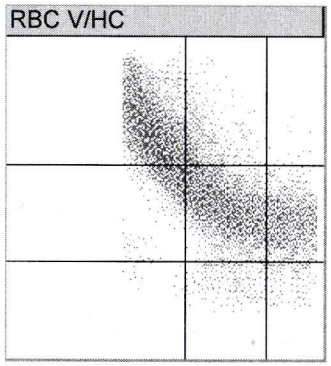


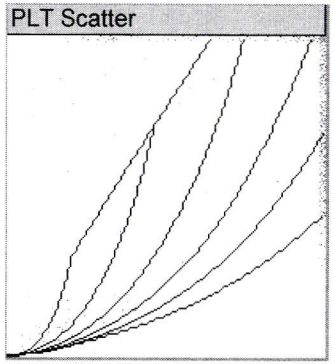


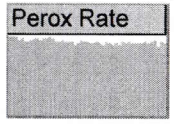












CBC					
WBC	H	510.72	*	x10⁹ cells/L	
RBC	L	2.19	*	x10¹² cells/L	
HGB	L	11.6	*	g/dL	
Cellular HGB		7.6		g/dL	
HCT	L	0.251	*	L/L	
MCV	H	114.7	*	fL	
MCH	H	53.0	*	pg	
MCHC	H	46.2	*	g/dL	
CHCM	L	30.5	*	g/dL	
CH		32.9	*	pg	
RDW	H	27.1	*	%	
HDW	H	8.24	*	g/dL	
PLT	L	5	*	x10⁹ cells/L	
MPV		9.1	*	fL	
PDW	L	16.5	*	%	
PCT	L	0.00	*	%	

Additional Routine Parameters		
%Blast Suspect	0.0	
%Hyper	13.4	
%Hypo	45.2	
%Macro	41.4	
%Micro	3.7	
RBC Fragments	0.00	x10¹² cells/L
RBC Ghosts	0.01	x10¹² cells/L
Neut X		
Neut Y		
MNx	8.5	
MNy	6.0	
%MN	0.0	
%PMN	99.5	
Cellular HGB	7.6	g/dL

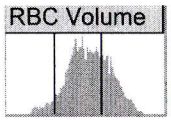
Routine WBC Differential							
		%			#		
WBC				H	510.72	*	x10⁹ cells/L
Neut	L	0.0	*	L	0.02	*	x10⁹ cells/L
Lymph	H	95.3	*	H	486.49	*	x10⁹ cells/L
Mono	L	2.7	*	H	13.73	*	x10⁹ cells/L
Eos		0.0	*		0.00	*	x10⁹ cells/L
Baso		0.0	*		0.04	*	x10⁹ cells/L
LUC		2.0	*	H	10.44	*	x10⁹ cells/L
NRBC			*			*	x10⁹ cells/L
LI					2.22	*	
MPXI				L	-56.1		
WBCP					296.03	*	x10⁹ cells/L

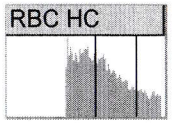
Uncorrected WBC and Differential							
		%			#		
WBCu				H	510.72	*	x10⁹ cells/L
NEUTu	L	0.0	*	L	0.02	*	x10⁹ cells/L
LYMPHu	H	95.3	*	H	486.49	*	x10⁹ cells/L
MONOu	L	2.7	*	H	13.73	*	x10⁹ cells/L
EOSu		0.0	*		0.00	*	x10⁹ cells/L
BASOu		0.0	*		0.04	*	x10⁹ cells/L
LUCu		2.0	*	H	10.44	*	x10⁹ cells/L
%MNu		0.0					%
%PMNu		99.5					%

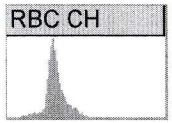
Morphology Flags	
MICRO	+
MACRO	+++
HYPO	+++
HYPER	+++
ANISO	+++
HC VAR	+++
LARGE PLT	+
PLT CLUMPS	+

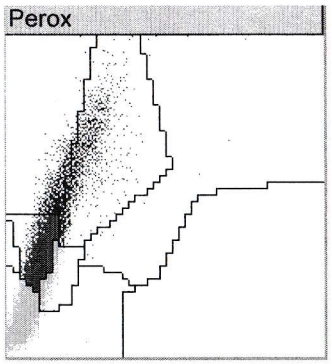
Sample/System Flags	
B-NO	#BASO %Baso WBC WBCB WBCu %BASOu #BASOu
B-NV	LI
CHCMCE	CHCM HCT HDW HGB MCH MCHC MCV RBC RDW CH CHDW
WBC-CE	WBC WBCu
PX-NO	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PXIFR	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PX-NV	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PLT-CL	MPV PCT PDW PLT WBCP
PLT-NO	MPV PCT PDW PLT
NRCELL	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC
NR-LPD	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC

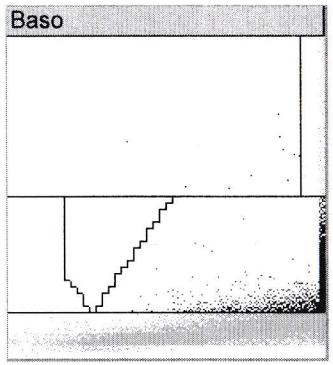
NHLS Universitas ADVIA 2120i B	
SID	HU00398547
Patient Name	CHICKEN 2 , NO NAM
Aspiration Date/Time	03-May-17 12:22:16 P
Sample Type	PATIENT
Rack & Position	0 0
Instrument Number	IR35111412
Sample Selectivity	CBC\DIFF
Age & Sex	U
FOR LABORATORY USE ONLY	

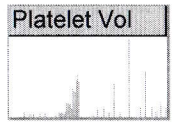


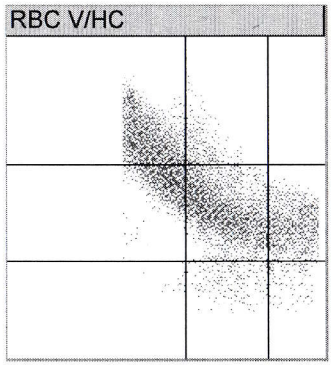


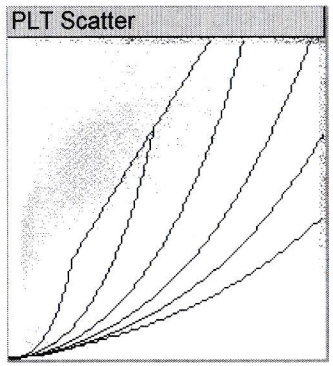


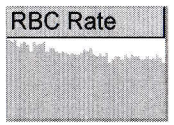


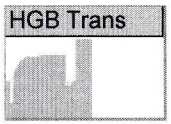


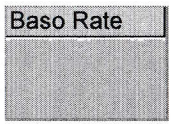












CBC					
WBC	H	257.41	*	x10⁹ cells/L	
RBC	L	1.81	*	x10¹² cells/L	
HGB	L	7.7	*	g/dL	
Cellular HGB		5.8		g/dL	
HCT	L	0.191	*	L/L	
MCV	H	105.4	*	fL	
MCH	H	42.7	*	pg	
MCHC	H	40.5	*	g/dL	
CHCM	L	30.5	*	g/dL	
CH		30.6	*	pg	
RDW	H	25.0	*	%	
HDW	H	8.06	*	g/dL	
PLT	L	24	*	x10⁹ cells/L	
MPV	H	35.2	*	fL	
PDW		36.6	*	%	
PCT	L	0.09	*	%	

Additional Routine Parameters		
%Blast Suspect	0.0	
%Hyper	12.6	
%Hypo	44.4	
%Macro	28.5	
%Micro	4.3	
RBC Fragments	0.00	x10¹² cells/L
RBC Ghosts	0.26	x10¹² cells/L
Neut X		
Neut Y		
MNx	3.7	
MNy	6.0	
%MN	0.0	
%PMN	99.6	
Cellular HGB	5.8	g/dL

Routine WBC Differential							
		%			#		
WBC				H	257.41	*	x10⁹ cells/L
Neut	L	0.1	*	L	0.14	*	x10⁹ cells/L
Lymph	H	84.9	*	H	218.59	*	x10⁹ cells/L
Mono	H	9.9	*	H	25.59	*	x10⁹ cells/L
Eos		0.0	*		0.00	*	x10⁹ cells/L
Baso		0.0	*		0.10	*	x10⁹ cells/L
LUC	H	5.0	*	H	12.99	*	x10⁹ cells/L
NRBC			*			*	x10⁹ cells/L
LI					2.56	*	
MPXI				L	-34.2		
WBCP					299.74	*	x10⁹ cells/L

Uncorrected WBC and Differential							
		%			#		
WBCu				H	257.41	*	x10⁹ cells/L
NEUTu	L	0.1	*	L	0.14	*	x10⁹ cells/L
LYMPHu	H	84.9	*	H	218.59	*	x10⁹ cells/L
MONOu	H	9.9	*	H	25.59	*	x10⁹ cells/L
EOSu		0.0	*		0.00	*	x10⁹ cells/L
BASOu		0.0	*		0.10	*	x10⁹ cells/L
LUCu	H	5.0	*	H	12.99	*	x10⁹ cells/L
%MNu		0.0					%
%PMNu		99.6					%

Morphology Flags	
MICRO	+
MACRO	+++
HYPO	+++
HYPER	+++
ANISO	+++
HC VAR	+++
ATYPS	+
LARGE PLT	+++
PLT CLUMPS	+
RBC Ghosts	+

Sample/System Flags	
B-NO	#BASO %Baso WBC WBCB WBCu %BASOu #BASOu
B-NV	LI
CHCMCE	CHCM HCT HDW HGB MCH MCHC MCV RBC RDW CH CHDW
RBCIFR	CHCM HCT HDW MCH MCHC MCV MPV PCT PDW PLT RBC RDW CH CHDW
WBC-CE	WBC WBCu
PX-NV	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PLT-CL	MPV PCT PDW PLT WBCP
PLT-NO	MPV PCT PDW PLT
NRCELL	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC
NR-LPD	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC

NHLS Universitas ADVIA 2120i B	
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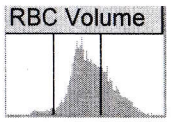
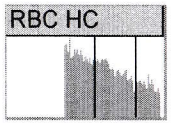
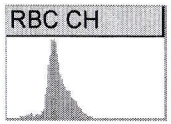
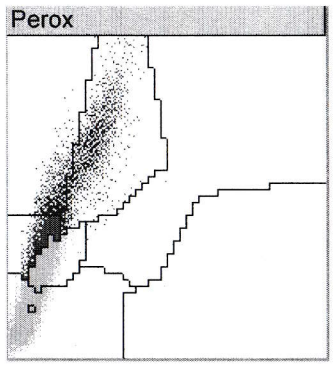
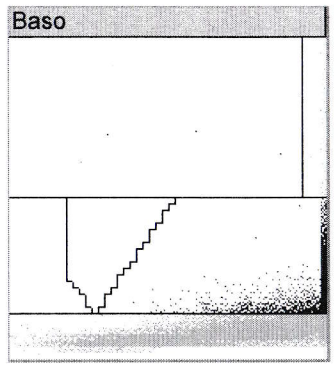
Patient Name	CHICKEN 3 , NO NAM
Aspiration Date/Time	03-May-17 12:22:52 P
Sample Type	PATIENT
Rack & Position	0 0
Instrument Number	IR35111412
Sample Selectivity	CBC\DIFF
Age & Sex	U
FOR LABORATORY USE ONLY	

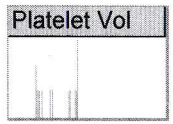


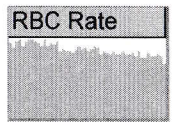


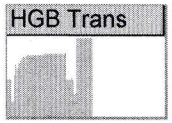


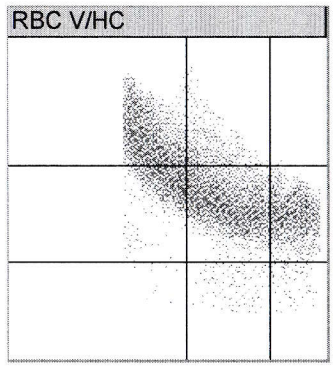


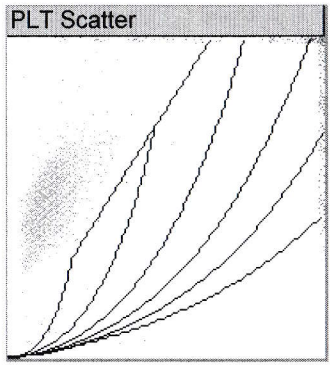


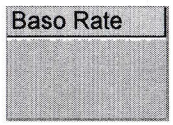


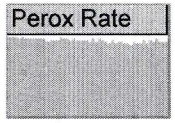












CBC					
WBC	H	222.19	*	x10⁹ cells/L	
RBC	L	1.73	*	x10¹² cells/L	
HGB	L	7.9	*	g/dL	
Cellular HGB		5.9		g/dL	
HCT	L	0.189	*	L/L	
MCV	H	109.1	*	fL	
MCH	H	45.4	*	pg	
MCHC	H	41.6	*	g/dL	
CHCM	L	31.4	*	g/dL	
CH		32.8	*	pg	
RDW	H	22.6	*	%	
HDW	H	8.48	*	g/dL	
PLT	L	1	*	x10⁹ cells/L	
MPV	H	19.2	*	fL	
PDW		34.7	*	%	
PCT	L	0.00	*	%	

Additional Routine Parameters		
%Blast Suspect	0.0	
%Hyper	16.4	
%Hypo	40.3	
%Macro	29.6	
%Micro	2.3	
RBC Fragments	0.00	x10¹² cells/L
RBC Ghosts	0.24	x10¹² cells/L
Neut X		
Neut Y		
MNx	4.0	
MNy	6.0	
%MN	0.0	
%PMN	99.8	
Cellular HGB	5.9	g/dL

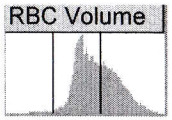
Routine WBC Differential		%			#		
WBC				H	222.19	*	x10⁹ cells/L
Neut	L	0.0	*	L	0.09	*	x10⁹ cells/L
Lymph	H	84.3	*	H	187.34	*	x10⁹ cells/L
Mono	H	9.5	*	H	21.18	*	x10⁹ cells/L
Eos		0.0	*		0.00	*	x10⁹ cells/L
Baso		0.0	*		0.03	*	x10⁹ cells/L
LUC	H	6.1	*	H	13.54	*	x10⁹ cells/L
NRBC			*			*	x10⁹ cells/L
LI					2.46	*	
MPXI				L	-47.1		
WBCP					74.39	*	x10⁹ cells/L

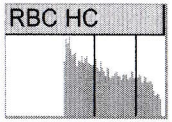
Uncorrected WBC and Differential		%			#		
WBCu				H	222.19	*	x10⁹ cells/L
NEUTu	L	0.0	*	L	0.09	*	x10⁹ cells/L
LYMPHu	H	84.3	*	H	187.34	*	x10⁹ cells/L
MONOu	H	9.5	*	H	21.18	*	x10⁹ cells/L
EOSu		0.0	*		0.00	*	x10⁹ cells/L
BASOu		0.0	*		0.03	*	x10⁹ cells/L
LUCu	H	6.1	*	H	13.54	*	x10⁹ cells/L
%MNu		0.0					%
%PMNu		99.8					%

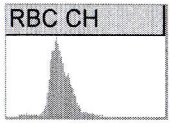
Morphology Flags	
MACRO	+++
HYPO	+++
HYPER	+++
ANISO	+++
HC VAR	+++
ATYPS	+
LARGE PLT	+++
PLT CLUMPS	+
RBC Ghosts	+

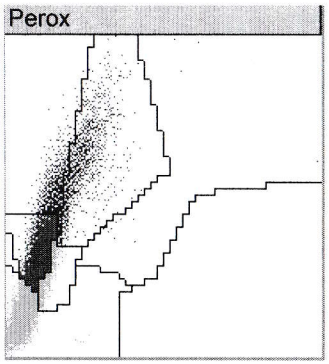
Sample/System Flags	
B-NO	#BASO %Baso WBC WBCB WBCu %BASOu #BASOu
B-NV	LI
CHCMCE	CHCM HCT HDW HGB MCH MCHC MCV RBC RDW CH CHDW
RBCIFR	CHCM HCT HDW MCH MCHC MCV MPV PCT PDW PLT RBC RDW CH CHDW
WBC-CE	WBC WBCu
PX-NO	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PXIFR	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PX-NV	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PLT-CL	MPV PCT PDW PLT WBCP
PLT-NO	MPV PCT PDW PLT
NRCELL	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC
NR-LPD	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC

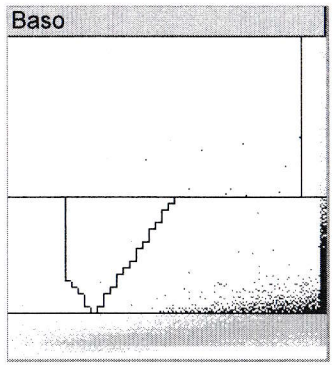
NHLS Universitas ADVIA 2120i B	
SID	HU00398551
Patient Name	CHICKEN 4 , NO NAM
Aspiration Date/Time	03-May-17 12:23:24 P
Sample Type	PATIENT
Rack & Position	0 0
Instrument Number	IR35111412
Sample Selectivity	CBC\DIFF
Age & Sex	U
FOR LABORATORY USE ONLY	

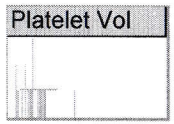


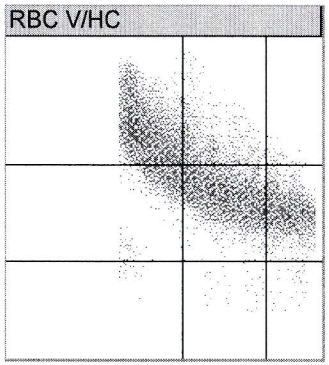


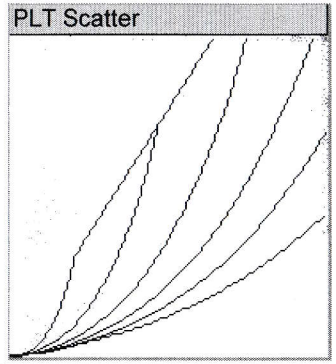


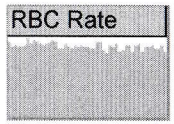


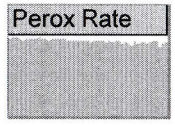












CBC					
WBC	H	242.23	*	x10⁹ cells/L
RBC	L	1.84	*	x10¹² cells/L
HGB	L	8.1	*	g/dL
Cellular HGB		6.7		g/dL
HCT	L	0.213	*	L/L
MCV	H	115.9	*	fL
MCH	H	44.1	*	pg
MCHC	H	38.0	*	g/dL
CHCM	L	31.5	*	g/dL
CH		34.8	*	pg
RDW	H	21.7	*	%
HDW	H	8.56	*	g/dL
PLT	L	3	*	x10⁹ cells/L
MPV	H	13.3	*	fL
PDW	L	13.6	*	%
PCT	L	0.00	*	%

Additional Routine Parameters		
%Blast Suspect	0.0	
%Hyper	16.9	
%Hypo	40.8	
%Macro	38.7	
%Micro	0.8	
RBC Fragments	0.00	x10¹² cells/L
RBC Ghosts	0.01	x10¹² cells/L
Neut X		
Neut Y		
MNx	6.7	
MNy	6.5	
%MN	0.0	
%PMN	99.7	
Cellular HGB	6.7	g/dL

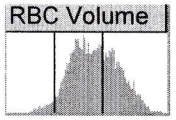
Routine WBC Differential							
		%			#		
WBC				H	242.23	*	x10⁹ cells/L
Neut	L	0.1	*	L	0.12	*	x10⁹ cells/L
Lymph	H	85.3	*	H	206.61	*	x10⁹ cells/L
Mono	H	9.5	*	H	23.13	*	x10⁹ cells/L
Eos		0.0	*		0.00	*	x10⁹ cells/L
Baso		0.0	*		0.09	*	x10⁹ cells/L
LUC	H	5.1	*	H	12.27	*	x10⁹ cells/L
NRBC			*			*	x10⁹ cells/L
LI					2.47	*	
MPXI				L	-30.5		
WBCP					279.59	*	x10⁹ cells/L

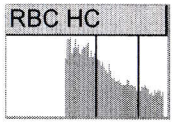
Uncorrected WBC and Differential							
		%			#		
WBCu				H	242.23	*	x10⁹ cells/L
NEUTu	L	0.1	*	L	0.12	*	x10⁹ cells/L
LYMPHu	H	85.3	*	H	206.61	*	x10⁹ cells/L
MONOu	H	9.5	*	H	23.13	*	x10⁹ cells/L
EOSu		0.0	*		0.00	*	x10⁹ cells/L
BASOu		0.0	*		0.09	*	x10⁹ cells/L
LUCu	H	5.1	*	H	12.27	*	x10⁹ cells/L
%MNu		0.0					%
%PMNu		99.7					%

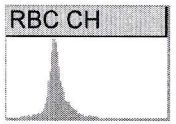
Morphology Flags	
MACRO	+++
HYPO	+++
HYPER	+++
ANISO	++
HC VAR	+++
ATYPS	+
PLT CLUMPS	+

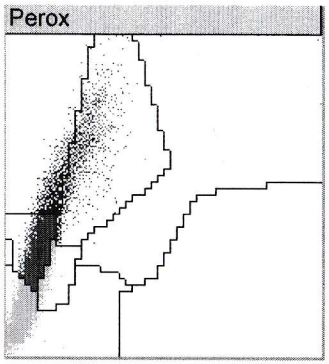
Sample/System Flags	
B-NO	#BASO %Baso WBC WBCB WBCu %BASOu #BASOu
B-NV	LI
CHCMCE	CHCM HCT HDW HGB MCH MCHC MCV RBC RDW CH CHDW
WBC-CE	WBC WBCu
PX-NO	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PX-NV	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PLT-CL	MPV PCT PDW PLT WBCP
NRCELL	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC
NR-LPD	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC

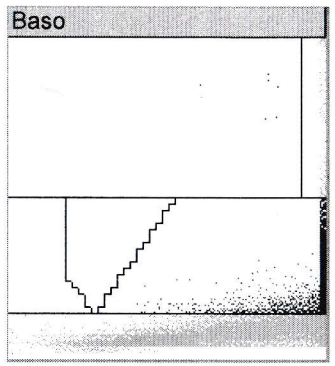
NHLS Universitas ADVIA 2120i B	
SID	HU00398553
Patient Name	CHEICKEN 5 , NO NAM
Aspiration Date/Time	03-May-17 12:23:57 PM
Sample Type	PATIENT
Rack & Position	0 0
Instrument Number	IR35111412
Sample Selectivity	CBC\DIFF
Age & Sex	U
FOR LABORATORY USE ONLY	

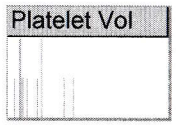


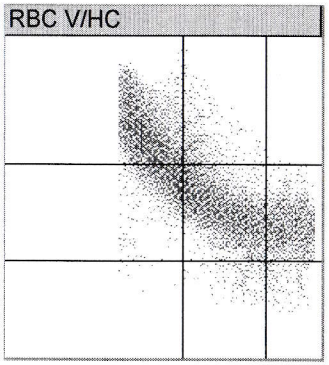


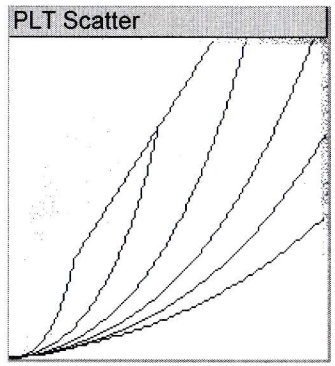


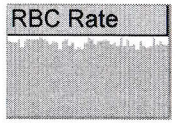


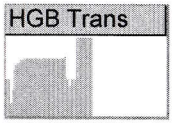


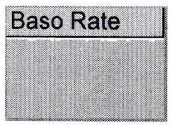


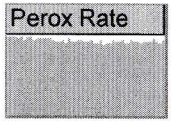












CBC					
WBC	H	420.82	*	x10⁹ cells/L	
RBC	L	2.14	*	x10¹² cells/L	
HGB	L	9.8	*	g/dL	
Cellular HGB		7.2		g/dL	
HCT	L	0.237	*	L/L	
MCV	H	110.9	*	fL	
MCH	H	45.8	*	pg	
MCHC	H	41.3	*	g/dL	
CHCM	L	30.4	*	g/dL	
CH		31.8	*	pg	
RDW	H	25.8	*	%	
HDW	H	8.34	*	g/dL	
PLT	L	1	*	x10⁹ cells/L	
MPV		9.8	*	fL	
PDW	H	69.0	*	%	
PCT	L	0.00	*	%	

Additional Routine Parameters		
%Blast Suspect	0.0	
%Hyper	13.9	
%Hypo	47.2	
%Macro	36.1	
%Micro	2.9	
RBC Fragments	0.00	x10¹² cells/L
RBC Ghosts	0.01	x10¹² cells/L
Neut X		
Neut Y		
MNx	10.7	
MNy	6.0	
%MN	0.0	
%PMN	99.5	
Cellular HGB	7.2	g/dL

Routine WBC Differential							
		%			#		
WBC				H	420.82	*	x10⁹ cells/L
Neut	L	0.0	*	L	0.05	*	x10⁹ cells/L
Lymph	H	91.8	*	H	386.44	*	x10⁹ cells/L
Mono		4.1	*	H	17.14	*	x10⁹ cells/L
Eos		0.0	*		0.00	*	x10⁹ cells/L
Baso		0.0	*		0.07	*	x10⁹ cells/L
LUC	H	4.1	*	H	17.12	*	x10⁹ cells/L
NRBC			*			*	x10⁹ cells/L
LI					2.39	*	
MPXI					-6.2		
WBCP					338.28	*	x10⁹ cells/L

Uncorrected WBC and Differential							
		%			#		
WBCu				H	420.82	*	x10⁹ cells/L
NEUTu	L	0.0	*	L	0.05	*	x10⁹ cells/L
LYMPHu	H	91.8	*	H	386.44	*	x10⁹ cells/L
MONOu		4.1	*	H	17.14	*	x10⁹ cells/L
EOSu		0.0	*		0.00	*	x10⁹ cells/L
BASOu		0.0	*		0.07	*	x10⁹ cells/L
LUCu	H	4.1	*	H	17.12	*	x10⁹ cells/L
%MNu		0.0					%
%PMNu		99.5					%

Morphology Flags	
MICRO	+
MACRO	+++
HYPO	+++
HYPER	+++
ANISO	+++
HC VAR	+++
LARGE PLT	+++
PLT CLUMPS	+

Sample/System Flags	
B-NO	#BASO %Baso WBC WBCB WBCu %BASOu #BASOu
B-NV	LI
CHCMCE	CHCM HCT HDW HGB MCH MCHC MCV RBC RDW CH CHDW
WBC-CE	WBC WBCu
PX-NV	#LUC %LUC #LYMPH %LYMPH WBCP #NEUT %NEUT #MONO %MONO #EOS %EOS %NEUTu #NEUTu %LYMPHu #LYMPHu %MONOu #MONOu %EOSu #EOSu %LUCu #LUCu
PLT-CL	MPV PCT PDW PLT WBCP
PLT-NO	MPV PCT PDW PLT
NRCELL	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC
NR-LPD	#BASO %Baso #LUC %LUC #LYMPH %LYMPH WBC #NEUT %NEUT #MONO %MONO #EOS %EOS %NRBC #NRBC

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527228 - Rekening No : F6019652

Dokter
AFSKRIF VERSLAG BFN (OTHER)
BLOEMFONTEIN LABORATORIUM

9300 BLOEMFONTEIN

Pasient
AVIAN CHICKEN BOUCHER AVIAN
DEPT OF MICROBIAL,BIOCHEMICAL
P O BOX 339
9300 BLOEMFONTEIN

Tel (H)	0514013253
Tel (S)	0783634531
Tel (W)	0514012274

Monster	0503:HA05267U
Versameldatum	2017-05-03 Nie verskaf nie
Ontvangsdatum	2017-05-04 20:10
Verslagdatum	2017-05-13 11:26

ID Nommer	NIE BESKIKBAAR NIE
Oud:Gslg:DvG	144y:M
Leer Nr	NIE BESKIKBAAR NIE
Hooflid	DR C BOUCHER
Mediese Fonds	VET ACC'S DIRECT TO OWNER
Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
URGENT !!!
FAX RESULTS TO: CHARLOTTE / POOJAH
E-MAIL RESULTS TO: jawallapersand@gmail.com
CONTROL(NOT CHALLENGED) CHICKEN BLOOD. TOTAL OF 5 SAMPLES IN TOTAL - HEALTHY CHICKENS
BREED: LEGHORN CHICKENS, AGE: 21weeks

Toetse Aangevra:
PLAATJIETELLING, HAEM SIGN QUEUE TRIGGER 1, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets	Resultaat	Wyser	Verwysing
BLOEDTELLING-GEEN PLAATJIES			
Rooiseltelling	1.81		1.8 - 2.3 x10E12/L
Hemoglobien	8.1	L	9.0 - 13.0 g/dL
Hematokrit	0.21	L	0.27 - 0.37 L/L
Gkv	118		fl
Gkh	45		pg
Gkhk	38		g/dL
Rdw	24.0	*H	10 - 15 %
Witseltelling	235.8	H	5.0 - 10.0 x10E9/L
	Let asseblief daarop dat as gevolg van rooibloedselle met kerne, is hierdie witseltelling nie korrek nie. Stuur asseblief 'n bloedsmeer aan die VetLab om te evalueer.		
Neutrofiele	5.7 % 13.44		x10E9/L
Limfosiete	92.8 % 218.82		x10E9/L
Monosiete	1.3 % 3.07		x10E9/L
Eosinofiele	0.1 % 0.24		x10E9/L
Vbt Kommentaar	.		
PLAATJIETELLING			
Plaatjietelling	15		x10E9/L

Goedgekeur deur: DR LUCIA LANGE op 2017-05-05 13:03:00
Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527227 - Rekening No : F6019643

Dokter		**Pasient**	
AFSKRIF VERSLAG BFN (OTHER)		**AVIAN CHICKENS BOUCHER AVIAN**	
BLOEMFONTEIN LABORATORIUM		DEPT OF MICROBIAL,BIOCHEMICAL	
		P O BOX 339	
9300 BLOEMFONTEIN		9300 BLOEMFONTEIN	
		Tel (H)	0514013253
		Tel (S)	0783634531
		Tel (W)	0514012274
Monster	0503:HA05266U	**ID Nommer**	NIE BESKIKBAAR NIE
Versameldatum	2017-05-03 Nie verskaf nie	**Oud:Gslg:DvG**	144y:M
Ontvangsdatum	2017-05-04 20:12	**Leer Nr**	NIE BESKIKBAAR NIE
Verslagdatum	2017-05-13 11:27	**Hooflid**	DR C BOUCHER
		Mediese Fonds	VET ACC'S DIRECT TO OWNER
		Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
URGENT !!!
FAX RESULTS TO:CHARLOTTE/POOJAH
E-MAIL RESULTS TO:JAWALLAPERSAND@GMAIL.COM
BREED: LEGHORN CHICKENS AGE: 21 WEEKS
CONTROL(NOT CHALLENGED) CHICKEN BLOOD
TOTAL OF 5 SAMPLES IN TOTAL. HEALTHY CHICKENS

Toetse Aangevra:
PLAATJIETELLING, HAEM SIGN QUEUE TRIGGER 1, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets		Resultaat	Wyser	Verwysing
BLOEDTELLING-GEEN PLAATJIES				
Rooiseltelling		2.16		1.8 - 2.3 x10E12/L
Hemoglobien		9.8		9.0 - 13.0 g/dL
Hematokrit		0.23	L	0.27 - 0.37 L/L
Gkv		108		fl
Gkh		45		pg
Gkhk		42		g/dL
Rdw		27.8	*H	10 - 15 %
Witseltelling		399.3	H	5.0 - 10.0 x10E9/L
	Please note - due to nucleated red cells the white cell count is incorrect			
	Please send blood smear to the VetLab for Diff count			
Neutrofiele	0.1 %	0.40		x10E9/L
Limfosiete	89.5 %	357.37		x10E9/L
Monosiete	10.4 %	41.53		x10E9/L
Eosinofiele		0.01		x10E9/L
Basofiele		0.01		x10E9/L
Morfologie Kommentaar				
	On the blood smear there is a relative decrease in white cells. Those present are mostly (granulocytes) heterophils and lymphocytes.			
Vbt Kommentaar				
PLAATJIETELLING				
Plaatjietelling		9		x10E9/L

Vir konsultasie: Dr Lucia Lange Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527226 - Rekening No : F6020518

Dokter
AFSKRIF VERSLAG BFN (OTHER)
BLOEMFONTEIN LABORATORIUM

9300 BLOEMFONTEIN

Pasient
AVIAN CHICKENS BOUCHER AVIAN
DEPT OF MICROBIAL, UFS
P O BOX 339
9300 BLOEMFONTEIN

Tel (H)	0514013253
Tel (S)	0783634531
Tel (W)	0514012274

Monster	0503:HA05268U
Versameldatum	2017-05-03 Nie verskaf nie
Ontvangsdatum	2017-05-04 20:13
Verslagdatum	2017-05-13 11:28

ID Nommer	NIE BESKIKBAAR NIE
Oud:Gslg:DvG	144y:M
Leer Nr	NIE BESKIKBAAR NIE
Hooflid	DR C BOUCHER
Mediese Fonds	VET ACC'S DIRECT TO OWNER
Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
URGENT !!!
FAX RESULTS TO:CHARLOTTE/POOJAH
E-MAIL RESULTS TO:JAWALLAPERSAND@GMAIL.COM
BREED: LEGHORN CHICKENS AGE: 21 WEEKS
CONTROL(NOT CHALLENGED) CHICKEN BLOOD
TOTAL OF 5 SAMPLES IN TOTAL. HEALTHY CHICKENS

Toetse Aangevra:
PERIPHERAL BLOOD FILM, PLAATJIETELLING, HAEM SIGN QUEUE TRIGGER 1, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets	Resultaat	Wyser	Verwysing
BLOEDTELLING-GEEN PLAATJIES			
Rooiseltelling	1.50	L	1.8 - 2.3 x10E12/L
Hemoglobien	6.1	L	9.0 - 13.0 g/dL
Hematokrit	0.16	L	0.27 - 0.37 L/L
Gkv	107		fl
Gkh	41		pg
Gkhk	38		g/dL
Rdw	27.0	*H	10 - 15 %
Witseltelling	166.1	H	5.0 - 10.0 x10E9/L
	Please note: Due to the nucleated red cells the white cell count is incorrect. Please send a blood smear to the VetLab for evaluation		
Neutrofiele	6.5 % 10.80		x10E9/L
Limfosiete	90.7 % 150.65		x10E9/L
Monosiete	2.4 % 3.99		x10E9/L
Eosinofiele	0.3 % 0.50		x10E9/L
Basofiele	0.1 % 0.17		x10E9/L
Vbt Kommentaar	.		
PLAATJIETELLING			
Plaatjietelling	24		x10E9/L

Vir konsultasie: Dr Lucia Lange Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527265 - Rekening No : F6020530

Dokter		**Pasient**	
AFSKRIF VERSLAG BFN (OTHER)		**AVIAN CHICKENS BOUCHER AVIAN**	
BLOEMFONTEIN LABORATORIUM		DEPT OF MICROBIAL,UFS	
		P O BOX 339	
9300 BLOEMFONTEIN		9300 BLOEMFONTEIN	
		Tel (H)	0514013253
		Tel (S)	0783634531
		Tel (W)	0514012274
Monster	0503:HA05269U	**ID Nommer**	NIE BESKIKBAAR NIE
Versameldatum	2017-05-03 Nie verskaf nie	**Oud:Gslg:DvG**	144y:M
Ontvangsdatum	2017-05-04 20:14	**Leer Nr**	NIE BESKIKBAAR NIE
Verslagdatum	2017-05-13 11:28	**Hooflid**	DR C BOUCHER
		Mediese Fonds	VET ACC'S DIRECT TO OWNER
		Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
URGENT !!!
FAX RESULTS TO:CHARLOTTE/POOJAH
E-MAIL RESULTS TO:JAWALLAPERSAND@GMAIL.COM
BREED: LEGHORN CHICKENS AGE: 21 WEEKS
CONTROL(NOT CHALLENGED) CHICKEN BLOOD
TOTAL OF 5 SAMPLES IN TOTAL. HEALTHY CHICKENS

Toetse Aangevra:
PERIPHERAL BLOOD FILM, PLAATJIETELLING, HAEM SIGN QUEUE TRIGGER 1, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets		Resultaat	Wyser	Verwysing
BLOEDTELLING-GEEN PLAATJIES				
Rooiseltelling		1.77	L	1.8 - 2.3 x10E12/L
Hemoglobien		7.2	L	9.0 - 13.0 g/dL
Hematokrit		0.20	L	0.27 - 0.37 L/L
Gkv		111		fl
Gkh		41		pg
Gkhk		37		g/dL
Rdw		23.4	*H	10 - 15 %
Witseltelling		209.1	H	5.0 - 10.0 x10E9/L
	Please note: Due to the nucleated red blood cells the machine count for the white cells is incorrect. Please send blood smear to the VetLab for evaluation.			
Neutrofiele		11.5 % 24.05		x10E9/L
Limfosiete		85.9 % 179.62		x10E9/L
Monosiete		2.4 % 5.02		x10E9/L
Eosinofiele		0.1 % 0.21		x10E9/L
Vbt Kommentaar		.		
PLAATJIETELLING				
Plaatjietelling		20		x10E9/L

Vir konsultasie: Dr Lucia Lange Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527264 - Rekening No : F6020545

Dokter
AFSKRIF VERSLAG BFN (OTHER)
BLOEMFONTEIN LABORATORIUM

9300 BLOEMFONTEIN

Pasient
AVIAN CHICKENS BOUCHER AVIAN
DEPT OF MICROBIAL, UFS
P O BOX 339
9300 BLOEMFONTEIN

Tel (H)	0514013253
Tel (S)	0783634531
Tel (W)	0514012274

Monster	0503:HA05270U	**ID Nommer**	NIE BESKIKBAAR NIE
Versameldatum	2017-05-03 Nie verskaf nie	**Oud:Gslg:DvG**	144y:M
Ontvangsdatum	2017-05-04 20:16	**Leer Nr**	NIE BESKIKBAAR NIE
Verslagdatum	2017-05-13 11:30	**Hooflid**	DR C BOUCHER
		Mediese Fonds	VET ACC'S DIRECT TO OWNER
		Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
URGENT !!!
FAX RESULTS TO:CHARLOTTE/POOJAH
E-MAIL RESULTS TO:JAWALLAPERSAND@GMAIL.COM
BREED: LEGHORN CHICKENS AGE: 21 WEEKS
CONTROL(NOT CHALLENGED) CHICKEN BLOOD
TOTAL OF 5 SAMPLES IN TOTAL. HEALTHY CHICKENS

Toetse Aangevra:
PERIPHERAL BLOOD FILM, PLAATJIETELLING, HAEM SIGN QUEUE TRIGGER 1, BLOEDTELLING-GEEN PLAATJIES

Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets		Resultaat	Wyser	Verwysing
BLOEDTELLING-GEEN PLAATJIES				
Rooiseltelling		2.67	H	1.8 - 2.3 x10E12/L
Hemoglobien		13.5	H	9.0 - 13.0 g/dL
Hematokrit		0.32		0.27 - 0.37 L/L
Gkv		118		fl
Gkh		50		pg
Gkhk		43		g/dL
Rdw		25.2	*H	10 - 15 %
Witseltelling		570.2	H	5.0 - 10.0 x10E9/L
	Please note: Due to the nucleated red cells the machine white cell count is incorrect. Please send blood smear to the VetLab for comment and evaluation			
Neutrofiele	4.2 %	23.95		x10E9/L
Limfosiete	95.3 %	543.40		x10E9/L
Monosiete	0.4 %	2.28		x10E9/L
Vbt Kommentaar		.		
PLAATJIETELLING				
Plaatjietelling		6		x10E9/L

Vir konsultasie: Dr Lucia Lange Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750528629 - Rekening No : F6035142

Dokter		**Pasient**	
AFSKRIF VERSLAG BFN (OTHER)		**AVIAN CHICKENS E19 BOUCHER AVIAN**	
BLOEMFONTEIN LABORATORIUM		DEPT OF MICROBIAL,BIOCHEMICAL	
		P O BOX 339	
9300 BLOEMFONTEIN		9300 BLOEMFONTEIN	
		Tel (H)	0514012274
		Tel (S)	0783634531
		Tel (W)	0514013253
Monster	0506:HA01627U	**ID Nommer**	NIE BESKIKBAAR NIE
Versameldatum	2017-05-06 17:00	**Oud:Gslg:DvG**	145y:M
Ontvangsdatum	2017-05-06 17:23	**Leer Nr**	NIE BESKIKBAAR NIE
Verslagdatum	2017-05-13 11:32	**Hooflid**	DR C BOUCHER
		Mediese Fonds	VET ACC'S DIRECT TO OWNER
		Med.Fnds Nr	VET ACCOUNT

Kliniese Data:
URGENT !!!
E-MAIL RESULTS TO:jawallapersendp@gmail.com
CHICKENS INFECTED WITH BACTERIAL CULTURE
Versameldatum nie aangedui op aanvraag vorm en kon dus nie deur PathCare bevestig word nie.

Toetse Aangevra:
PLAATJIETELLING, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets	**Resultaat**	**Wyser**	**Verwysing**
BLOEDTELLING-GEEN PLAATJIES			
Hemoglobien	4.4	*L	9.0 - 13.0 g/dL
Hematokrit	0.12	L	0.27 - 0.37 L/L
Vbt Kommentaar			
Geen bloed parasiete waargeneem nie.			

Goedgekeur deur: DR LUCIA LANGE op 2017-05-12 17:04:00
Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527253 - Rekening No : F6074971

Dokter		**Pasient**	
AFSKRIF VERSLAG BFN (OTHER)		**AVIAN CHICKENS EU BOUCHER AVIAN**	
BLOEMFONTEIN LABORATORIUM		DEPT OF MICROBIAL,BIOCHEMICAL	
		P O BOX 339	
9300 BLOEMFONTEIN		9300 BLOEMFONTEIN	
		Tel (H)	0514012274
		Tel (S)	0783634531
		Tel (W)	0514013253
Monster	0510:HA04211U	**ID Nommer**	NIE BESKIKBAAR NIE
Versameldatum	2017-05-10 Nie verskaf nie	**Oud:Gslg:DvG**	145y:M
Ontvangsdatum	2017-05-10 19:29	**Leer Nr**	NIE BESKIKBAAR NIE
Verslagdatum	2017-05-13 11:33	**Hooflid**	DR C BOUCHER
		Mediese Fonds	VET ACC'S DIRECT TO OWNER
		Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
BREED: LEGHOMS AGE:NOT SUPPLIED
BLOOD SAMPLES TAKEN ON THE 10TH MAY 2017. INFECTED BIRDS, 8
DIFFERENT CHICKENS(FROM DIFFERENT ANIMALS)
FAX RESULTS TO:DR
E-MAIL RESULTS TO:JAWALLAPERSANDP@GAMIL.COM
Please note that the collection date was not provided and
could not be verified by PathCare.

Toetse Aangevra:
PLAATJIETELLING, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets	**Resultaat**	**Wyser**	**Verwysing**
BLOEDTELLING-GEEN PLAATJIES			
Hemoglobien	2.4	*L	9.0 - 13.0 g/dL
Hematokrit			0.27 - 0.37 L/L
HCT = 0.065			
Morfologie Kommentaar			
Geen parasiete waargeneem.			
Vbt Kommentaar			
Volbloedtellings word nie op hoenders gedoen nie.			
Kern bevattende rooibloedselle teenwoordig.			
EDTA veranderinge met slegs naakte witselle teenwoordig			

Goedgekeur deur: DR LUCIA LANGE op 2017-05-12 17:56:00
Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527252 - Rekening No : F6074996

Dokter		**Pasient**	
AFSKRIF VERSLAG BFN (OTHER)		**AVIAN CHICKENS E20 BOUCHER AVIAN**	
BLOEMFONTEIN LABORATORIUM		DEPT OF MICROBIAL,BIOCHEMICAL	
		P O BOX 339	
9300 BLOEMFONTEIN		9300 BLOEMFONTEIN	
		Tel (H)	0514012274
		Tel (S)	0783634531
		Tel (W)	0514013253
Monster	0510:HA04229U	**ID Nommer**	NIE BESKIKBAAR NIE
Versameldatum	2017-05-10 Nie verskaf nie	**Oud:Gslg:DvG**	145y:M
Ontvangsdatum	2017-05-10 19:32	**Leer Nr**	NIE BESKIKBAAR NIE
Verslagdatum	2017-05-13 11:34	**Hooflid**	DR C BOUCHER
		Mediese Fonds	VET ACC'S DIRECT TO OWNER
		Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
E-MAIL RESULTS TO: JAWALLAPERSANDP@GMAIL.COM
FAX RESULTS TO:
BREED: LEGHORNS
AGE: 22 WEEKS
BLOOD SAMPLES TAKEN ON THE 10TH MAY 2017, INFECTED BIRDS.
8 DIFFERENT CHICKENS (FROM DIFFERENT ANIMALS).
Versameldatum nie aangedui op aanvraag vorm en kon dus nie
deur PathCare bevestig word nie.

Toetse Aangevra:
PERIPHERAL BLOOD FILM, PLAATJIETELLING, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------HEMATOLOGIE----------

Toets	**Resultaat**	**Wyser**	**Verwysing**
BLOEDTELLING-GEEN PLAATJIES			
Hemoglobien	6.4	L	9.0 - 13.0 g/dL
Hematokrit	0.17	L	0.27 - 0.37 L/L
Morfologie Kommentaar			
Geen parasiete waargeneem.			
Vbt Kommentaar			

Vir konsultasie: Dr Lucia Lange Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

Drs Voigt & Vennote
Medi-Clinic Hospitaal
Derde Laan, Bloemfontein
Tel : 051 401 4616

PathCare

Prac.No. :5200539

FINALE VERSLAG - Lab Verw : 750527247 - Rekening No : F6075011

Dokter		**Pasient**	
AFSKRIF VERSLAG BFN (OTHER)		**AVIAN CHICKENS E28 BOUCHER AVIAN**	
BLOEMFONTEIN LABORATORIUM		DEPT OF MICROBIAL,BIOCHEMICAL	
		P O BOX 339	
9300 BLOEMFONTEIN		9300 BLOEMFONTEIN	
		Tel (H)	0514012274
		Tel (S)	0783634531
		Tel (W)	0514013253
Monster	0510:HA04239U	**ID Nommer**	NIE BESKIKBAAR NIE
Versameldatum	2017-05-10 Nie verskaf nie	**Oud:Gslg:DvG**	145y:M
Ontvangsdatum	2017-05-10 19:39	**Leer Nr**	NIE BESKIKBAAR NIE
Verslagdatum	2017-05-13 11:35	**Hooflid**	DR C BOUCHER
		Mediese Fonds	VET ACC'S DIRECT TO OWNER
		Med.Fnds Nr	NOT AVAILABLE

Kliniese Data:
INFECTED BIRDS,8 DIFFERENT CHICKENS
FAX RESULTS
E-MAIL RESULTS TO: JAWALLAPERSANDP@GMAIL.COM
BREED: LEGHORNS
AGE : 22 WEEKS

Toetse Aangevra:
PERIPHERAL BLOOD FILM, PLAATJIETELLING, BLOEDTELLING-GEEN PLAATJIES
Primere ICD10 Kode(s) : Z76.9

----------**HEMATOLOGIE**----------

Toets	**Resultaat**	**Wyser**	**Verwysing**
BLOEDTELLING-GEEN PLAATJIES			
Hemoglobien	6.6	L	9.0 - 13.0 g/dL
Hematokrit	0.20	L	0.27 - 0.37 L/L

Morfologie Kommentaar

Geen parasiete waargeneem.

Vbt Kommentaar

Volbloedtellings word nie op hoenders gedoen nie.

Kern bevattende rooibloedselle teenwoordig.

EDTA veranderinge met slegs naakte witselle teenwoordig

Goedgekeur deur: DR LUCIA LANGE op 2017-05-12 17:56:00
Veearts Patoloog: 082 8088773
~ H=Hoog, L=Laag, *H=Kritiek Hoog, *L=Kritiek Laag
~ Liasseer [ ] Bel Pasient [ ] Maak Afspraak [ ] Voorskrif [ ] Trek Leer [ ]

APPENDIX A

Scoring System used for Infectious Coryza Disease Progression

	Matsumoto & Yamamoto (1971, 1975)		Bragg & Greyling (1999)
Scoring system	**No clinical symptoms** (not in literature, adaptation of M&Y[1971,1975])		**Score 0:** No clinical symptoms
	Mild: Nasal discharge with/without mild facial oedema		**Score 1:** Mild clinical symptoms
	Moderate: Nasal discharge on both left and right sides with slight facial oedema		**Score 2:** Moderate clinical symptoms
	Severe: Severe bilateral oedema with/without haemorrhage and conjunctivitis		**Score 3:** Severe clinical signs
Notes*			B&G[1999] is an adaptation and summarised form of M&Y[1971,1975]

Infectious Coryza Progression : Daily Clinical Score Data Sheet of Experimental Chickens [Veterinary Biotechnology MSc. Study]

Group:			Date:	Species:			Age:_____ weeks	Bacteria:				Additional comments	Body Weight (kg)	Body Temperature (°C)
Experimental Chickens	Day :		Challenge study:			Pre-	Post-	Infection	Serovar:					
	Sex		Number of eggs/day	Matsumoto & Yamamoto (1971, 1975)				Bragg & Greyling (1999)						
	Male	Female		No signs	Mild	Moderate	Severe	Score 0	Score 1	Score 2	Score 3			
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														
14														
15														
16														
17														
18														
19														
20														
21														
22														
23														
24														
25														
26														
27														
28														
29														
30														
Mean Score														

Injection site:	

Infectious Coryza Progression : Daily Clinical Score Data Sheet of Control Chickens [Veterinary Biotechnology MSc. Study]

Group:			Date:			Species:		Age:_____ weeks	Bacteria:			Additional comments	Body Weight (kg)	Body Temperature (°C)
Control Chickens	Day :		Challenge study :			Pre-	Post-	Infection	Serovar:					
	Sex		Number of eggs/day	Matsumoto & Yamamoto (1971, 1975)				Bragg & Greyling (1999)						
	Male	Female		No signs	Mild	Moderate	Severe	Score 0	Score 1	Score 2	Score 3			
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
Mean Score														

APPENDIX B

PathCare VETLAB

COMPANION ANIMAL REQUEST FORM

Cape Town - 021 596 3636 Bloemfontein - 051 401 4720/1

VET

RESULT PRIORITY URGENT

Contact Person

Please tick no. supplied (✓) Tel Fax Cell

Referring Veterinarian | Veterinarian's Code | Copies to Vets (initials & surname) | File No.

Address

Tel | Fax | Cell | E-mail

ANIMAL INFORMATION

Name | Age | Gender M F

Breed

Species: CANINE 121 DOG | FELINE 126 CAT | EQUINE 136 HORSE | AVIAN 145 BIRD | OTHER 150

Number of Specimens | Specimen type: BLOOD URINE FAECES SWAB OTHER

Specimen Collected by | Date DD MM YY | Time :

RELEVANT CLINICAL DETAILS / HISTORY

ACCOUNT TO OWNER VET

OWNER'S INFORMATION

Name and Surname Mr | Mrs | Miss | Dr | Prof

ID No.

Postal Address

C O D E

Tel. (h) | Cell

Tel. (w) | Fax

E-mail

LOCATION 7500

Practice No. 5200539

PATHCARE STEYER

Site Priority: U | U URGENT

Received by | Date DD MM YY | Time :

SPECIMEN INFORMATION AND COUNT

URINE	HEPARIN	EDTA 4ml	CITRATE	GEL	CLOTTED	FLUORIDE	OTHER - please specify	TEST COUNT

SCREENING PANELS

FOR MORE INFORMATION ON THE SCREENING PANELS, PLEASE REFER TO THE INSIDE COVER OF THE REQUEST FORM PAD

Test Code	✓		Sample Type
D2815	☐	GENERAL SCREENING PANEL	
M2811	☐	EMERGENCY PANEL	
C1929	☐	GENERAL CANINE SCREENING	
G1913	☐	FELINE VIRAL PANEL (FeLV, FIV & FIP)	
G1591	☐	GENERAL FELINE SCREENING	
Z2820	☐	BASIC RENAL PANEL	
B2817	☐	BASIC HEPATIC PANEL	
L1928	☐	PRE-ANAESTHETIC SCREENING	
X2816	☐	GERIATRIC PANEL	
E3886	☐	DIABETIC PANEL	

HAEMATOLOGY

Test Code	✓		Sample Type
Y1110	☐	FULL BLOOD COUNT	
P1227	☐	HAEMATOCRIT	
P1112	☐	HAEMOGLOBIN	
B1115	☐	RETICULOCYTE COUNT	
D1113	☐	WHITE CELL COUNT & DIFF. (%)	
M2857	☐	WHITE CELL COUNT (only)	
F1226	☐	PLATELET COUNT	
E1724	☐	CANINE COOMBS TEST	
E1724	☐	EQUINE COOMBS TEST	
F1134	☐	FIBRINOGEN	
Y1455	☐	BABESIA PARASITES	
A1130	☐	PT (PROTHROMBIN TIME) *	
M1132	☐	PTT (PARTIAL THROMBOPLASTIN TIME) *	
		*NB! Remember control sample	
P5275	☐	IN SALINE AGGLUTINATION	

CHEMISTRY

Test Code	✓		Sample Type
J1002	☐	SODIUM	
Z1003	☐	POTASSIUM	
V1650	☐	CHLORIDE	
P1572	☐	CALCIUM	
C1009	☐	MAGNESIUM	
E1011	☐	SERUM INORGANIC PHOSPHATE	
C1262	☐	UREA (BUN)	
L1261	☐	CREATININE	
E2483	☐	TSP (TOTAL SERUM PROTEINS)	
R3382	☐	ALBUMIN	
Q3374	☐	TSP, ALBUMIN & GLOBULIN	
J1025	☐	ALT (ALANINE AMINO TRANSFERASE)	
X1022	☐	ALP (ALKALINE PHOSPHATASE)	
N1024	☐	AST (ASPARTATE AMINO TRANSFERASE)	
B1023	☐	GAMMA GLUTAMYL TRANSPEPTIDASE	
X1505	☐	TOTAL BILIRUBIN	
D1021	☐	TOTAL + CONJUGATED BILIRUBIN	
C1492	☐	BILE ACIDS (pre & post)	

CHEMISTRY cont.

Test Code	✓		Sample Type
R1036	☐	CK (CREATININE KINASE)	
BHBA1G	☐	GLYCATED HB	
D1044	☐	GLUCOSE (FASTING)	
X1045	☐	GLUCOSE (RANDOM)	
Z3211	☐	INSULIN (RANDOM)	
S1050	☐	FRUCTOSAMINE	
Y1041	☐	CHOLESTEROL	
F1042	☐	TRIGLYCERIDES	
H1007	☐	URATE (URIC ACID)	
K1240	☐	CANINE SPECIFIC PANCREATIC LIPASE	
K1240	☐	FELINE SPECIFIC PANCREATIC LIPASE	
S1027	☐	AMYLASE	
V1029	☐	LIPASE	

DRUG MONITORING

Test Code	✓		Sample Type
W1152	☐	PHENOBARBITONE	
Q4938	☐	POTASSIUM BROMIDE	
T1150	☐	DIGOXIN	

TRACE ELEMENT BLOOD

Test Code	✓		Sample Type
H2364	☐	SELENIUM	
W1727	☐	COPPER	
L2871	☐	ZINC	
K3241	☐	IRON	

ENDOCRINOLOGY

Test Code	✓		Sample Type
S1119	☐	VIT B12 (Cyanocobalamine)	
W1060	☐	FREE T4 (THYROXINE)	
L4918	☐	TOTAL T4	
Y2766	☐	CANINE TSH	
H4917	☐	TESTOSTERONE	
L1077	☐	PROGESTERONE	
J2819	☐	VET PROGESTERONE (wildlife)	
R4049	☐	EQUINE PREGNANCY	
A2763	☐	TLI (TRYPSIN-LIKE IMMUNOREACTIVITY)	
A1084	☐	CORTISOL	
A2809	☐	ACTH STIMULATION	
R2807	☐	DEXAMETHAZONE SUPPRESSION	

URINE

Test Code	✓		Sample Type
A1015	☐	CALCULUS ANALYSIS	
G2511	☐	URINE (SPECIFIC GRAVITY)	
C2412	☐	URINE ANALYSIS	
B1736	☐	URINE CORTISOL: CREATININE	
3627	☐	URINE PROTEIN: CREATININE RATIO	

PARASITOLOGY / FAECAL ANALYSIS

Test Code	✓		Sample Type
W3383	☐	PARASITE SCREEN (Internal & external)	
N3393	☐	PARASITE IDENTIFICATION	
S3396	☐	GIARDIA Ag	
V3559	☐	ELECTRON MICROSCOPY ON FAECES	

MICROBIOLOGY

Test Code	✓		Sample Type
N7027	☐	BACTERIAL CULTURE (AEROBIC & ANAEROBIC) & ID	

SPECIMEN TYPE: ☐ SWAB ☐ EAR ☐ OTHER

Specify: _____

D2585	☐	ANTIBIOGRAM (General)	
F2836	☐	BACTERIAL TYPING	
T3381	☐	FUNGI	

SPECIMEN TYPE: ☐ SWAB ☐ HAIR ☐ OTHER

Specify: _____

TOXICOLOGY

Test Code	✓		Sample Type
B3530	☐	PESTICIDE SCREEN ONLY	
H1030	☐	CHOLINESTERASE	
C4919	☐	MONOFLUOROACETATE (1080)	
B3277	☐	LEAD POISONING	

SEROLOGY, MOLECULAR DIAGNOSTICS

EQUINEANTIBODY TESTS

Test Code	✓		Sample Type
V1880	☐	EQUINE ENCEPHALOSIS (Ab)	
X1298	☐	AFRICAN HORSE SICKNESS (Ab)	
K1884	☐	EQUINE INFLUENZA (Ab)	
L1882	☐	EQUINE HERPES (EHV 1 + 4) (Ab)	
T1886	☐	EQUINE VIRAL ARTERITIS (Ab)	
C1883	☐	EQUINE INFECTIOUS ANAEMIA (Ab)	
P4562	☐	WEST NILE VIRUS (Ab)	
Q1879	☐	B.CABALLI & T EQUI (Ab)	
W1842	☐	DOURINE Ab CFT #	

Must be accompanied by the DOURINE Test & Agreement form.

SEROLOGY, MOLECULAR DIAGNOSTICS

EQUINEANTIGEN TESTS

Test Code	✓		Sample Type
V2938	☐	PCR EQUINE ENCEPHALOSIS (Ag)	
D1504	☐	PCR AFRICAN HORSE SICKNESS (Ag)	
K3724	☐	PCR EQUINE HERPES (EHV 1 + 4) (Ag)	
W3912	☐	PCR WEST NILE VIRUS (Ag)	
F2606	☐	PCR B.CABALLI & T EQUI (Ag) Screening	

CANINEANTIBODY TESTS

Test Code	✓		Sample Type
S3649	☐	DISTEMPER (Ab)	
X2540	☐	LEPTOSPIROSIS (Ab)	
A3867	☐	BRUCELLA CANIS (Ab)	
E1862	☐	EHRLICHIA (Ab)	
M2581	☐	TOXOPLASMA (Ab)	
H2663	☐	NEOSPORA (Ab)	

CANINEANTIGEN TESTS

Test Code	✓		Sample Type
Z1831	☐	DISTEMPER (Ag)	
W2992	☐	CANINE PARVO (Ag) (stool sample)	
W3659	☐	EHRLICHIA (Ag)	
L2664	☐	PCR TOXOPLASMA (Ag)	

FELINEANTIBODY TESTS

Test Code	✓		Sample Type
N4727	☐	FIV (Ab)	
B1920	☐	FIP (corona) (Ab)	
F2491	☐	TOXOPLASMA (Ab) & CHLAMYDOPHILA (Ab)	
M1914	☐	FeLV (Ag) & FIV (Ab)	
G1913	☐	FELINE VIRAL PROFILE (Ab)	

FELINEANTIGEN TESTS

Test Code	✓		Sample Type
B4726	☐	FeLV (Ag) (serum)	
H3652	☐	PCR FELINE CORONA (Ag)	
G1913	☐	FELINE VIRAL PROFILE (Ab+Ag)	
L2664	☐	PCR TOXOPLASMA (Ag)	
V3651	☐	PCR CHLAMYDOPHILA (Ag)	

OTHER

Test Code	✓		Sample Type
C4919	☐	PCR TESTING (EDTA or FRESH TISSUE or DRY SWAB) Contact Vetlab	

Please Specify:

HISTO / CYTO (p.t.o.)

CHAPTER 4

INFECTIOUS CORYZA AS AN INFECTION MODEL TO MONITOR IMMUNE CELLS AND MOLECULES DURING DISEASE PROGRESSION

Sections of Chapter 4 have been used for manuscript for submission in a peer-reviewed journal, with the title “Omens and Remnants of Infectious Coryza: A Macabre Tale of Necropsy and Immunohistopathology of Chicken Lymphatic Tissues after Infection with *Av. paragallinarum serovar C-3*” and “The Infectious Coryza Diaries: Disease Monitoring of Immune Cells and Molecules during *Av. paragallinarum* C-3 serovar Infection”.

4.1. Introduction

Infectious coryza (IC) occurs wherever chickens are raised, however it is still a major problem for the intensive chicken industry (Blackall and Soriano, 2008). In the district of Kurnool of India, IC was found to be the second most important bacterial disease of chickens associated with high mortality rates after salmonellosis (Srinivasa *et al*. 1989). Epidemiological data from Morocco, indicated from 10 IC outbreaks, showed a significant decline in egg production of 14 to 41% and a mortality rate of 0.7 to 10% (Thitisak *et al*. 1988). In Thailand, IC was reported to be the common cause of mortality in chickens less than 2 months of age and those older than 6 months of age (Blackall, 1999). Moreover, IC

had a massive economic impact on chicken meat when two states, San Joaquin Valley of California and Alabama, were affected by an outbreak in the United States of America (Droual *et al*. 1990a; Droual *et al*. 1990b; Hoerr *et al*. 1994). As such, continuous outbreaks of IC, have highlighted the importance of this poultry disease internationally.

Commercial vaccines for infectious coryza are based on killed *A. paragallinarum* and an extensive review of the literature on inactivated IC vaccines has also been published (Blackall, 1995; Dungu *et al*. 2009). However, failed vaccination attempts against *Av. paragallinarum* have been reported dating to the mid-1980s (Blackall, 1999; Bragg, 2002). There has been evidence of a dramatic shift in the incidence of serovar C-3 of *Av. paragallinarum* in South Africa in the recent years. Bragg *et al*. (1996) have reported on the serovars of *Av. paragallinarum* using a partial Kume serotyping scheme during the 1970s-1990s (Blackall, 1999). It was shown that the incidence of Kume serovar C-3 had increased by 40%, during the 1970s (30%) to the early 1990s (70%) (Blackall, 1999). Moreover, Bragg *et al*. (1996) have suggested that the apparent failure of commercial vaccines in South Africa (which do not contain Kume serovar C-3) has occurred, because the dominant serovar in the field is Kume serovar C-3. Furthermore, Kume serovar C-3 is antigenically distinct from other Kume C serovars (C-1 and C-2), which implies that there is no cross-protection between these serovars. Moreover, it was suspected that the emergence of new *Av. paragallinarum* serovars or serovar variants are the main culprits for vaccination failures, as these new serovars or serovar variants do not provide cross-protection (Blackall, 1999). However, these reports are purely speculative, as there has been no evidence from vaccination trials to support these propositions. Hence, there is a definitive need for such studies, including research on examining the level of cross-protection within Kume serogroups A and C (Blackall, 1999).

From the above reports, there is a gap in knowledge that correlates solutions to the problem of IC, which is that both the innate and adaptive avian immune responses as well as host-pathogen interactions are critical in defining the severity and physiological outcome of the bacterial infection (Boucher *et al*. 2014). Without knowledge pertaining to immunity against IC, vaccination attempts and disease-control against IC will be a constant struggle. Chapter 4 focuses mainly on how the avian immune system equips itself against *Av. paragallinarum* strain serovar C-3 (SA-3 strain) infection. Our interest was mainly on disease progression with regards to *Av. paragallinarum* SA-3 infection and the immune mechanisms employed by the avian innate and adaptive responses through the recruitment of immune cells and the activation of immune molecules such as cytokines using different techniques. This study is novel and the first of its kind in IC infection, since very little is known about the exact immune response with regards to IC infection, especially serovar C-3 (SA-3 strain) infection.

4.2. Materials and methods

4.2.1. Ethics approval, animal husbandry and study design

Ethics approval, animal husbandry and study design were conducted as mentioned in Section 3.2.1, Chapter 3, with a few adjustments and modifications. A movement permit was issued by the poultry supplier Deltamune (Lyttleton, Centurion, Pretoria, South Africa) at the end of July 2017 for veterinary clearance of chickens obtained as proof, that the chickens being transported were specific-pathogen-free (SPF) and disease-free, as there was an emergence of highly pathogenic avian influenza A (H5N8) in South Africa during the period June to July 2017. A total of 40 SPF/unvaccinated White Leghorn chickens at 25 weeks of age, were obtained and purchased from Deltamune (Lyttleton, Centurion, Pretoria, South Africa). However, during the transportation from Pretoria to Bloemfontein, 2 chickens might

have died before collection or along the way due to unknown and unforeseen circumstances therefore there were only 38 chickens as experimental subjects.

The chickens were separated into two cohorts; the experimental group consisting of 30 birds and the control group consisting of 8 birds. The same experimental set-up, feeding, drinking systems, care and disinfection routines were used as Section 3.2.1, Chapter 3. Moreover, regular check-ups were conducted to ensure that the well-being of the chickens was taken care of. Proper cleaning and disinfection of cages were conducted on a regular basis. Additionally, precautions were taken to ensure that there is minimum bacterial aerosol carry-over to the control group in isolators, the control group was fed before the experimental group, and the respective isolators were cleaned first before that of experimental chickens was conducted to avoid cross-contamination. Likewise, a laboratory animal technician was always on standby for any emergencies or assistance needed, even during weekends. The chickens were kept under observation and were monitored over a week before the experimental phase commenced.

4.2.2. Bacterial isolate used for challenge

The same bacterial isolate (*Av. paragallinarum* SA-3 (serovar C-3)) from the same supplier, using the same clearance procedures in accordance with the Department of Agriculture, Forestry and Fisheries (DAFF), for the importation of the bacterial strain were conducted as in Section 3.2.2, Chapter 3. The bacterial strain was not re-imported since it would have further delayed the project and there was also no need, as it was confirmed from our previous experiment (Chapter 3) that the correct bacterial isolate was being used for experimentation and there were also several back-up freeze-dried isolates. However, a new freeze- dried vial containing the bacterial isolate of *Av. paragallinarum* serovar C-3 (SA-3

strain) was used to ensure that the culture was not passaged several times to prevent any potential loss of virulence.

4.2.3. Microbial cultivation and identification

The same techniques and methodology as in Chapter 3 were used for microbial cultivation of *Av. paragallinarum* serovar C-3 (Section 3.2.3.1), genomic DNA extraction (Section 3.2.3.2), identification of bacterial strain (Section 3.2.3.3), agarose gel electrophoresis and visualisation of correct DNA fragment size (Section 3.2.3.4); and the sequencing of 16S rDNA PCR products (Section 3.2.3.5) for Chapter 4. A few modifications were made pertaining to the challenge dose to be administered and the holding temperature of the challenge dose containing *Av. paragallinarum* serovar C-3 (SA-3 strain) before and while injecting the chickens, to keep the bacterial colonies alive and in favourable conditions.

The bacterial culture containing *Av. paragallinarum* serovar C-3 (SA-3 strain) was standardised to an OD_{600} of 1.0. Contamination checks were carried out throughout each cultivation step, to prevent cross-contamination. However, the minimum requirement of 10^8 colony forming units (CFUs) per ml needed to be injected into the chickens to reach the threshold host density for disease to occur, which implies that there needs to be a significant concentration of the bacteria to cause infection.

Sterile TSA plates supplemented with 0.2% NAD^+ (v/v) (Merck) were prepared, whereby TSB (Merck) was mixed with bacteriological agar (Sigma-Aldrich) and autoclaved at 121°C for 15-20 min. After the media had been autoclaved for sterilisation, the TSA medium was placed at 55°C, until the agar cooled down. After cooling, NAD^+ (v/v) was added by filter

sterilisation to a final concentration of 0.2% (v/v), mixed and plates were poured. Standard cultivation techniques were used, whereby a pre-inoculum was prepared that contained a bacterial culture of less than 24 h, which was then inoculated into a flask containing 500 ml of TSB supplemented with 0.2% NAD^+ (v/v) for a further 10-14 h with continuous shaking of the flask at 120 rpm at 37°C (Labwit Scientific), grown to an OD_{600} of 1.0. Using 600µl of the bacterial culture obtained, a ten-fold serial dilution (10^0-10^{-8}) using a volume of 60 µl of the bacterial sample and 540 µl of diluent (sterile TSB supplemented with 0.2% NAD^+ (v/v)). Once the serial dilution was prepared, 100 µl (0.1 ml) of each serial dilution sample was pipetted onto the TSA supplemented with 0.2% NAD^+ (v/v) surface and spread around using a sterile glass rod, in the presence of a Bunsen burner. Only a volume of 100 µl of each sample dilution was pipetted onto a plate. The plate spreading was performed in triplicate for the original sample and for each of the dilutions. Once all the plates had been prepared, they were left to dry and were then moved to the incubator at 37°C for 48 h for the microorganism being studied. The incubation time depends on the organism and the growth medium, however during the incubation, each viable cell that was spread to a discrete position on the agar surface would grow and divide many times to form a visible colony of microorganisms.

Following the incubation period, the number of colonies was counted to determine how many microorganisms were present in the original sample. Depending on the dilution of the sample, the plates would have different numbers of colony forming units. If there were too many colonies it was impossible or very difficult to count them and the count was designated too many to count (TMTC). If there were only a small number of colonies it was easy to count the number of colonies, but the results were prone to error and the count was designated as too few to count (TFTC). Colonies between 30 and 300 were counted. The results were recorded noting the dilutions that had between 30 and 300 colonies and how many colonies there were on these plates. To determine how many viable microorganisms

were in the original sample, the number of CFU per plate, the amount by which the sample was diluted and the volume that was added onto the plate was needed. Hence, the cell count per ml= (cell count ($30 \leq CFU \leq 300$) x dilution factor x factor of 10 (to convert a 100 µl sample to a 1000 µl sample)). From the cell count, the bacterial culture volume was estimated to produce enough bacteria to infect the total number of chickens at 10^8 CFU/ml or more.

A fresh and sterile batch of bacterial culture was then cultivated at a volume of 500 ml in flask, according to standard cultivation techniques, whereby a pre-inoculum was prepared that contained a bacterial culture of less than 24 h, which was then inoculated into a flask containing 500 ml of TSB supplemented with 0.2% NAD^+ (v/v) for a further 10-14 h with continuous shaking of the flask at 120 rpm at 37°C (Labwit Scientific), grown to an OD_{600} of 1.0. The 500 ml bacterial culture was then centrifuged at 3000 x *g* for 10 min, to obtain a pellet which was re-suspended in 5 ml of sterile TSB supplemented with 0.2% NAD^+ (v/v) which was kept at 37°C. A volume of 1 ml of the bacterial suspension was kept for bacterial identification and the rest was used for the infection of chickens on the same day.

4.2.4. Challenge methods and clinical scoring

The challenge methods, disease and clinical scoring and egg production indices for this study were conducted as in Section 3.2.4, Chapter 3. For this study, the control group was injected using 100 µl of sterile TSB supplemented with 0.2% NAD^+ (v/v) only and the experimental group was injected with 100 µl of TSB supplemented with 0.2% NAD^+ (v/v) containing a suspension of *Av. paragallinarum* C-3 serovar (SA-3) bacterial cells at an optical density OD_{600} of 1.0 and containing at least 10^8 CFU/ml injection. The 16 h old bacterial culture was prepared and constantly kept at 37°C before being injected via the

infra-orbital route in chickens. The preparation of the challenge dose and administration of the challenge dose to chickens was conducted on the same day. Following infection, the signs and symptoms were monitored, to ensure that the chickens had been exposed and infected to solely *Av. paragallinarum* serovar C-3 (SA-3). Clinical scoring was conducted daily on a monitoring sheet for both groups (Appendix A), for a total period of 21 days. Blood was collected after every 2-4 days, based on the disease score and symptoms observed in chickens. On Day 8, 5 chickens were randomly selected for re-infection with a 48 h bacterial culture as described (Section 3.2.3.1 and 4.2.3). For each scoring obtained, chickens were unbiasedly/randomly selected from the total population of experimental subjects, where 2-4 chickens showing the same disease score were sacrificed for post-mortem examination and histopathology of lymphoid organs (Chapter 5), whereby the rest of the chickens were left for the disease to progress further. This process was repeated until a score of 3 was observed. The total number of eggs produced, and the total number of eggs laid per bird per day from the control and experimental group were also carefully recorded.

4.2.5. Blood collection and processing

Blood collection and processing was conducted as described in Section 3.2.5, Chapter 3. Whole blood samples were collected and processed within 6-8 h. The collected blood (maximum 4 ml) was then separated into three parts into separate 1 ml EDTA coated SGVac PET Blood Collection Tubes (The Scientific Group) with a maximum volume of approximately 1 ml per tube for flow cytometry, blood microscopy and to obtain plasma to perform sandwich enzyme-linked immunosorbent assays (ELISAs). Plasma was obtained by centrifugation at 3000 x *g* for 15 min, to separate the blood components from plasma and was then stored at -80°C for long-term storage and until further use.

4.2.6. Avian blood smears and microscopy

EDTA tubes containing a volume of 1 ml of the blood sample collected from each experimental subject, were sent to the National Health Laboratory Service (NHLS, Universitas, Bloemfontein, South Africa). Blood smears were conducted according to the SOP described by the NHLS as in Section 3.2.6, Chapter 3. Visualisation of the blood smears was conducted with the Eclipse 50i microscope, DS-Fi1 digital microscope camera and NIS-Elements F 4.00.06 Build 786 microscope imaging software (Nikon), whereby images were taken at a 100X magnification.

4.2.7. Flow cytometry and antibodies

Flow cytometric analysis was performed as described in Section 3.2.7, Chapter 3. The following mouse anti-chicken antibodies were used: CD4-FITC (2-35 clone, MCA2164F), CD8-RPE (11-39 clone, MCA2166PE), CD45-RPE (UM16-6 clone, MCA2413PE) (Bio-Rad). A CD45 pan-leukocyte marker was used in this study to render the gating of the leukocyte population easier, as the RBCs in avian blood are nucleated and could overlap with the leukocyte population making it difficult to differentiate between cell populations and gate the leukocyte population. Moreover, the CD45 pan-leukocyte marker will only bind to leukocytes (lymphocytes, granulocytes, and monocytes) and will not bind to erythrocytes (RBCs) or thrombocytes (platelets) expressing the CD45 marker.

4.2.8. Sandwich enzyme-linked immunosorbent assay (ELISA)

Following collection of chicken plasma from Section 4.2.5, a sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of IL-8 (interleukin 8) was performed according to manufacturer's instructions (E-EL-Ch1234, Elabscience®). The micro ELISA

plate that was provided had been pre-coated with an antibody specific to IL-8. All reagents and samples were brought to room temperature before use. The Reference Standard was prepared within 15 minutes before use. The Reference Standard was centrifuged at 10 000 x *g* for 1 min and was reconstituted with 1 ml of the Reference Standard and Sample Diluent (which is the name of the diluent). The lid was tightened, and the sample was left to incubate at room temperature for 10 minutes and inverted several times. After the pellet dissolved, a pipette was used to mix the contents thoroughly. The reconstituted sample produced a stock solution of 1000 pg/ml. From the stock a two-fold serial dilution was prepared in 1.5 ml tubes at different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.63, 0 pg/ml), whereby the Reference Standard and Sample Diluent (which is the name of the diluent) served as a zero (0 pg/ml).

A volume of 100 µl of the Standard, blank or chicken plasma was added per well, whereby each sample was conducted in duplicate. Reference Standard and Sample Diluent were added to the blank wells. Wells containing a negative control serum, no plasma control, no avidin-horseradish peroxidase (HRP) conjugate and empty wells were included in the assay as quality controls and for troubleshooting purposes. There was no known positive control serum available, however the Reference Standard at a concentration of 1000 pg/ml for IL-8 served as a positive control as it had a known concentration of IL-8 and also gave a positive colour change during the assay. A plate sealer was used to cover the plate, which was then followed by gentle mixing with the Mini BioMixer (Benchmark Scientific). The plate was then incubated for 90 min at 37°C. The removal of liquid was conducted by shaking-off liquid by decanting in one motion or by complete aspiration of the well contents. A volume of 100 µl of Biotinylated Detection Ab working solution was added to each well and covered with a plate sealer. The side of the plate was then gently tapped to ensure thorough mixing and incubated for 1 h at 37°C. Following incubation, the complete removal of liquid was conducted by decanting in one motion or by complete aspiration of the well contents. The

plate was then washed with a volume of 350 µl of Wash Buffer, followed by decantation of the plate. The process was repeated three times. After the last wash, the plate was inverted and patted against a thick clean absorbent paper. A volume of 100 µl of HRP Conjugate working solution was added to each well and covered with a plate sealer. The plate was then incubated for 30 min at 37°C. Following the incubation step, the plate contents were removed by decantation and the plate was washed with a volume of 350 µl of Wash Buffer, whereby this process was repeated five times. After the last wash, the plate was inverted and patted against a thick clean absorbent paper. A volume of 90 µl of Substrate Solution was added to each well, the plate was covered with plate sealer and was incubated for approximately 15 minutes at 37°C. Once, an apparent blue colour gradient appeared in the wells, the reaction was terminated by addition of 50 µl of Stop Solution to each well. The colour of the solution changed from blue to yellow after the Stop Solution was added. The absorbance values of the wells at a wavelength of 450 nm was measured using the ELx800™ plate reader with Gen5™ software (BioTek). Statistical analysis was performed to determine the statistical significance of the data, whereby the mean and standard deviation of the data were calculated and a $p<0.05$ value was found to be statistically significant, from which a graph was plotted. The unused reagents were then stored at -20 °C.

4.3. Results and discussion

4.3.1. Microbial cultivation and identification

Tiny dewdrop colonies and satellitic behaviour adjacent the *S. epidermidis* "feeder" cultures on the BTA plates were observed, typical of *Av. paragallinarum* (Figure 4.1) as described in literature (Blackall *et al*. 1997). As bacterial cultures were grown in 2-5 flasks, only flasks with an OD_{600} closest to 1.0 was selected for the first (1IC: $OD_{600\ (flask)}$= 0.958) and second

injection (2IC: $OD_{600\ (flask)}$= 0.964). Turbidity in cultures was checked for any bacterial contamination, whereby no contamination was found.

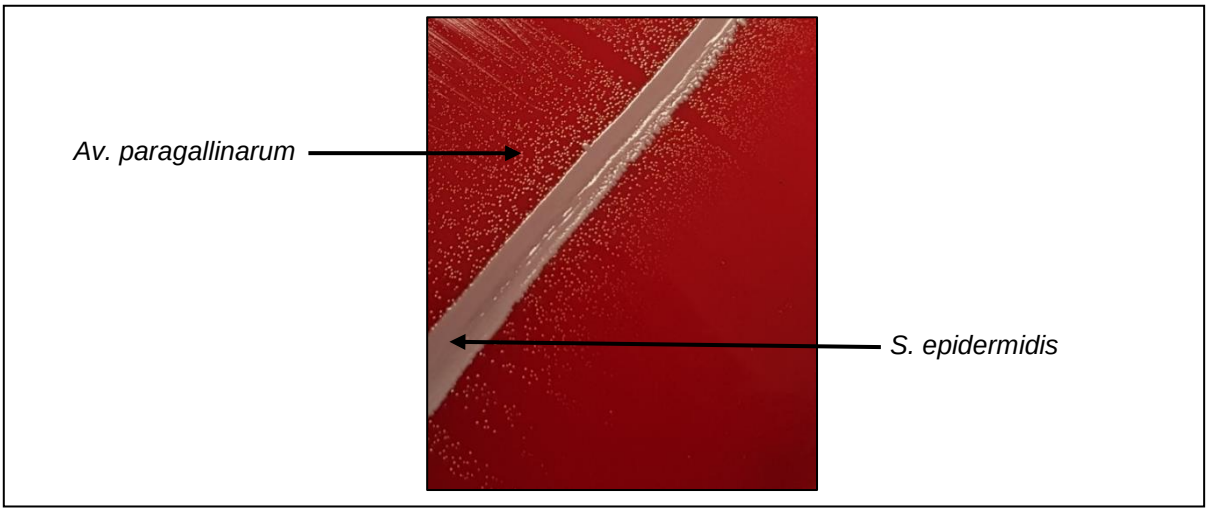


Figure 4.1: Cattle blood tryptose agar plate with *Av. paragallinarum*. Tiny dewdrop colonies are observed near the cross-streaked "feeder" cultures thus displaying satellitic behaviour.

Table 4.1: Cell count and CFU/ml results for triplicate plating of bacterial culture for the first injection.

Serial dilution	Average plate count/cell count	Within Range of 30 and 300 cells	CFU/ml
10^{0}	>300	TMTC	-
10^{-1}	>300	TMTC	-
10^{-2}	>300	TMTC	-
10^{-3}	>300	TMTC	-
10^{-4}	>300	TMTC	-
10^{-5}	>300	TMTC	-
10^{-6}	109	30<109<300	1.09×10^{9}
10^{-7}	12	TFTC	-
10^{-8}	2	TFTC	-

Table 4.2: Cell count and CFU/ml results for plating of bacterial culture for the second injection.

Serial dilution	Average plate count/cell count	Within Range of 30 and 300 cells	CFU/ml
10^{0}	>300	TMTC	-
10^{-1}	>300	TMTC	-
10^{-2}	>300	TMTC	-
10^{-3}	>300	TMTC	-
10^{-4}	>300	TMTC	-
10^{-5}	>300	TMTC	-
10^{-6}	69	30<69<300	6.90×10^{8}
10^{-7}	2	TFTC	-
10^{-8}	0	TFTC	-

*TMTC: Too many to count; TFTC: Too few to count

The cell count from 100 μl from each of the cell cultures for the first and second injection was determined by plating out (Figure 4.2) and calculated, whereby the results were recorded (Table 4.1 and Table 4.2). Hence, using the formula for cell count per ml= (cell count (30≤CFU≤300) x dilution factor x factor of 10 (to convert a 100 µl sample to a 1000 µl sample)), the cell count per ml was determined (Table 4.1 and Table 4.2). Since, 500 ml of the bacterial culture was grown and was then concentrated to a volume of 5 ml, the cell concentration increased 100x. From the cell count per ml results obtained, it was evident that there was greater than 10^{8} CFU/ml of viable cells to invade the upper respiratory system of the host to cause disease following infra-orbital injection, especially since the microorganism is also a highly virulent serovar (Table 4.1 and Table 4.2). The cell count also enabled us to know approximately how many viable cells were injected into the chickens,

instead of relying solely on the OD_{600} based on the turbidity of bacterial growth (Table 4.1 and Table 4.2).

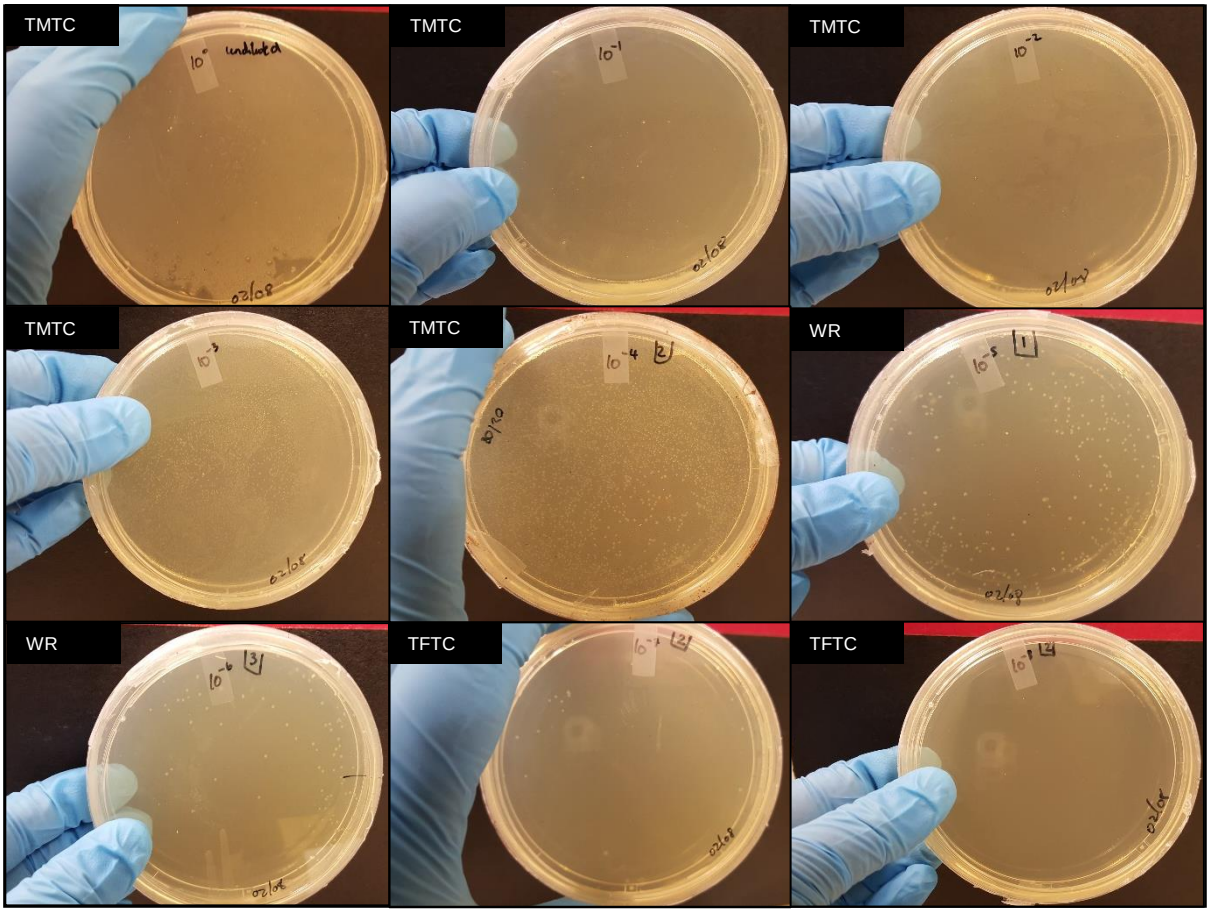


Figure 4.2: Tryptic soy agar supplemented with 0.2% (v/v) NAD^+ (TSA) plates showing growth of *Av. paragallinarum* serovar C-3 at different concentrations 10^0-10^{-8} when a serial dilution was performed in triplicate, however only one of the plates from each dilution was shown. Tiny colonies visible to the naked eye can be seen and the cell count can be determined. *TMTC: Too many too count; WR: Within the range of 30 and 300 colonies; TFTC: Too few to count.

A species-specific PCR was performed, using the DNA extracted from 800 µl of the bacterial culture obtained from the flasks with broth (TSB) (Merck) supplemented with 0.2% (v/v) NAD^+ (Merck) containing a bacterial suspension of C-3, for the first and second injection

respectively. The species-specific PCR/ HPG2-PCR was performed with the DNA extracted from the reference isolate *Av. paragallinarum* serovar C-3 (SA-3 strain) cultivated, whereby an expected amplicon size of 500 bp was obtained indicative that the desired microorganism was present (Figure 4.3).

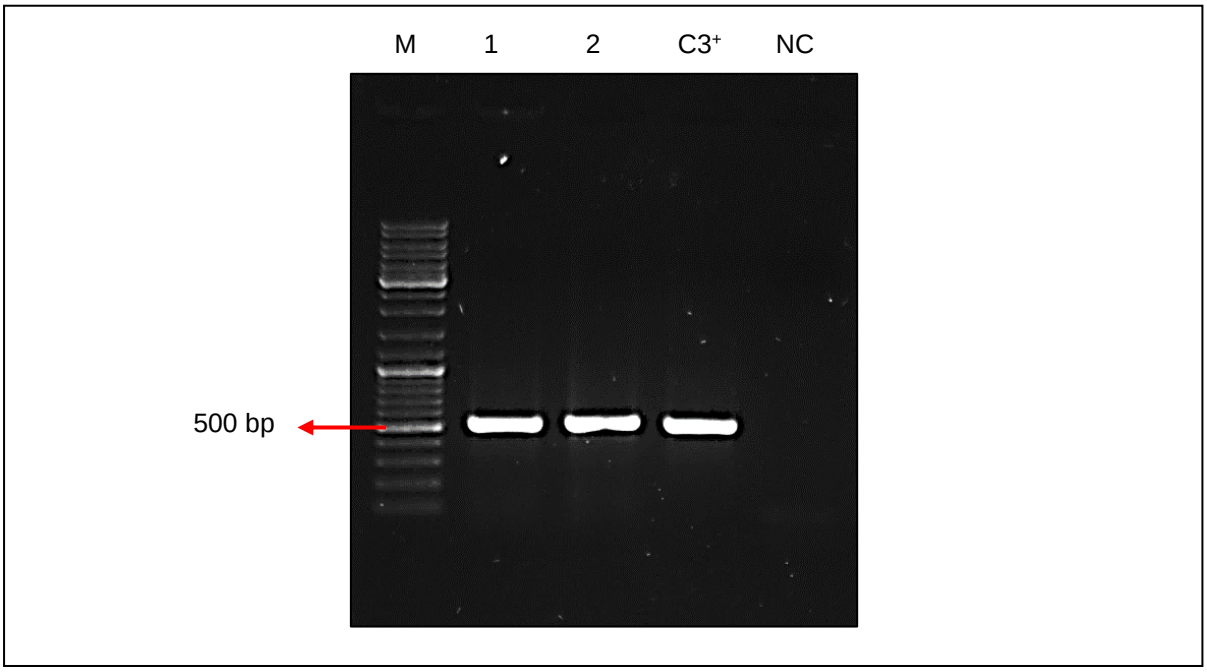


Figure 4.3: HPG2-PCR for the SA-3 (C-3) reference isolate cultivated for the experimental trial, whereby amplification was observed for all samples, with an expected band size of 500 bp. Lane M- molecular marker O'GeneRuler™ DNA Ladder; lane 1: SA-3 strain used for first injection (1IC); lane 2: SA-3 strain used for second injection (2IC); $C3^+$: SA-3 strain serovar C-3 positive control; lane NC: negative control.

The identity of the *Av. paragallinarum* serovar C-3 (SA-3 strain) reference isolate was confirmed using 16S rDNA amplification (Section 3.2.3.5, Chapter 3; Section 4.2.3, Chapter 4). PCR products of an amplicon size of 1500 bp were obtained for all samples (Figure 4.4), indicating positive amplification of the DNA template. Following amplification of samples with 16S rDNA PCR, the gel slices (Figure 4.5) which were excised were then purified (Section 3.2.3.5, Chapter 3; Section 4.2.3, Chapter 4) and Sanger sequencing was conducted on the purified DNA samples. Sequences obtained, were analysed using Geneious® 9.8.1

(Biomatters Ltd.) (Kearse *et al*. 2012). Sequences of each sample were aligned and the consensus sequence was compared with known sequences in GenBank, using a nucleotide BLAST analysis program (Appendix C) (Altschul *et al*. 1990). A homology of 100% between reference sequences and the queried sequences, indicated DNA-DNA relatedness and as such the identity of the bacterial strain of interest could be known to a species level.

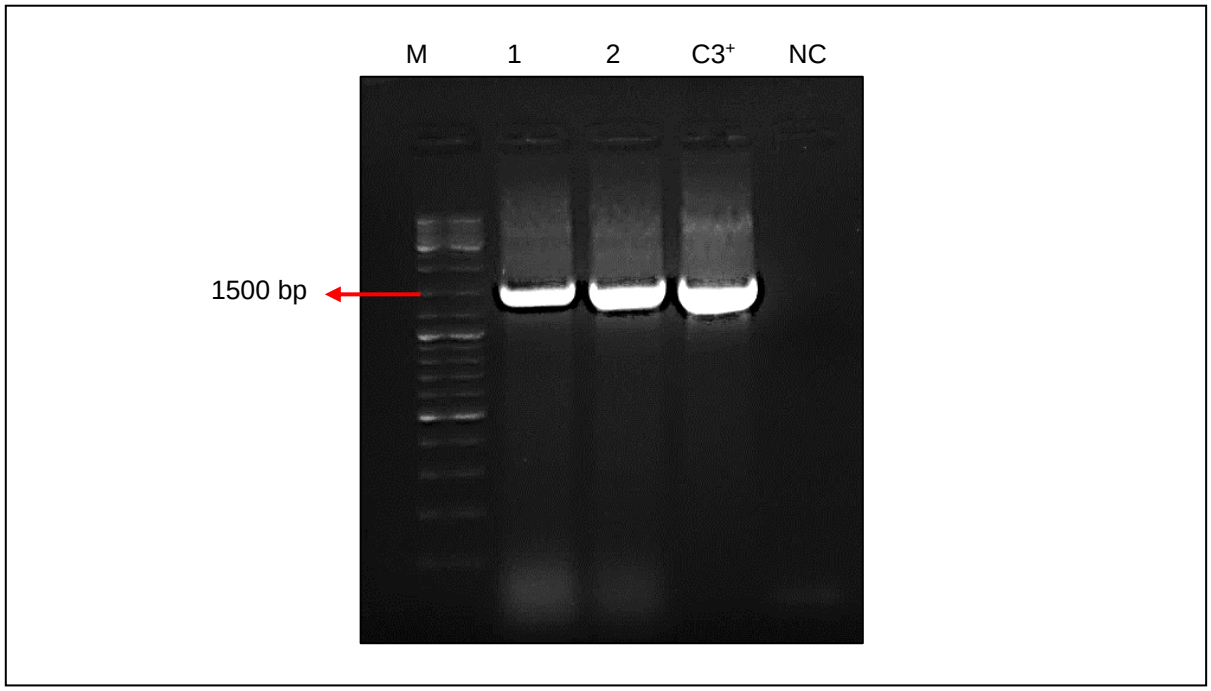


Figure 4.4: 16S rDNA PCR for the SA-3 (C-3) reference isolate cultivated for the experimental trial, whereby amplification was observed for all samples, with an expected band size of 1500 bp. Lane M-molecular marker O'GeneRuler™ DNA Ladder; lane 1: SA-3 strain used for the first injection (1IC); lane 2: SA-3 strain used for the second injection (2IC); lane $C3^+$: SA-3 strain positive control; lane NC: negative control.

The sequencing results for the samples were recorded in Table 4.3. Finally, using the results from both the species-specific PCR and the BLASTn analysis of the 16S rDNA sequencing results, it was found that the data corresponds to that of *Av. paragallinarum* strain SA-3 in the database. BLASTn results show that the strain of interest shares 100% correlation to the

Av. paragallinarum strain SA-3 16s rDNA gene from the database, hence the results coincide and support that the strain used for the study was that of *Av. paragallinarum* serovar C-3 (SA-3 strain) (Table 4.3). The identification of the bacterial species of interest was very important, since during the infection of the chickens it was crucial that the *Av. paragallinarum* SA-3 strain be the main avian pathogenic organism, as having other microorganisms in the bacterial culture injected might have led to secondary infections or no infection, and could have jeopardised the validity of the study, since clinical symptoms of IC might have been masked or not seen at all.

Table 4.3: Nucleotide BLAST results for all 16S rDNA PCR products for the experimental trial with species identification, GenBank® accession numbers, query length, query coverage, E-value and high sequence identities.

Sample	Isolate/Species	Accession number	Query length (bp)	Query coverage	E-value	Identity
1IC	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	640	100%	0.0	100%
2IC	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	649	100%	0.0	100%
C3^{+}	*Avibacterium paragallinarum* strain SA-3 16S ribosomal RNA gene, partial sequence	KC951277.1	644	100%	0.0	100%

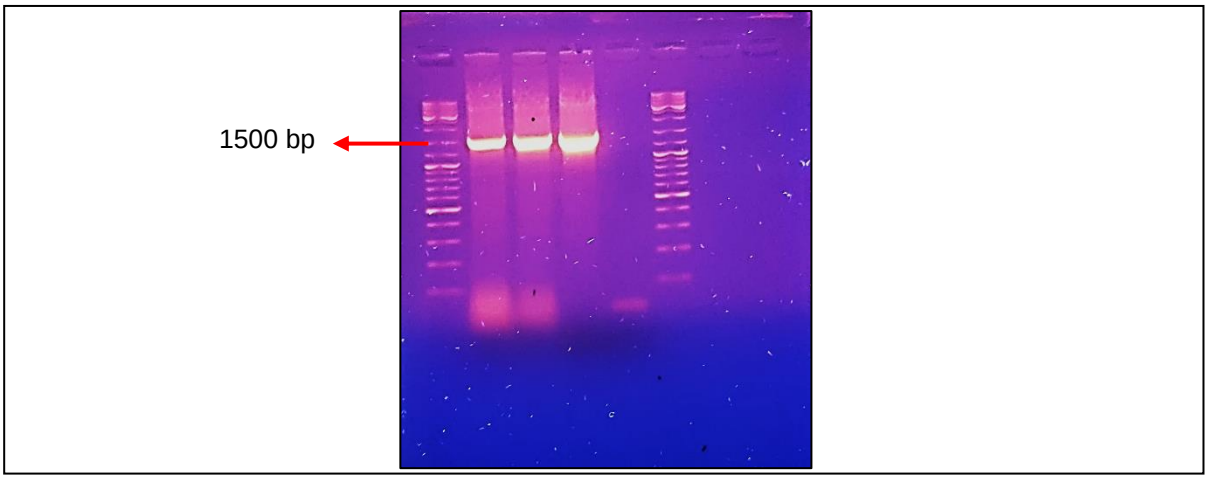


Figure 4.5: Agarose gel visualisation of 16S rDNA PCR products for the SA-3 (C-3) reference isolate cultivated for the experimental trial when viewed using a UV transilluminator (Spectroline®). The correct gel fragments were cut out using a sterile surgical scalpel blade, the excised gel slices were then purified, whereby the purified DNA samples were used for Sanger sequencing.

4.3.2. Challenge methods and clinical scoring

Once all chickens were injected accordingly, they were closely monitored for a period of 21 days for any visible IC related symptoms, the daily mean disease score was recorded during disease progression for both the experimental and control groups and a disease profile was plotted (Figure 4.6) and a summary of the total number of chickens and scores can be viewed (Table 4.4). After 24 h (Day 1), facial oedema as a result of an initial inflammatory response, was observed around the site of injection with all experimental chickens. Thus, all experimental chickens reached a mean disease score of 1. Symptoms associated with a score of 1 in the experimental chickens also included lethargy and diarrhoea, however the most spot-on indication of a score of 1 was the serous to mucoid nasal discharge observed on the left side of the facial area, which corresponds to the side which was injected (Figure 4.7).

Contrary to the study in Chapter 3, the signs and symptoms of the experimental chickens infected with IC did progress from mild (score 1) to moderate (score 2) to severe (score 3) within a period of 18 days similar to the disease progressions as described in literature (Bragg, 2004; Blackall and Soriano, 2008). After Day 4, some chickens recovered, while the rest progressed to a score of 2. Affected chickens with score 2 had a cheese-like mucoid nasal discharge on both sides of the nasal cavity with slight facial oedema, lethargy, diarrhoea and swollen combs and wattles (swollen head-like syndrome). Chicken E14 at a score of 2, had difficulty in breathing, rales and disorientation in addition with symptoms such as bilateral nasal discharge, poor appetite and facial oedema. Chicken E29 was presented with rales and sneezing in addition to facial oedema and bilateral nasal discharge On Day 8 and 9 respectively, the mean disease score was 1.9, which implies that the majority of the experimental cohort reached a score 2 and score 3 respectively (Figure 4.6). After Day 14, some chickens remained on a score 1 and 2, while very few chickens progressed to a score 3. Only a minority of chickens reached a score of 3, which can be observed on Day 18, where the daily mean disease score was 2.5, whereby 12 out of the remaining 23 chickens had a score 3, 10 out of 23 had a score 2 and 1 out of 23 had a score 1. Chickens presented with score 3 had bilateral facial oedema with or without haemorrhage, poor appetite and conjunctivitis (Figure 4.7). There were no mortalities from IC in this study.

On two occurrences, on the disease profile there was a sharp decline or drop observed in the disease progression trend, resulting in recovering chickens (Figure 4.6). The first decline on the disease profile was seen on Day 4 where the mean disease score changed from a score 1 on Day 3 to a score 0.6 on Day 4, which is then followed by an increase in the mean disease score on Day 5 to a score of 1.7 followed by a relatively stable disease progression trend over the next 8 days (Figure 4.6). The reason for this sharp decline on Day 4, may be attributed to the innate immune response that is active during those first few days following infection, whereby the first-line of defence includes release of immune cells (phagocytes,

dendritic cells, heterophils and natural killer cells) and immune molecules (complement, collectins, acute phase proteins, cytokines) that have antibacterial properties, that are involved in inflammatory responses and that interact with the adaptive phase of the avian immune system (Playfair and Bancroft, 2013). The second significant drop on the disease profile was observed on Day 14, whereby from Day 11 the mean disease score gradually declined from a score of 1.9 to a score of 1.1, over the course of 3 days (Figure 4.7). After Day 14, the mean disease score then increases from a score of 1.1 to a score of 2 on Day 15 (Figure 4.7).

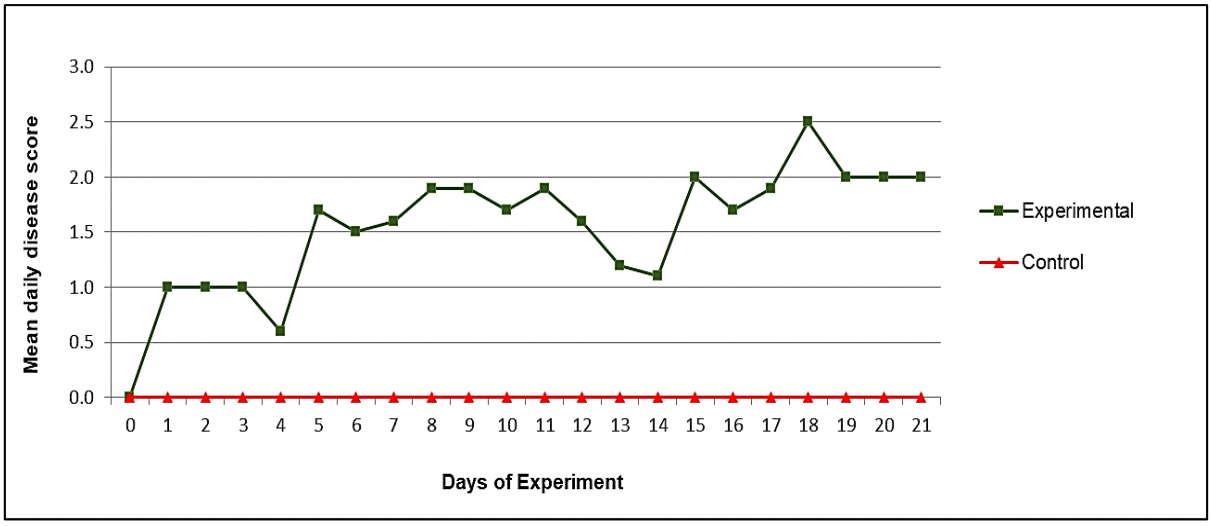


Figure 4.6: The disease profile showing the daily mean disease score of the control (red) and experimental (green) cohorts. The trend of the control group was consistent at a score 0 throughout the duration of 21 days, as they were not challenged or exposed to C-3 isolate. The experimental group had a different trend whereby an initial innate immune response was observed on Day 4 and an active adaptive immune response occurred on Day 14, resulting in recovering chickens given that IC does not cause mortality.

Table 4.4: Summary of disease progression over the course of 21 days (3 weeks) and the total number of chickens with clinical scores from each cohort. Chickens randomly selected were sacrificed after a clinical score was obtained. At the end of the study the control chickens, with no IC related symptoms at a score 0 were sacrificed.

Day	Experimental chickens (N_E= 30)				Control chickens (N_C=8)			
	Score 0	Score 1	Score 2	Score 3	Score 0	Score 1	Score 2	Score 3
0	30/30				8/8			
1		30/30			8/8			
2		30/30			8/8			
3		29/29			8/8			
4	11/29	18/29			8/8			
5		9/26	17/26		8/8			
6		13/26	13/26		8/8			
7		11/26	15/26		8/8			
8		8/26	12/26	6/26	8/8			
9		11/26	8/26	7/26	8/8			
10		10/26	13/26	3/26	8/8			
11		8/24	11/24	5/24	8/8			
12	1/23	11/23	8/23	3/23	8/8			
13	2/23	15/23	5/23	1/23	8/8			
14	6/23	9/23	8/23		8/8			
15		7/23	9/23	7/23	8/8			
16	4/23	6/23	6/23	7/23	8/8			
17	2/23	5/23	9/23	7/23	8/8			
18		1/23	10/23	12/23	8/8			
19	1/23	1/23	17/23	4/23	8/8			
20	1/23	1/23	18/23	3/23	8/8			
21	1/23	1/23	18/23	3/23	8/8			

*N_E= Initial total number of chickens in the experimental group; N_C= Initial total number of chickens in the control group

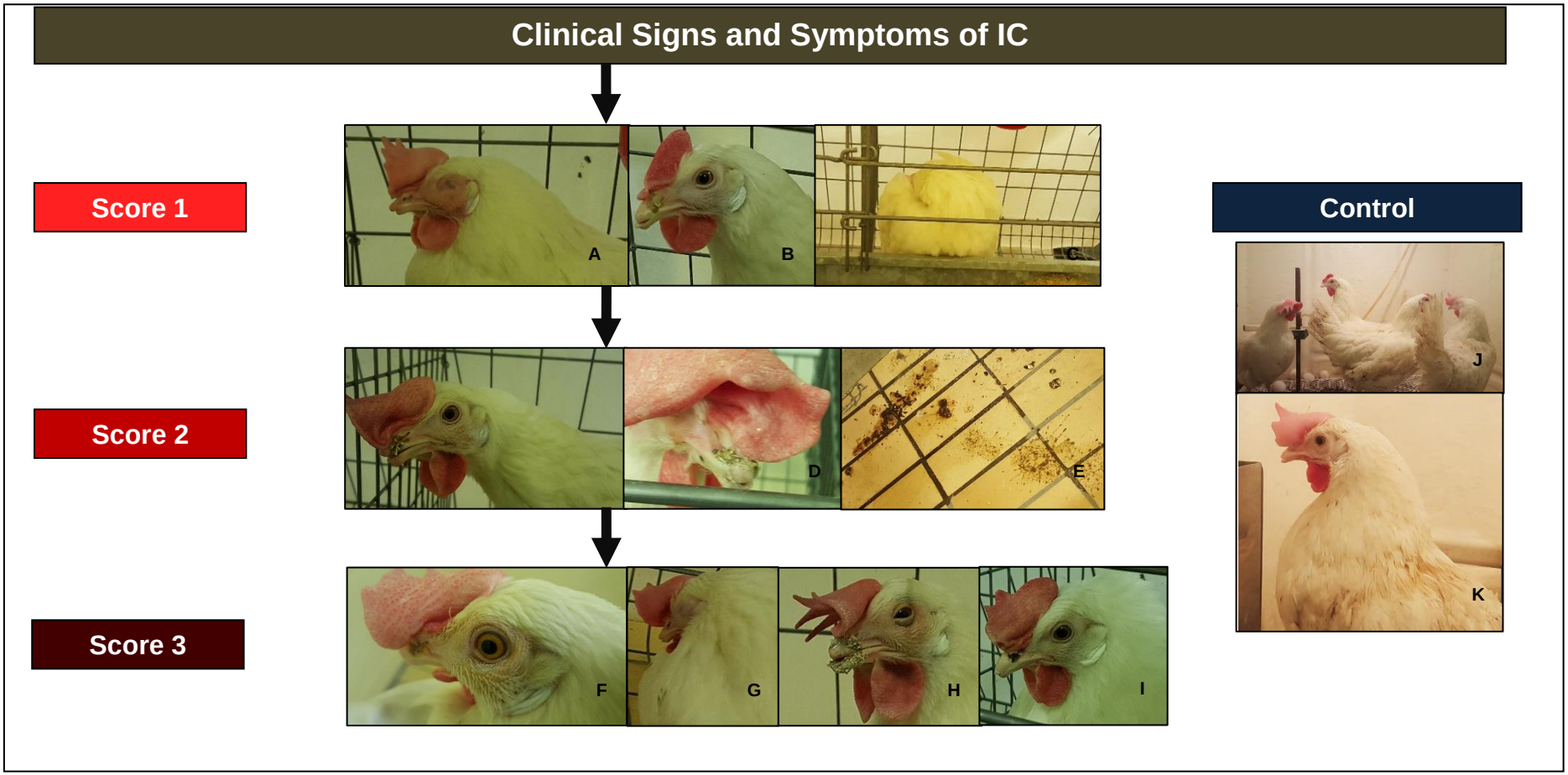


Figure 4.7: Clinical signs and symptoms observed in chickens presented with IC. Score 1 chickens displayed mild clinical signs and symptoms such as (A) facial oedema, (B) nasal discharge on one side of the nasal cavity and (C) lethargy. Score 2 chickens showed moderate symptoms such as (D) bilateral nasal discharge with swollen comb and (E) diarrhoea. Score 3 chickens were afflicted with (F) severe bilateral facial oedema, (G and H) conjunctivitis and bloody nasal exudates due to haemorrhage. Control chickens were kept separate in isolators and did not have any IC related symptoms, hence they were given a score 0 (J and K).

This second decline in the disease trend is due to the adaptive immune system that is highly active during the last stages of IC infection following the innate immune response, whereby the humoral response associated with B-cells and antibody production, and the cell-mediated response involving T-cells associated with cell proliferation, cell differentiation, cytotoxic activity and cytokine secretion; have a vital and active role to play (Playfair and Bancroft, 2013). The adaptive immunity also leads to memory B and T cells, important since a second exposure to IC would lead to a more rapid immune response or even resistance to IC infection, as a result of an already well-established immune response.

The scoring for the control group over the course of 21 days, was stable at a constant mean disease score of 0. No clinical signs and symptoms of IC were observed, with all the control chickens being healthy, as they were not challenged with or exposed to *Av. paragallinarum* serovar C-3 (SA-3 strain). This was a good indication that no cross-contamination occurred between the two cohorts and that all necessary measures taken while handling the two cohorts was effective. The control chickens also had a good appetite with feed and water provided on a daily basis, compared to the infected chickens that had a poor appetite for feed with increased water uptake.

Following the first injection, 5 chickens E15 (score 2), E16 (score 2), E24 (score 3), E26 (score 2) and E28 (score 1) respectively, were injected a second time on Day 8. The chickens were injected a second time to observe whether the second dose of bacterial culture would lead to disease escalation. However, after 24 h (Day 9), the chickens which received the second injection of *Av. paragallinarum* serovar C-3 (SA-3 strain) only suffered from inflammation at the site of injection and diarrhoea. Re-injection of the 5 chickens did not cause the disease to progress any further, and as such the disease progression of the re-infected chickens was the same as the rest of the experimental group that were at score 2.

Chicken E24 stayed at a score 3, 2 days after being injected. Chickens E15 and E16 did reach a score of 3, 2 days after being injected, with the rest of the experimental group. Chicken E26 remained on score 2 and E28 on score 1, were there was no disease progression. A probable explanation for disease progression not becoming severe could be due to an actively running innate immune response that has already activated the adaptive immune response within the first week of infection, since these two systems are integrated. The innate immune system makes an important contribution to the activation of adaptive immunity via the inflammatory response caused by macrophages that secrete cytokines that increase vascular permeability when they encounter bacteria (Janeway *et al*. 2005). Vascular permeability allows antigen to flow into lymphoid tissues, thereby activating lymphocytes (Janeway *et al*. 2005). Moreover, the induction of the adaptive immune system starts when the bacteria is phagocytosed by an immature dendritic cell (Janeway *et al*. 2005). Upon phagocytosis, the dendritic cell becomes activated and matures into an antigen presenting cell (APC), that presents antigen to T lymphocytes (Janeway *et al*. 2005).

The total number of eggs laid per chicken per day was recorded for both experimental and control groups. On Day 9 and 18, there was a slight drop in the egg-laying trend for the experimental group (Figure 4.8). However, no statistically significant decline in egg production with infected chickens was observed, as mentioned in literature, as the chicken sample size in this study was too small to deduce the effect of IC on egg production, whereas in the extensive chicken industry farmers have larger flocks to work with (Blackall and Soriano, 2008). Moreover, as the study was conducted for only 21 days, the long-term effects on egg production caused by IC was not investigated. For the control group, over the course of 3 days (Days 1 to 3), it was observed that the chicken egg production was higher than expected, resulting in no eggs being laid on Day 4. However, after Day 4, a normal egg laying trend was observed resulting in 1 egg laid per day per chicken. The reason for this phenomenon could be attributed to human error, as it was possible that eggs laid the night

before could have remained hidden from view and counted with the rest of the eggs laid on the next day, resulting in more eggs being counted, as four chickens were kept together in one isolator. Compared to the control group, the experimental group had a more stable egg-laying trend, whereas the control group had a highly variable egg-laying trend (Figure 4.8).

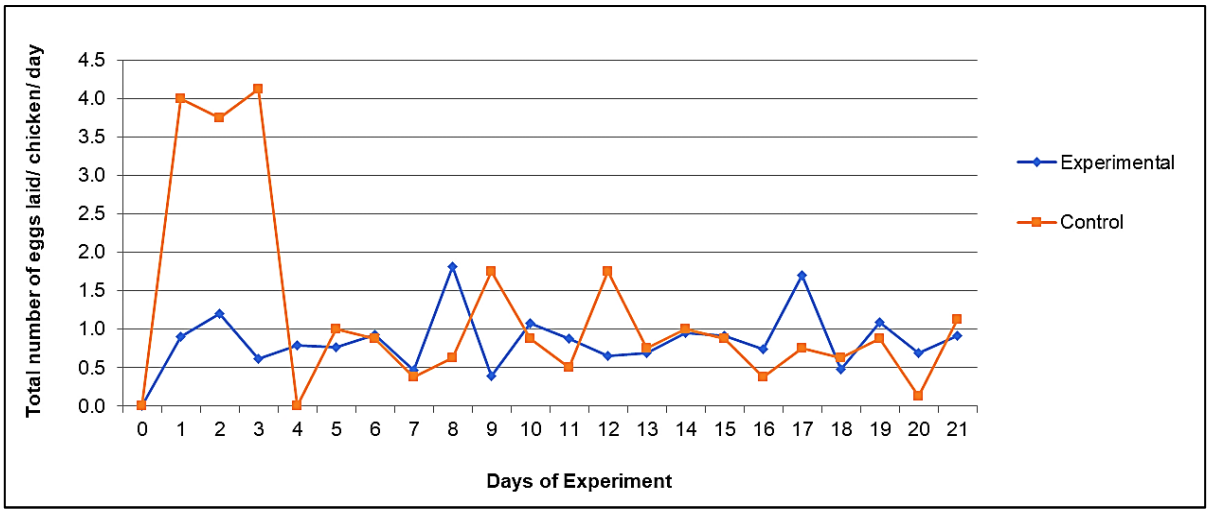


Figure 4.8: Egg production indicated as the total number of eggs laid/chicken/day in the experimental and control groups. The experimental group had a more consistent egg-laying trend in comparison to the control group that had a highly variable egg-laying trend, thus making it difficult to deduce any significant egg-laying patterns.

4.3.3. Avian blood smears and microscopy

Following the principles of the "Three Rs" in animal research (Reduction, Refinement and Replacement), to minimise stress, harm and pain to the chickens during blood collection, blood was drawn from the chickens every 1-4 days and the blood was collected in EDTA coated SGVac PET Blood Collection Tubes (The Scientific Group). Unfortunately, the chickens could not be bled every day, this was due to a haematoma being formed each time a needle was inserted into a vein and the vein collapsed, during blood collection. The haematoma formed takes approximately a week to recover. Hence, to overcome this

problem chickens were randomly selected for bleeding based on the clinical scoring usually from one wing. In case the same chicken had to be bled again the other wing would be available for phlebotomy.

The experimental chickens were bled on Days 0, 1, 3, 4, 7, 10, 11, 15, 18 and 21. For the control group, as there were only a few chickens and we did not want to risk *Av. paragallinarum* serovar C-3 (SA-3 strain) exposure by opening and closing the doors sealing the incubators frequently as well as causing hematoma formation in a few chickens, hence blood was drawn on Days 0, 11, 15, 18 and 21. To collect the blood, an additional volume of EDTA (0.5 M, Merck) of 1 ml was supplemented to the EDTA tubes to prevent quick coagulation of the blood collected, as chicken blood coagulates quickly due to high levels of calcium present. Following blood collection, the EDTA tubes were quickly inverted 3-4 times to thoroughly mix the blood and EDTA together to prevent coagulation and to minimise clots. Whole blood samples were collected and processed within 6-8 h, so that the samples were not compromised and thus making them unsuitable for flow cytometry analysis (Section 4.3.4) and blood smears.

Blood smears were prepared by and obtained from the NHLS. The blood smears were visualised using the Eclipse 50i microscope, DS-Fi1 digital microscope camera and NIS-Elements F 4.00.06 Build 786 microscope imaging software (Nikon), whereby images were taken at a 100X magnification. The blood smears prepared and obtained from the blood of chickens at different clinical scores on Day 0, 1, 3, 4, 7, 10, 11, 15, 18 and 21, were compiled (Annexure C). From the blood smears, cell morphology of different blood cells based on the staining technique used could be evaluated (Hematek Modified Wright's Stain and Hematek Wright-Giemsa was used) for the classification and differentiation of those blood cells. Figure 4.9 (A-F) shows some of the blood cells observed under the microscope

that have interesting features and functions in the avian immune response following infection with *A. paragallinarum* serovar C-3 (SA-3 strain).

A toxic heterophil (h) was observed with a lymphocyte (ly) indicated by an arrow and a teardrop-shaped red blood (tds) cell in chicken E8 with score 1 on Day 1 (Figure 4.9 A), after 24 h following infection. Heterophils are characterised by brick-red granules found in the cytoplasm and possessing a bilobed nucleus (Fudge, 1998; Harrison and Lightfoot, 2006). However, with the toxic heterophil shown there was a loss of nuclear lobulation and the cytoplasm shows very few granules (Figure 4.9 A). During pathogenic invasion, induction of the innate immune response occurs, which triggers heterophils as the main effector cells to respond to the site of infection via chemokines released and that are actively involved in the phagocytosis of these invading pathogens (Kaiser, 2010), which is also the reason why the toxic heterophil on Day 1 was observed. Moreover, heterophils are the counterparts of the mammalian neutrophil and are polymorphonuclear cells (along with basophils and eosinophils) (Harrison and Lightfoot, 2006). In addition to phagocytosis, heterophils are involved in bactericidal activity in processes such as respiratory burst and degranulation (Kaiser, 2010). Teardrop-shaped red blood cells observed are usually an indication of toxicosis, septicaemia or anaemia due to bacterial endotoxins released from gram-negative bacteria (lipopolysaccharide-LPS) (Fudge and Joseph, 2000; Raetz and Whitfield, 2002).

On Day 7, the blood sample from chicken E22 with score 2, there was an infiltration of lymphocytes (ly) indicated by several arrows and monocytes (mo), and a heterophil (h) as seen in (Figure 4.9 B). The infiltration of lymphocytes and monocytes which were observed, was an indication of an infection, but it could also explain the adaptive immune response that was actively at play on Day 7, as lymphocytes consist of B and T cells (Figure 4.9 B). Lymphocytes are round in shape with a centrally positioned nucleus with a pale blue

cytoplasm when stained with Wright-Giemsa stain. Monocytes are large sized and typically have a round shape with a ratio of 3:1 cytoplasm to nucleus. A normal monocyte with a kidney shaped nucleus eccentrically positioned was observed on the blood smear of chicken E17 with score 3 on Day 10 (Figure 4.9 C).

On Day 11, for chicken E15 with score 3, a bilobed heterophil (h) with lymphocytes (ly) in the periphery blood smear along with teardrop-shaped red blood cells (tds), was observed (Figure 4.9 D). During the activation of heterophils by pathogens or cytokines, there is expression of pro-inflammatory cytokines such as interleukins (IL) (IL-1, IL-6 and IL-8) (Kogut *et al*. 2005; Kogut *et al*. 2006). IL-1 and IL-6 target T and B cells, thus causing acute phase responses, recruitment of other immune cells, phagocyte activation and proliferation of antibody secreting B cells (Playfair and Bancroft, 2008). In addition to this, IL-8 is a potent pro-inflammatory cytokine and stimulator of neutrophil activation and chemotaxis leading to inflammatory reactions in humans, not much is known about IL-8 in avian immunology except that it is involved in mucosal immunity (Borrmann *et al*. 2007). Perhaps this explains why there are lymphocytes in the vicinity of heterophils for Day 1, 7 and 11, which could be due to cytokines acting as chemoattractants for B and T lymphocytes. Thus, this shows that there is an interaction of both the innate immune system via heterophils and cytokines; and the adaptive immune system through the recruitment of B and T lymphocytes leading to the antibody and cell-mediated responses.

A basophil (ba) was shown with an unlobed nucleus for chicken E22 with score 3 on Day 11 (Figure 4.9 E). The basophil observed was characterised according to the presence of variable large and small, round and dark purple granules widespread across the cytoplasm, with an unlobed nucleus. Basophils have a role in early inflammatory and immediate hypersensitivity responses (Maxwell and Robertson, 1995). Moreover, there seems to be a

correlation between severe stress, an increased heterophil/lymphocyte (H/L) ratio, heteropenia and basophilia, which may be a physiological response unique to birds (Maxwell and Robertson, 1995). If that was the case, the chickens during the experimental procedure were also stressed (other than being infected), especially when bled, it is possible that basophilia or leucocytosis could be an indicator of stress. The blood sample from chicken E22 at score 3 on Day 11 (Annexure C), basophilia can be observed by numerous basophils in the blood periphery, which is not normally seen.

In the blood smear prepared from blood of chicken E13 with score 3 on Day 15, there were several blood cells such as a monocyte (mo), an eosinophil (eo), thrombocytes (th), hypochromic (hc) and teardrop-shaped (tds) red blood cells (Figure 4.9 F). The hypochromic and teardrop-shaped red blood cells are indicative of anaemia and gram-negative septicaemia respectfully. Thrombocytes are smaller than lymphocytes and monocytes and are oval to rectangular in appearance (Figure 4.9 F). Thrombocytes are the haemostatic counterparts of mammalian platelets (Ferdous *et al*. 2016). Thrombocytes have an important function in the immune response such as phagocytic ability, inflammation mediation, antimicrobial activity and other immune modulating activities, as well as haemostatic function and blood coagulation (Ferdous *et al*. 2016). Monocytes are naïve precursors having limited effector and regulatory capabilities, however upon stimulation they develop into macrophages (Klasing, 1998). Macrophages are more capable of phagocytic activity and mediating the host defence mechanism that is crucial in defining the type and intensity of specific innate immune responses (Klasing, 1998). The disrupted eosinophil observed were of a medium size and there was loss of nuclear lobulation, as the cytoplasm stained pale blue with red-orange granules (Figure 4.9 F). The role of avian eosinophils in literature is still unclear, however avian eosinophils may have a role in delayed hypersensitivity responses and as such could have similar function as their mammalian counterparts such as immediate

hypersensitivity responses and modulation against parasitic infestation (Montali, 1988; Grasman, 2002).

Unfortunately, the automatic slide stainer Hematek® 3000 System (Siemens Healthineers) with Hematek Modified Wright's stain and Hematek Wright-Giemsa stain used for staining of human blood smears, did not produce adequate quality with the staining of avian blood smears, for the differentiation of subtle blood structures. Although, the avian blood smears were of poor quality, the different morphologies of red blood cells could still be distinguished. However, the morphological characteristics of heterophils, monocytes, leukocytes and granulocytes were very difficult to classify and thus could not be properly distinguished. This indicates that the Hematek Modified Wright's stain and Hematek Wright-Giemsa stain, which are rapid stains, are not suited for staining of avian blood samples for morphological characterisation and classification, as they yield poor quality staining. Therefore, stains such as Wright stain, Giemsa stain, Wright-Giemsa stain, Leishman stain, Wright-Leishman stain, May-Grünwald stain and May-Grünwald-Giemsa stain, are highly recommended for future studies.

Moreover, the avian blood slides analysed, cannot be used to interpret disease progression solely on their own, as they do not provide adequate information on the immune response in the affected organism except the morphological characteristics of blood cells and possible causes. Thus, in future studies, the blood slides need to be supported by complete/full blood counts (CBC/FBC) and white blood cell differential counts (WBC Diff) to obtain an overall view in making a diagnosis and prognosis. In our study, CBC/FBC and WBC Diff could not be performed, however the data from the flow cytometry results with the blood smears obtained was used to show the change in blood morphology and the evolution of the immune response during IC disease progression in the chickens.

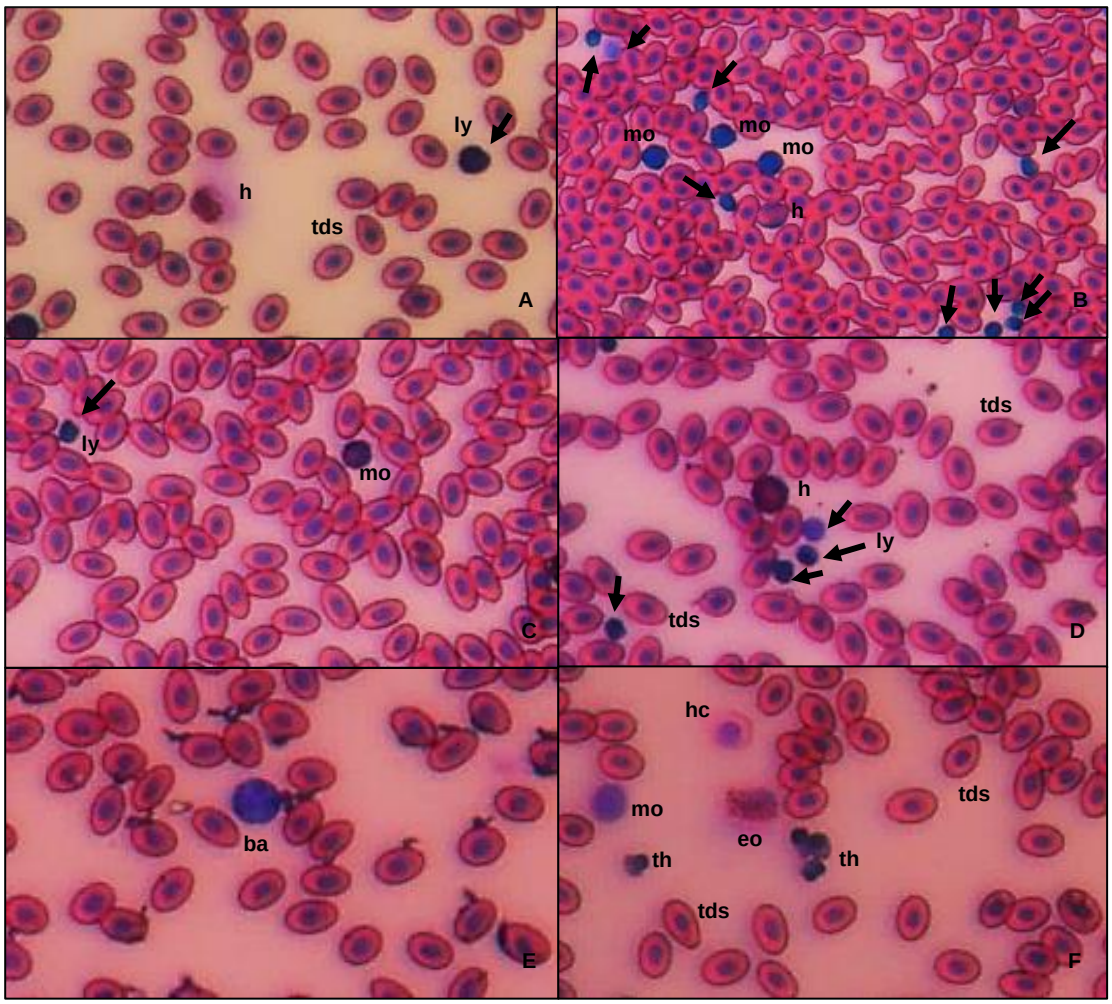


Figure 4.9: Haematological observations for blood slides showing different blood cells observed for infected chickens. (A) A heterophil (h) was observed with a lymphocyte (ly) indicated by an arrow and a teardrop-shaped red blood (tds) cell in chicken E8 with score 1 on Day 1. (B) For the blood sample from chicken E22 with score 2 on Day 7, there was an infiltration of lymphocytes (ly) indicated by arrows and monocytes (mo), and a heterophil (h) was seen. (C) The blood smear of chicken E17 with score 3 on Day 10, showed a normal monocyte (mo) with a kidney-shaped nucleus and a lymphocyte (ly) indicated by an arrow. (D) A bilobed heterophil (h) was seen, with lymphocytes (ly) in the periphery blood smear along with teardrop-shaped red blood cells (tds) for chicken E15 with score 3 on Day 11. (E) A basophil was shown with an unlobed nucleus for chicken E22 with score 3 on Day 11. (F) For this particular blood smear belonging to chicken E13 with score 3 on Day 15, there were several blood cells such as a monocyte (mo), an eosinophil (eo), thrombocytes (th), hypochromic (hc) and teardrop-shaped (tds) red blood cells. Magnification X100.

4.3.4. Flow cytometry and antibodies

The cell population profiles of the leukocytes (composed of lymphocytes and granulocytes) using a CD45 pan-leukocyte marker were studied and generated; to evaluate a specific lymphocyte population, the T-cell population mainly the CD4 T cells (T helper cells) and CD8 T cells (cytotoxic T cell) using the CD4 and CD8 markers respectively. Flow cytometry was conducted as described (Section 3.2.7, Section 4.2.7) using the BD FACSCanto II (Becton Dickinson) with analysis on the BD FACSDiva 8.0.1 software (Becton Dickinson), however two separate flow cytometric tubes were run on the BD FACSCanto II (Becton Dickinson) simultaneously for each chicken blood sample, whereby the first tube consisted of CD45 (total leukocyte) and CD4 (T helper cell) markers and the second tube consisted of CD4 (T helper cell) and CD8 (cytotoxic T cell) markers respectively. All three mouse anti-chicken antibodies: CD4-FITC (2-35 clone, MCA2164F), CD8-RPE (11-39 clone, MCA2166PE), CD45-RPE (UM16-6 clone, MCA2413PE) (Bio-Rad) could not be added, into one tube as both CD8 and CD45 antibodies had the same fluorophore RPE (R-phycoerythrin), whereas the CD4 antibody had a different fluorophore FITC (Fluorescein isothiocyanate). Having the same fluorophore would imply that the fluorescent labels would have the same peak excitation (496 nm) and emission wavelength (578 nm, green in colour) with a 488 nm blue argon laser from the BD FACSCanto II (Becton Dickinson), which can be problematic as $CD8^+$ cells are also $CD45^+$ cells, thus the differentiation between $CD8^+/CD45^+$ (cytotoxic T cells) cells from $CD45^+$ cells would not be possible. Moreover, $CD8^+$ cells would be masked and be solely shown as $CD45^+$ cells due to the same fluorescent labels being used, therefore analyses of each chicken blood sample in two separate tubes (Annexure D) had to be conducted, which was very laborious.

The flow cytometry profiles were generated from the excitation of labelled cells with fluorescent antibodies and the interrogation point with the laser, whereby light scatter is produced that could be measured and correlated with relative cell size and structures inside the cell. The measurements were termed forward angle scatter (FSC) which was based on

the size of the cell and side angle scatter (SSC) which was based on the granular complexity of the cell. All the flow cytometry profiles of both control and experimental chickens were compiled (Annexure D) that were bled on the days mentioned in Section 4.3.3.

In the previous study (Chapter 3, Section 3.2.7 and Section 3.3.4), during our runs, it was difficult to locate and gate the precise location of the entire leukocyte population and lymphocyte population. This was due to the nucleated RBCs that overlapped with the leukocyte and lymphocyte population, thus making it difficult to gate the leukocyte population, which was also where the lymphocyte population was containing both CD4 and CD8 cells. However, the problem of overlapping RBCs with leukocytes, was overcome with the use of the CD45 pan-leukocyte marker as observed in Figure 4.10 compared to Figure 3.10 (Chapter 3), there is better separation of the RBCs from the leukocytes.

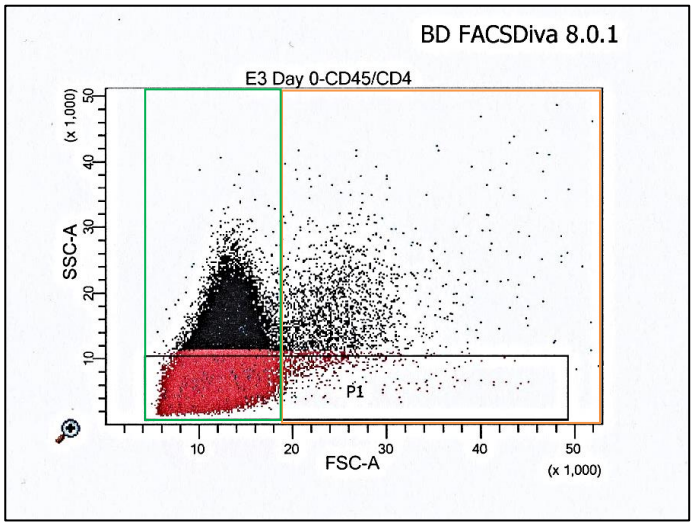


Figure 4.10: Flow cytometry profile of one of the experimental chickens (E3-score 0) showing the forward scatter (FSC-A) and side scatter (SSC-A) plot. Two distinct populations can be seen within the sequestered region gated as region P1 (shown in red) which is the approximate location of the lymphocyte population. The RBC population can be seen outlined in green and the leukocyte population can be seen outlined in orange.

Firstly, a CD45/CD4 run (whole blood stained with CD45 and CD4) was performed, to enable us to locate and gate the correct region where the total leukocyte cells were situated, whereby a forward scatter (FSC-A) and a side-scatter (SSC-A) plot (Figure 4.10) was generated. From there onwards, a SSC-A versus a CD45 PE-A plot was generated, whereby proper gating of the total leukocyte population (total CD45 count) could be performed, which consists of both lymphocytes and granulocytes (Figure 4.11). Once this was done, it was easier to locate and gate the region where the lymphocytes were situated within the total CD45 population, based on the size and complexity of the cells (Figure 4.11). Finally, after the lymphocyte population were gated, a CD45 PE-A vs CD4 FITC-A analysis was performed, to obtain the cell counts of the total lymphocytes, $CD45^+$ cells (CD8 T cells/B lymphocytes/ Natural killer (NK) cells), cells that are $CD4^+/CD45^+$ (T helper cells) and the total CD45 cells (total leukocytes) (Figure 4.12).

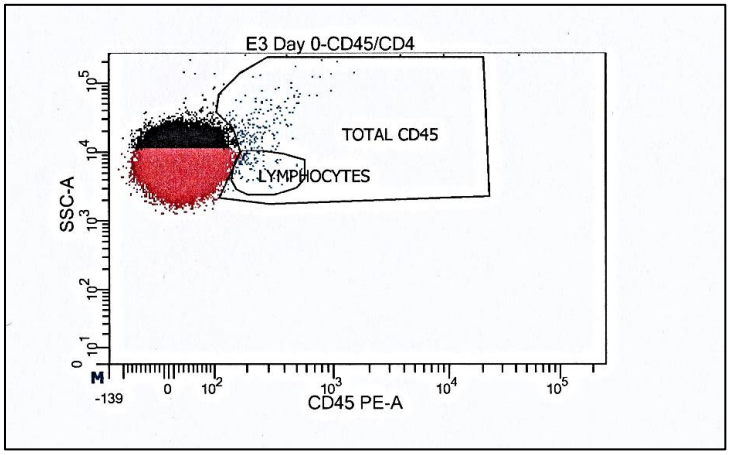


Figure 4.11: Flow cytometry profile of one of the experimental chickens (E3-score 0) showing the side scatter (SSC-A) vs CD45 PE-A plot. Two distinct populations can be seen the RBC population (shown in red) and the total CD45 (total leukocyte) population outlined (shown in blue). The lymphocyte population is outlined within the total CD45 population.

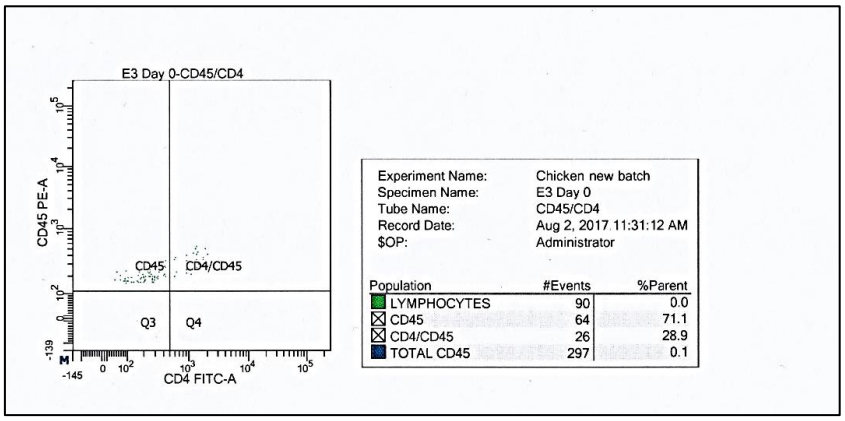


Figure 4.12: Flow cytometry profile of one of the experimental chickens (E3-score 0) showing the CD45 PE-A vs CD4 FITC-A plot. In the first quadrant (Q1) above quadrant three (Q3), $CD45^+$ cells are shown. In the second quadrant (Q2) above quadrant four (Q4), $CD4^+/CD45^+$ cells are shown. The cell counts (#Events) can be seen in the table for each of the cell components such as total lymphocytes, $CD45^+$ cells (CD8 T cells/B lymphocytes/ Natural killer (NK) cells), cells that are $CD4^+/CD45^+$ (T helper cells) and the total CD45 cells (total leukocytes).

Following the first run, a CD4/CD8 run (whole blood stained with CD4 and CD8), was conducted using the same chicken blood sample. The same steps as in the first run were performed where a FSC-A and a SSC-A plot (similar to Figure 4.10) was generated, to locate and gate the exact region where the lymphocytes were situated, known from the CD45/CD4 run. A SSC-A versus a CD8 PE-A plot was then generated, whereby proper gating of the CD8 cells could be performed, which was also the same region, where the CD4 cells could be located (Figure 4.13). Once this was done, the CD8 and CD4 populations based on the emission wavelength produced by the two different fluorophores: 525 nm (FITC, green in colour) and 578 nm (R-PE, yellow in colour) with a blue argon laser from the BD FACSCanto II (Becton Dickinson) were gated and analysed. A CD8 PE-A vs CD4 FITC-A plot was generated, to obtain the cell counts of $CD8^+$ cells (cytotoxic T cells) and $CD4^+$ (T helper cells) (Figure 4.14), whereby for chicken E3 with score 0 on Day 0, there were no $CD8^+$ cells but there were 10 $CD4^+$ cells. The whole process was then conducted and

repeated for all chicken blood samples for the CD45/CD4 and CD4/CD8 runs, as described above. However, the data obtained from the first and second analyses (CD45/CD4 and CD8/CD4 runs) were not reliable as is, as findings need to be reported as the percentage of total leukocytes. Hence, using the data from both the first and second analyses conducted, ratio calculations (Appendix D) were used to obtain the following: % total leukocytes, % CD8 of total leukocytes, % CD4 of total leukocytes, the CD4/CD8 ratio and % B and NK cells of total leukocytes. The data was graphically represented to understand the trend of the avian immune response elicited by *Av. paragallinarum* serovar C-3 via immune cells, which could be inferred or detected from the flow cytometry analysis (Figure 4.15 - Figure 4.20). Statistical analysis was performed to determine the statistical significance of the data. The mean and standard deviation of the data were calculated, and a Student's t-test was conducted to validate the results obtained, where a $p<0.05$ was found to be statistically significant.

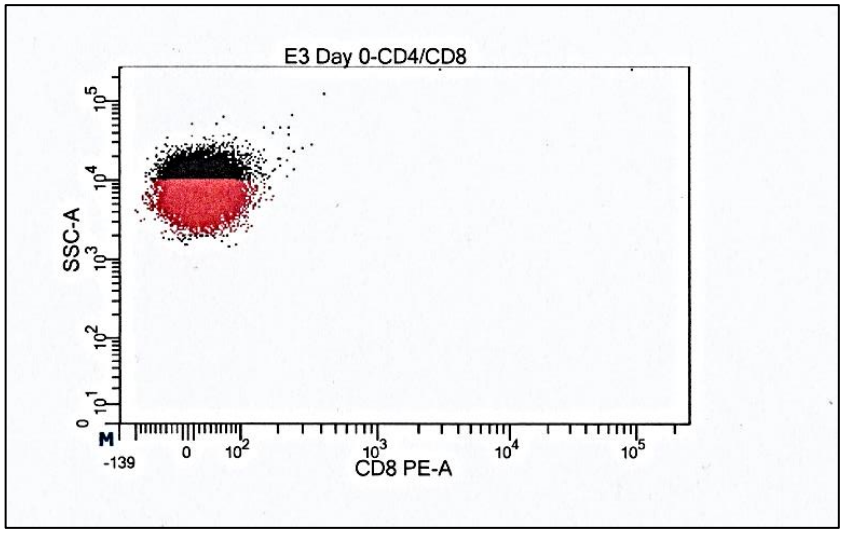


Figure 4.13: Flow cytometry profile of one of the experimental chickens (E3-score 0) showing the side scatter (SSC-A) vs CD8 PE-A plot. From the plot there were very few lymphocytes. However, once the same region as in Figure 4.11 was gated, lymphocytes of interest could be obtained.

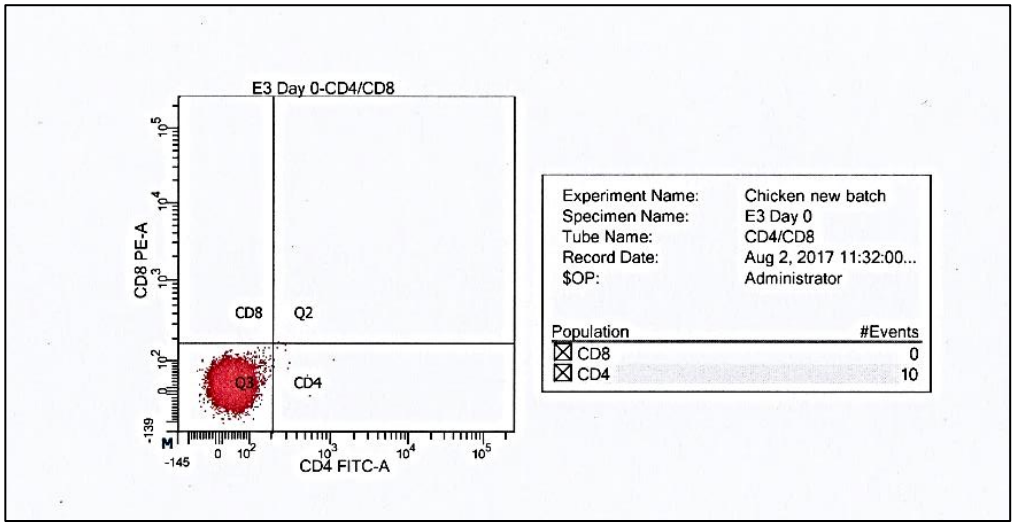


Figure 4.14: Flow cytometry profile of one of the experimental chickens (E3-score 0) showing the CD45 PE-A vs CD4 FITC-A plot. In the first quadrant (Q1) above quadrant three (Q3), $CD8^+$ cells would have been shown. In the fourth quadrant (Q4), $CD4^+$ cells are shown. In the third quadrant (Q3), RBCs are shown. The cell counts (#Events) can be seen in the table for the CD8 and CD4 count. However, as observed there are no $CD8^+$ cells.

The % total leukocytes (CD45+ cells) plot gives all $CD45^+$ cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens (p>0.05) (Figure 4.15). For better visualisation of how the leukocyte population looks like in whole blood, blood smears (Section 4.3.3) were compiled from each day from the control group (Days 11 and 21) and experimental group (Days 0-21), that correlates to the results obtained for the flow cytometry results as represented by the % total leukocyte plot (Figure 4.16). On Day 0, it seemed that the chickens had a high leukocyte percentage, which could be due to stress or previous exposure to microorganisms before the study. In the experimental group: On Day 1, following infection of experimental chickens, it was observed that there was a decrease in the percentage of total leukocytes, followed by an increase on Day 3 and a decrease on Day 4. The chickens were injected on Day 0, and a score 1 was observed in the majority of chickens, the innate immune system was triggered as an immediate response to infection and inflammation caused, that had a key role through innate immune cells such

as epithelial cells, dendritic cells, monocytes, macrophages, heterophils and natural killer (NK) cells. Monocytes matured into inflammatory macrophages, whereby both macrophages and heterophils were involved in phagocytosis of the Gram-negative bacteria, *Av. paragallinarum* serovar C-3 (Bellingan and Laurent, 2008). However, the fate of the innate immune cells was short-lived, hence the decline observed on Day 1 and 4, which could be due to necrosis, apoptosis and subsequent phagocytosis of these innate immune cells (Bellingan and Laurent, 2008). Moreover, macrophages might be involved in phagocytosis of apoptotic heterophils, thus causing their numbers to decline with the clearance of phagocytosed cells, allowing the tissue to return to their normal structure and function (Bellingan and Laurent, 2008). There could also be possible degranulation of the heterophils, as observed previously with Gram-negative bacteria such as *Salmonella enterica* serovar Typhimurium (Lam and Munn, 2002). Furthermore, the heterophil numbers might have peaked earlier than macrophages during the inflammatory response, with simultaneous activation of the adaptive immune response having a slow effect (requiring several days to even a few weeks to respond to inflammation via the B and T cells) (Davison *et al*. 2011). Hence, on Day 3, an increase in the total leukocyte population was observed, as infected macrophages increase MHC class II expression to activate additional antigen-specific Th cells and produce co-stimulatory cytokines (IL-12, IL-18) that help in the differentiation of Th1 effector cells, leading to an efflux of leukocytes as seen on Day 3 (Erf, 2004).

On Days 7 and 10, the percentage total leukocyte stayed stable. On Days 11 and 18, a high percentage of total leukocytes was observed, due to the adaptive immune system actively playing a role in the humoral and cell-mediated responses with a release of antibodies. This was followed by low percentages on Days 15 and 21, respectively, due to clearance of the immune cells following the cell-mediated response with cytotoxic T cells, natural killer (NK) cells and phagocytosis of infected cells via macrophages. In the control group: A stable trend

of percentage total leukocytes over the course of 11 days was observed, followed by a slight decrease in percentage leukocytes on Days 15, 18 and 21 respectively. On Day 11, there was a high leukocyte percentage, which could imply that there was possible exposure, however the bacterial load was not sufficient to cause disease, hence the chickens did not develop IC related signs or symptoms. The high leukocyte percentage on Day 11, could also be stress induced.

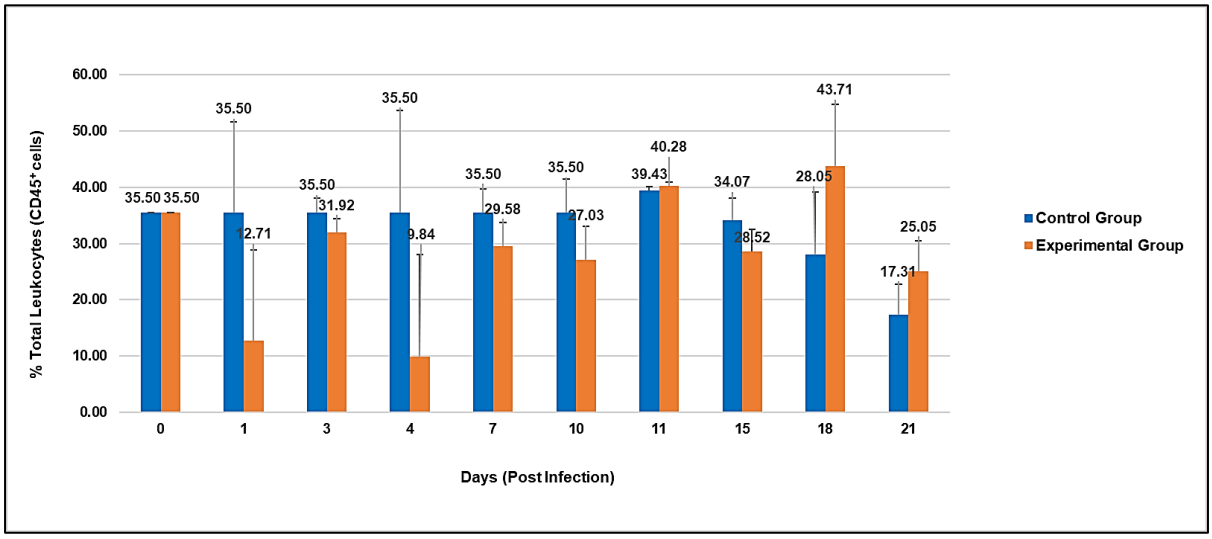


Figure 4.15: % Total leukocytes (CD45$^+$ cells) plot gave all CD45$^+$ cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens (p>0.05). In the experimental group: On Day 1, following infection of experimental chickens, it was observed that there was a decrease in the percentage of total leukocytes, followed by an increase on Day 3 and a decrease on Day 4. On Days 7 and 10, the percentage leukocyte stayed stable. On Days 11 and 18, a high percentage of leukocytes was observed. This was followed by low percentages on Days 15 and 21, respectively. In the control group: A stable trend of percentage leukocytes over the course of 11 days was observed, followed by a slight decrease in percentage leukocytes on Days 15, 18 and 21 respectively.

Antibodies can prevent disease and provide protection through mucosal surfaces (secretory IgA) and in the elimination of pathogens that are found in the extracellular environment of the host (Erf, 2004). However, when antigens have infiltrated cells (endocytic mechanisms; exogenous antigens) or are produced within the cell (viral or neoplastic proteins;

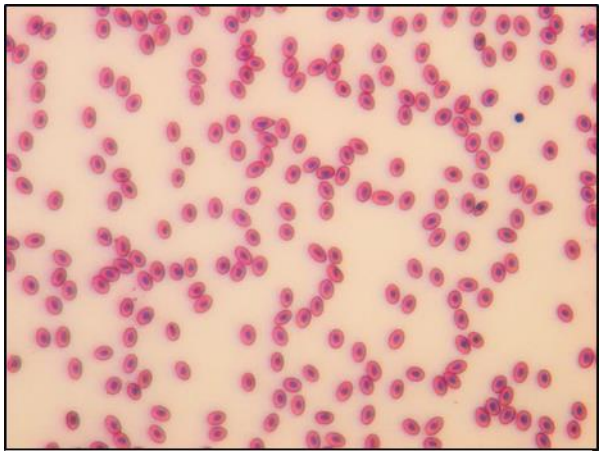
endogenous antigens) the humoral immune response which involves direct antibody-antigen contact is no longer effective in eliminating antigen (Abbas *et al*. 2014). In such a situation, the cell-mediated immune mechanisms that lead to intracellular elimination of the antigen or elimination of the host cell are the most promising strategies in antigen elimination (Erf, 2004).

In poultry, as in humans and other mammals, T cells are the antigen-specific components of the cell-mediated immunity (CMI) and express T-cell receptors (TCR) that are collectively able to recognise various antigens (Chen *et al*.1991). All T cells express CD3 complexes together with the TCR molecules, which makes CD3 a pan-T cell marker; whereby its presence on a cell indicates that the cell is a T cell (Erf, 2004). T cells, having a primary and regulatory role in the adaptive immune response, whether cell-mediated or humoral, are referred to as T helper (Th) cells and express CD4 molecules on their surface (Chen *et al*. 1991; Arstila *et al*. 1994). Thus, upon specific recognition of the antigen-peptide expressed on the cell surface of an antigen-presenting cell (APC) in association with a self-MHC class II complex to the TCR, Th cells are activated that secrete cytokines and express cell surface molecules providing crucial activation signals to cells of innate and adaptive immunity, thereby propelling the mechanisms of the immune response towards pathogen elimination (Arstila *et al*. 1994). There are 2 types of Th cells that are highly specialised and specific to the type of infection (Arstila *et al*. 1994). Type-1 Th (Th1) cells which are effective in directing the innate immune response towards a cell-mediated response for intracellular pathogens whereby macrophage activation is required (Playfair and Bancroft, 2013). Th1 cells secrete macrophage-activating cytokines such as interferon-γ (IFN-γ), tumour-necrosis factor-α (TNF-α) and interleukin- 2 (IL-2) (Erf, 2004; Playfair and Bancroft, 2013). Contrary to Th1, type-2 Th (Th2) cells favour the development of a humoral response for antibody production to deal with extracellular pathogens with further specialisation towards particular antibody subclasses such as IgA for mucosal infections (Arstila *et al*. 1994; Playfair and

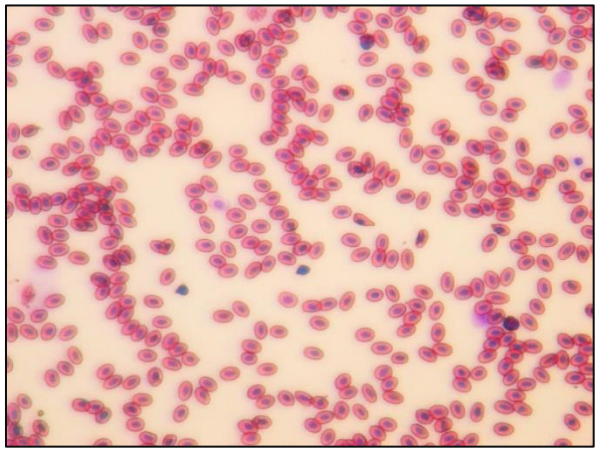
Bancroft, 2013; Abbas *et al*. 2014). Th2 cells secrete B cell-activating cytokines such as transforming growth factor-β (TGF-β), IL-4, IL-5 and IL-10 (Erf, 2004; Playfair and Bancroft, 2013).

The % $CD4^+$ cells of total leukocytes plot gave all $CD4^+$ cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens (p>0.05) (Figure 4.17). For the experimental group: On Day 3 and from Day 7 onwards, high $CD4^+$ percentages (Day 3- 11.41% and Day 7- 15.73%), which was slightly higher than the $CD8^+$ percentage (Day 3- 8.04% and Day 7- 5.94%), were observed (Figure 4.17 and Figure 4.18). This was mainly because $CD4^+$ cells upon being activated during IC infection, they proliferate and transform into effector Th1 and Th2 cells or memory cells, mediating both the innate and adaptive immune responses. Hence, Th cells are found in more numbers as they recruit macrophages, granulocytes, B-cells and $CD8^+$ cells in different tissue locations, compared to $CD8^+$ cells involved in cytotoxic activity and apoptosis.

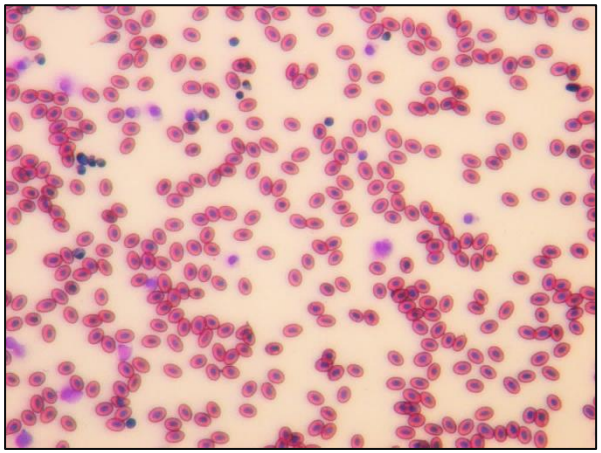
On Days 0, 1 and 4 low $CD4^+$ percentages (Day 0- 6.12%, Day 1- 6.87%, Day 4- 5.01%) were observed, with the same trend seen with $CD8^+$ percentages (Day 0- 2.06%, Day 1- 2.90%, Day 4- 1.73%) (Figure 4.17 and Figure 4.18), this was mainly due to the fact that T cells are mainly involved in the adaptive immune response requiring a few days to be fully functional following the innate immune response. Moreover, unlike B cells that secrete antibodies, T cells need to physically travel to the site of an infection to perform their respective functions, implying they will travel to peripheral tissues, hence their numbers in whole blood could decline as they disperse. In the control group: high $CD4^+$ percentages were observed on Days 11, 15 and 18, which could be due to previous exposure to microorganisms, thus the adaptive immune system was observed to already have been activated (Figure 4.17).



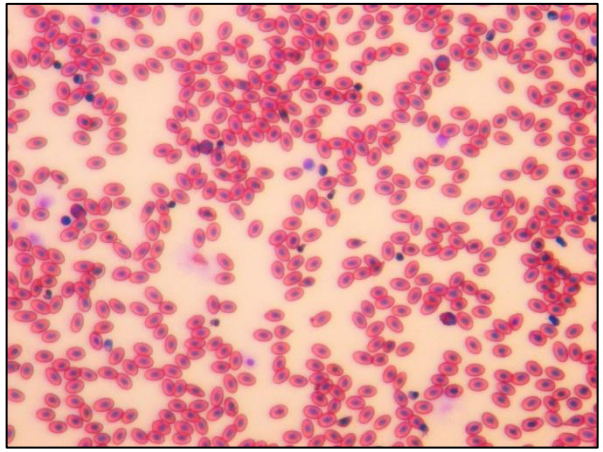
Day 0: E3-Score 0



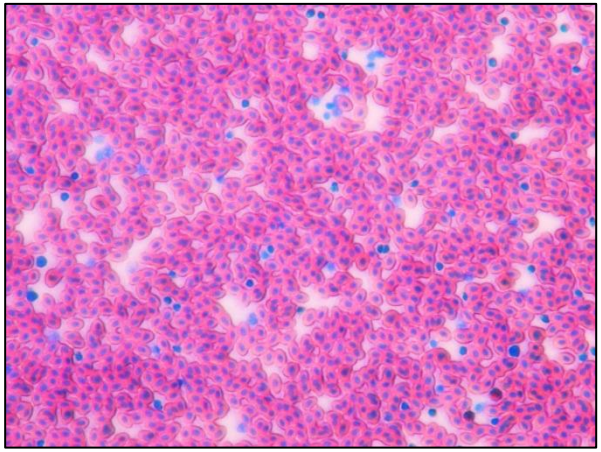
Day 1: E7-Score 1



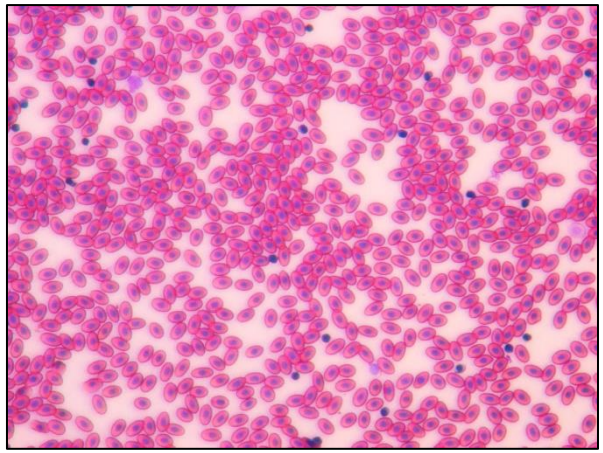
Day 3: E21-Score 1



Day 4: E4-Score 1

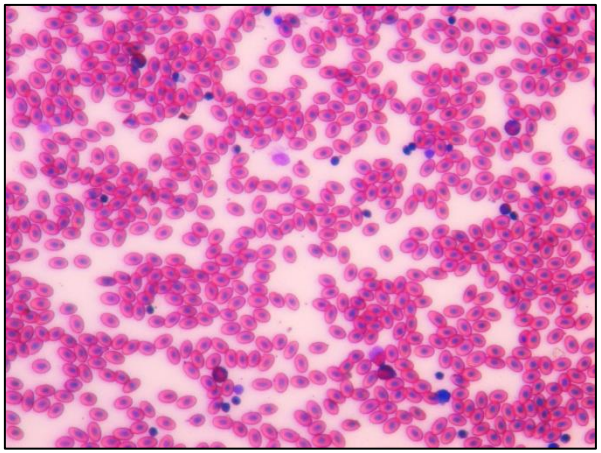


Day 7: E15-Score 2

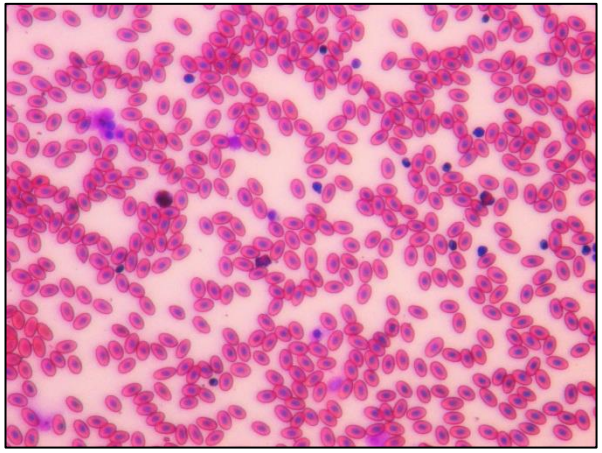


Day 10: E6-Score 1

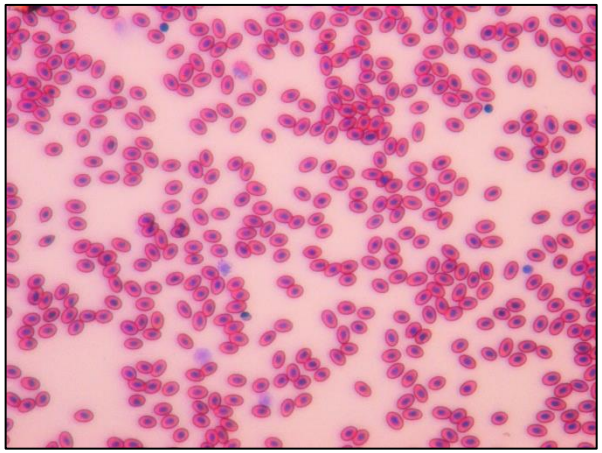
Figure 4.16: Blood smears used with the flow cytometry results obtained for % total leukocytes over 21 days showing cell morphology. (Continued on next page)



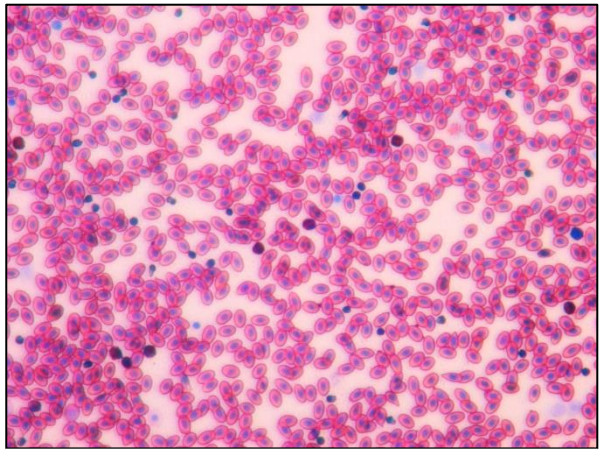
Day 11: E15-Score 3



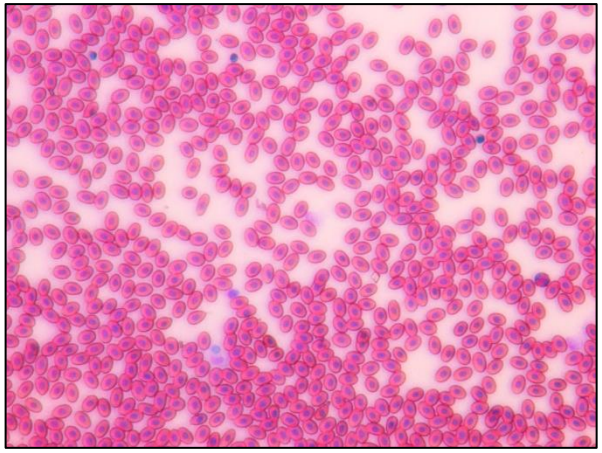
Day 11: Control 2-Score 0



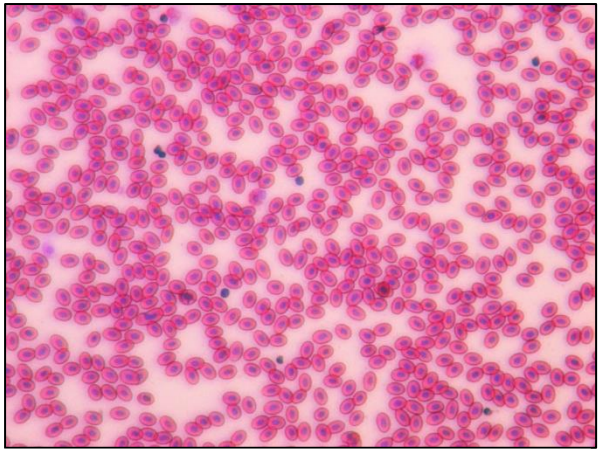
Day 15: E2-Score 3



Day 18: E4-Score 2



Day 21: E9-Score 2



Day 21: Control 12-Score 0

Figure 4.16: Blood smears used with the flow cytometry results obtained for % total leukocytes over 21 days showing cell morphology.

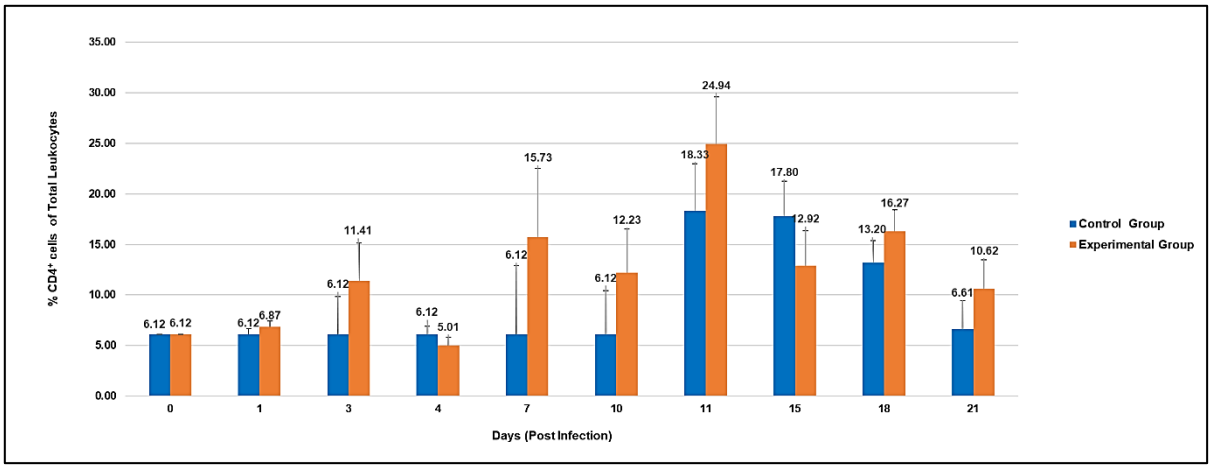


Figure 4.17: % CD4⁺ cells of total leukocytes plot gave all CD4⁺ cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens (*p*>0.05). For the experimental group: On Day 3 and from Day 7 onwards high CD4⁺ percentages were observed, which was slightly higher than the CD8⁺ percentage. On Days 0, 1 and 4 low CD4⁺ percentages were observed, with the same trend seen with CD8⁺ percentages. For the control group: high CD4⁺ percentages on Days 11, 15 and 18 were observed.

Cytotoxic T lymphocytes (CTLs) of this lineage, are specialised effector cells that will eliminate target cells such as virus-infected cells and neoplastic cells that contain endogenous antigen (Erf, 2004). CTLs typically express CD8 molecules on their cell-surface in the form of either αβ heterodimers or αα homodimers (Erf, 2004). CD8⁺ T cells are MHC class I restricted, unlike CD4⁺ Th cells that are MHC class II restricted and act by recognising antigen peptides bound to MHC class I molecules and APC and interacting with the complex, this in turn triggers the CD8⁺ cell causing the release or activation of molecules that gives a “suicide signal” (Playfair and Bancroft, 2013; Abbas *et al*. 2014). CD4⁺ Th cells may also activate CTLs via IL-2 that promotes cell proliferation and differentiation of functional CTLs (Playfair and Bancroft, 2013). CTLs kill in two ways. The first tactic is the most rapid and important mechanism, whereby granzymes (granule-derived enzymes) are transferred to the target cell through the actions of perforin whereby holes are punctured into the target cell cytoplasm, this in turn activates caspase enzymes that induce apoptosis or

"cell suicide" (Playfair and Bancroft, 2013). Secondly, a Fas ligand is present on the surface of CTLs, whereby they use this molecule to trigger apoptosis in the target cell via the Fas receptor ("death receptor") (Playfair and Bancroft, 2013). In these ways CTLs, are the "serial killers" of the adaptive immune response, as they bind and release cytotoxic mediators with the delivery of a lethal dose, after which they seek other infected target cells as their next victims (Playfair and Bancroft, 2013).

The % $CD8^+$ cells of total leukocytes plot shows all $CD8^+$ cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens (p>0.05) (Figure 4.18). For the experimental group: On Days 0, 1 and 4 very low $CD8^+$ percentages were observed (Figure 4.18), which shows that $CD8^+$ cells are part of the cell-mediated response and adaptive immunity, employed for cytotoxic killing of target cells and the clearance of infected cells, which is why on Days 7 to 21, high $CD8^+$ percentages were observed (Figure 4.18). However, on Day 3, high $CD8^+$ percentages were also observed which might be due to $CD8^+$ cells being recruited by $CD4^+$ Th cells and dendritic cells to become activated and fully functional, during the innate response. It is also possible that natural killer (NK) cells were present on Day 3 leading to an increase in $CD8^+$ percentage, as they share the same cell surface molecules as T cells such as CD8 marker, a putative interleukin-2 receptor, CD45 and a receptor for IgG, but do not express CD4, major histocompatibility complex class II or immunoglobulin (Göbel et al. 1994). On Day 11 and 15, the control group had high $CD8^+$ percentages, and it was evident from the results that there was previous exposure since cytotoxic T cells are primarily recruited to kill host cells that are infected. However, severe stress induction could also be a contributing factor.

It was found that 7% of the bacterial genome of *Av. paragallinarum* consisted of prophages and/or prophage remnants (Roodt *et al*. 2012). Moreover, in a study by Boucher *et al*.

(2014), TLR7 was found to be up-regulated, whereby they hypothesized that the prophages and/or prophage remnants could be stimulating TLR7 expression, thus a more infallible and potent immune response was triggered. $CD8^+$ cells are activated specifically for viral invasion, as viruses invade host cells and replicate, hence it is possible that due to the prophages and/or prophage remnants found in the *Av. paragallinarum* genetic make-up, that $CD8^+$ cells recognise *Av. paragallinarum* infections as viral, instead of bacterial. There might also be a correlation between TLR7 expression and $CD8^+$ T cells, as TLR7 has been highly expressed in $CD8^+$ cells of individuals with HIV (human immunodeficiency virus) type-1 infection compared with healthy control individuals (Song *et al*. 2009).

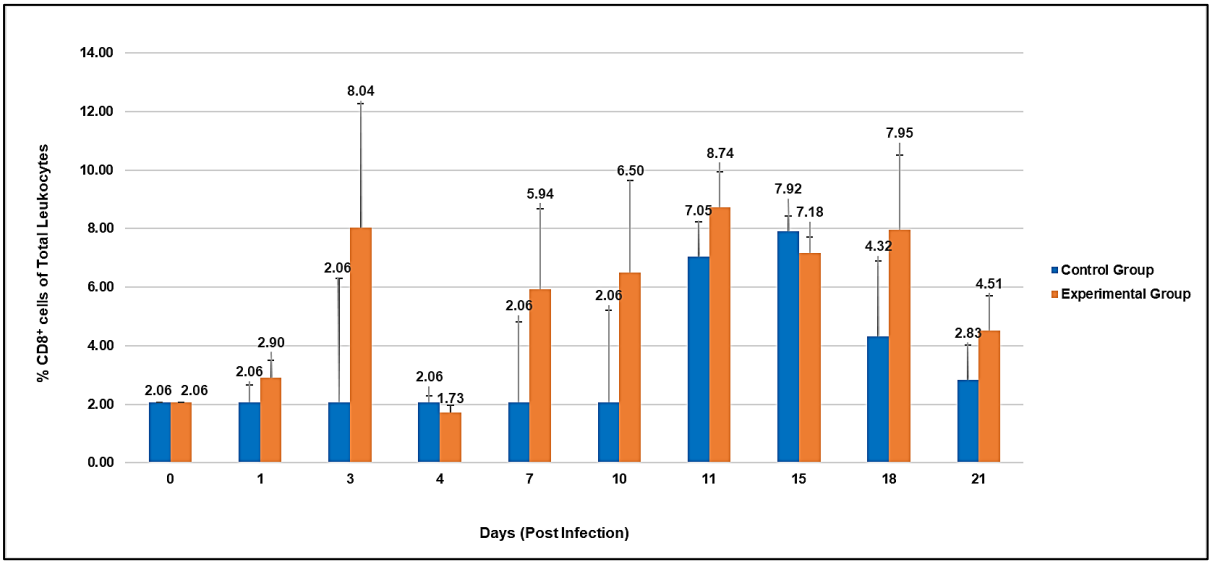


Figure 4.18: % $CD8^+$ cells of total leukocytes plot gave all $CD8^+$ cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens (p>0.05). On Day 3 and from Day 7 onwards high $CD8^+$ percentages were observed, in the experimental group. On Day 11 and 15, the control group had high $CD8^+$ percentages. Cells expressing the CD8 marker are cytotoxic T cells.

The $CD4^+/CD8^+$ ratio measures the ratio of CD4 helper/inducer cells and CD8 cytotoxic/suppressor cells which are 2 phenotypes of T lymphocytes. In humans, the CD4/CD8 ratio is routinely evaluated and used as a prognostic factor for disease progression in patients with AIDS (acquired immune deficiency syndrome) as well as in viral acute diseases such as cytomegalovirus, Epstein-Barr virus and influenza virus infections (Amadoni *et al*. 1995). Furthermore, it was found that MHC genes are known to determine the CD4/CD8 ratio in rats (Damoiseaux *et al*.1999). Similarly, the CD4/CD8 ratio of the peripheral T cells in various chicken lines had been detected which seemed to be dependent on the MHC haplotype (Hala *et al*. 1991). In chickens, as in humans, the normal CD4/CD8 ratio is still poorly established (Amadoni *et al*. 1995).

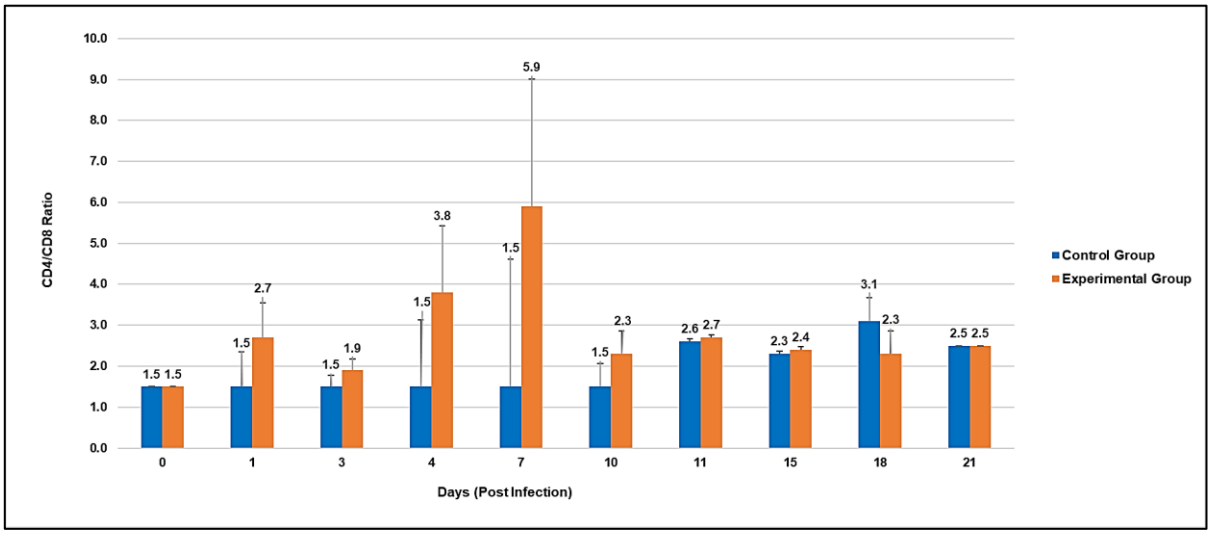


Figure 4.19: Flow cytometry profile showing the $CD4^+/CD8^+$ ratio of cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens (p>0.05). For the experimental group: On Days 1, 4 and 7 had high $CD4^+/CD8^+$ ratios compared to the rest of the days that had decreased to stable $CD4^+/CD8^+$ ratios. For the control group: The $CD4^+/CD8^+$ ratios throughout the days were stable with a consistent trend, with a slightly higher ratio on Day 18.

The CD4/CD8 ratios in Figure 4.19, were based on the estimated cell counts obtained from the flow cytometry calculations (Appendix D). Figure 4.19 shows the flow cytometry profile

with the $CD4^+/CD8^+$ ratio of cells detected via flow cytometry over 21 days in whole blood of both control and experimental chickens ($p>0.05$). For the experimental group: On Days 1, 4 and 7 had high $CD4^+/CD8^+$ ratios compared to the rest of the days that had decreased to stable $CD4^+/CD8^+$ ratios. The high ratio indicates that the $CD4^+$ percentage was higher than $CD8^+$ cell percentage and that there was an inflammatory response (Figure 4.17 and Figure 4.18). Therefore, the immune system was active and resilient towards IC infection due to Th cell mediation with cytotoxic T cells. For the control group: The $CD4^+/CD8^+$ ratios throughout the days were stable with a consistent trend, with a slightly higher ratio on Day 18. The slightly higher ratio could be attributed to an inflammatory response due to suspected exposure or stress.

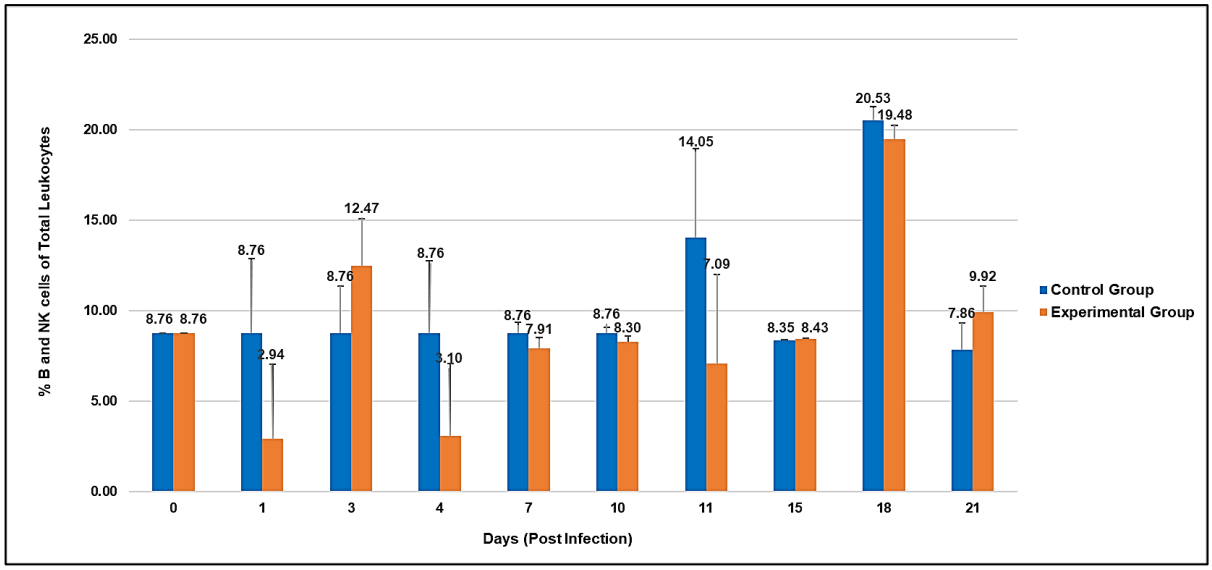


Figure 4.20: Plot with % B and NK (natural killer) cells of total leukocytes detected via flow cytometry over 21 days in whole blood of both control and experimental chickens ($p>0.05$). For the experimental group: Days 3, 18 and 21 had high percentages of B and NK cells compared to the rest of the days that had low to moderate percentages. For the control group: Days 11 and 18 had high percentages of B and NK cells, compared to the rest of the days that had a stable trend.

NK cells are large granular lymphocytes, also involved in the elimination of viral and neoplastic infected cells (Erf, 2004). However, unlike CTLs they do not have the classical TCR for specific antigen recognition and are not MHC restricted, hence being non-specific, NK cells are part of the innate immunity (Playfair and Bancroft, 2013). NK cells are activated via Th1-mediated activity with type-1 cytokines such as IFN-γ, IL-2 and IL-12, which enhances cell proliferation and cytotoxic activity of the NK cells (Erf, 2004; Abbas *et al*. 2014). Additionally, NK cells share similarities including phenotypic expression with T cells such as the cell surface molecules other than TCR-CD3 complexes (CD8 and CD25) and cytotoxic killing mechanisms (Abbas *et al*. 2014). NK cells target cells via activating receptors that recognise target-cell surface motifs such as viral products or stress-related proteins, thus initiating a kill signal (Playfair and Bancroft, 2013). However, upon recognition of MHC class I molecules, inhibitory receptors of the NK cells are put on hold or prevent, thus only when MHC molecules are absent or altered can NK cells carry out their functions (Playfair and Bancroft, 2013). Lysis of cells by NK cells is carried out by the granules of NK cells such as perforin and granzymes, whereas induction of apoptosis and intracellular killing are carried out by granulysin (Playfair and Bancroft, 2013).

B cells are produced in the bone marrow and mature in the bursa of Fabricius (Davison *et al*. 2011). Remarkably, only 5% of B cells from the bursa of Fabricius, survive to emigrate to the periphery and participate in active humoral immunity (Scott, 2004). B cells are activated in the lymphoid organs, depending on the route by which the antigen arrives. IC is associated with the upper respiratory tract of chickens, hence mucosal lymphoid tissue such as the paraocular Harderian glands (HG), conjunctiva-associated lymphoid tissue (CALT) and nasal-associated lymphoid tissue (NALT) are regions where activated B cells would be present (Davison *et al*. 2011). B cells possessing B cell receptors (BCRs) are activated in different ways: via costimulatory molecules (CD21, CD19 and CD81), through direct activation with mitogenic antigens (bacterial endotoxins), repeating antigens (bacterial

capsular polysaccharides) that cross-link the immunoglobulin (Ig) surface molecules on B cells eliciting recognition and lastly responses to typical proteins that need to cross-link the Ig surface with further stimuli or assistance from $CD4^+$ bearing T cells (T helper cells-Th2) for B cell proliferation and antibody production/secretion which may include IgM, IgA and IgY (Davison *et al*. 2011; Playfair and Bancroft, 2013). Thus, antibodies bind to antigen that have a principle biological functions such as agglutination, mucosal surface mediators via FcαR receptor, B cell triggering, opsonization for phagocytosis and can activate the classical complement pathway (Woof, 2004; Playfair and Bancroft, 2013).

Figure 4.20 represents a plot with % B and NK (natural killer) cells of total leukocytes detected via flow cytometry over 21 days in whole blood of both control and experimental chickens ($p>0.05$). For the experimental group: Days 3, 18 and 21 had high percentages of B and NK cells compared to the rest of the days that had low to moderate percentages. On Day 3, there were high percentages due to: NK cells that were recruited and proliferated due to cytokines released by Th1 cells thereby performing cytotoxic activity; and B cells being recruited by Th2 cells via secretion of B cell- activating cytokines such as IL-4, IL-5 and IL-10 that would be actively involved in the adaptive immunity mainly the humoral response. On Day 18 and 21, B cells proliferated, bound to antigen via the B-cell receptor (BCR) and secreted immunoglobulins whereby some B cells became plasma cells and others remained as memory cells. In chickens, there are only three classes of antibody IgA, IgM and IgY, whereby each of the antibodies have specialised functions. For the control group: Days 11 and 18 had high percentages of B and NK cells, compared to the rest of the days that had a stable trend. The control group showed both innate and adaptive immune responses since the plot shows the percentages of B and NK cells, hence the chickens were indeed exposed to pathogen before or during the study, however from our daily monitoring scores the control chickens did not develop IC related symptoms, hence exposure could be due to other microorganisms or the bacterial load of *Av. paragallinarum* serovar C-3 was not sufficient to

cause disease. Fortunately, the incident from the infection model was detected, showing that the infection model was correctly set-up and designed, and discrepancies could be accounted for despite the drawbacks. However, severe stress could also be a possible contributing factor in the overall immune responses observed, as the chickens were in isolation and when bled, their physical and mental wellness could have been compromised during the study. Moreover, birds may develop a leukopenia and lymphopenia in the initial stress response, however after 12h later may show leucocytosis and heterophila (Davison and Flack, 1981).

Unfortunately, for statistical analysis using the Student's t-test we failed to reject the null hypothesis ($p>0.05$), that there is no difference between the means of the experimental and control groups (Appendix D). Furthermore, the t-statistics were not statistically significant, hence it was concluded that there was not sufficient evidence available to suggest the null hypothesis false at a 95% confidence level. From our data analysis, a considerable variation between birds of the control and experimental groups was observed. This could be due to a small sample size used for the study (n=38), thus the sample size was not large enough to allow the null hypothesis to be rejected at the $p<0.05$ level. Hence a larger sample size should be chosen for future studies. Moreover, there were bird-to-bird differences as it seemed that some birds were previously exposed prior to the study despite being SPF, implying that their immune system was already well established.

4.3.5. Sandwich enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was carried out ($p<0.05$) and the results are shown in (Figure 4.21). Day 0 chickens that were SPF, were not yet infected when they were bled at a score 0, however moderate levels of IL-8 titres for chickens E5, Control 1 and 2 were observed; with a very

high IL-8 titre for chicken E2. A possible explanation for the Day 0 chickens with moderate to high IL-8 expressions could be attributed to previous exposure to microorganisms before the study started, which could have been transmitted before collection from suppliers, during transportation or while they were being housed at the experimental facility in spite of necessary precautions and disinfection routines which were implemented during transportation and while housing at the facility at the University of the Free State. The control groups had a stable trend over the duration of the study, showing low expression of IL-8 on Days 3, 7 and 15. However there were moderate levels of IL-8 titre at the beginning (Day 0) with Control 1 and 2 chickens at score 0 and at the end of the trial (Day 21) with Control 10 and 11. Control 1 and 2 chickens could have been previously exposed as mentioned. Control 10 and 11 (named after the nth time they were bled, where n is a number), could have also been exposed to IC during the study, however the threshold host density was not met and was not sufficient to cause disease, leading to a lowered infection rate. Although the Day 0 and control chickens had moderate to high cytokine titres of IL-8, they did not show any IC related signs or symptoms (asymptomatic).

For the experimental group it was observed that IL-8 expression from moderate to high titres was found across all IC clinical scores 1, 2 and 3. Chicken E13 with score 1 on Day 3 had a moderate IL-8 titre. High cytokine titres were observed for experimental chickens presented with score 3: E15 (Day 7), E16 (Day 7) and E2 (Day 15). Moderate levels of IL-8 production were observed, for chicken E19 with score 3 on Day 15. Only at a later stage were moderate levels of IL-8 production for chickens presented with score 2 observed: E11 (Day 15), E6, E9, E11 and E26 (Day 21); but not during the initial stages of score 2, as seen with chickens E8 and E26, having low cytokine titres.

In mammals, IL-8 is a CXC chemokine and has therefore been renamed CXCL8 and is involved in the recruitment, activation and movement of human neutrophils (Cacalano *et al*. 1994; Davison *et al*. 2011). The chicken orthologue of IL-8/CXCL8 was suggested to be 9E3/CEF4, known as cCAF (chicken chemotactic and angiogenic factor) (Martins-Green and Feugate, 1998; Kaiser *et al*, 1999). However, it was proposed that 9E3/CEF4 would be called chicken CXCLi2 (chCXCLi2, "i" denoting inflammatory function) (Kaiser *et al*. 2005).

To date, IL-8 has been mostly associated with mucosal and gut-associated immunity, linked to diseases, such as rheumatoid arthritis, inflammatory bowel disease and highly pathogenic avian influenza (H5N3 and H7N9) in human hosts, as well as *Salmonella enteritidis* and *Campylobacter jejuni* infections in avian hosts (Luster, 1998; Kogut, 2002; de Jong *et al*. 2006; Smith *et al*. 2008; Zhou *et al*. 2013). chCXCLi2/IL-8 is a potent chemoattractant and during an inflammatory response, there is a dramatic increase in the secretion of this cytokine resulting in the selective recruitment and influx of specific leukocytes into inflamed tissue or site of infection such as monocytes, heterophils and macrophages (Kaiser *et al*., 2008). IC is an upper respiratory disease, whereby for score 1 there is mild facial swelling, score 2 there is bilateral facial oedema and score 3 there is severe bilateral oedema and conjunctivitis with or without haemorrhage, whereby the chickens also develop diarrhoea.

The results with regards to the expression of IL-8 with sandwich ELISA, coincide with the observed clinical scores of IC in infected chickens, whereby as the disease becomes more severe the expression and cytokine titres of IL-8 increases. Moreover, the findings also correlate to a study by Boucher *et al*. (2015), where there was a significant up-regulation for IL-8 during disease score 2 with *Av. paragallinarum* serovar C-3 infection. Furthermore, the flow cytometry (Figure 4.15 - Figure 4.20) and IL-8 assay (Figure 4.20) results showed that although the chickens were supposedly SPF before the start of the study, the chickens

already had a well-established immune system possibly due to prior exposure, since they were able to generate innate and adaptive immune cells as well as produce cytokines even before being exposed to the experimental *Av. paragallinarum* serovar C-3 (SA-3 strain), fortunately this was detected. Hence, it was possible that due to prior exposure, severe symptoms as described in literature were not observed in this study, which might also be the reason why some chickens recovered quickly after a score was reached with only a minority of chickens reaching a score of 3. In future studies, it is recommended to screen for antibodies even if suppliers guarantee that the chickens provided are SPF, as the immunity of chickens for the study could be compromised, especially if the effect of a drug or challenge study is being conducted which could lead to misleading or even erroneous results.

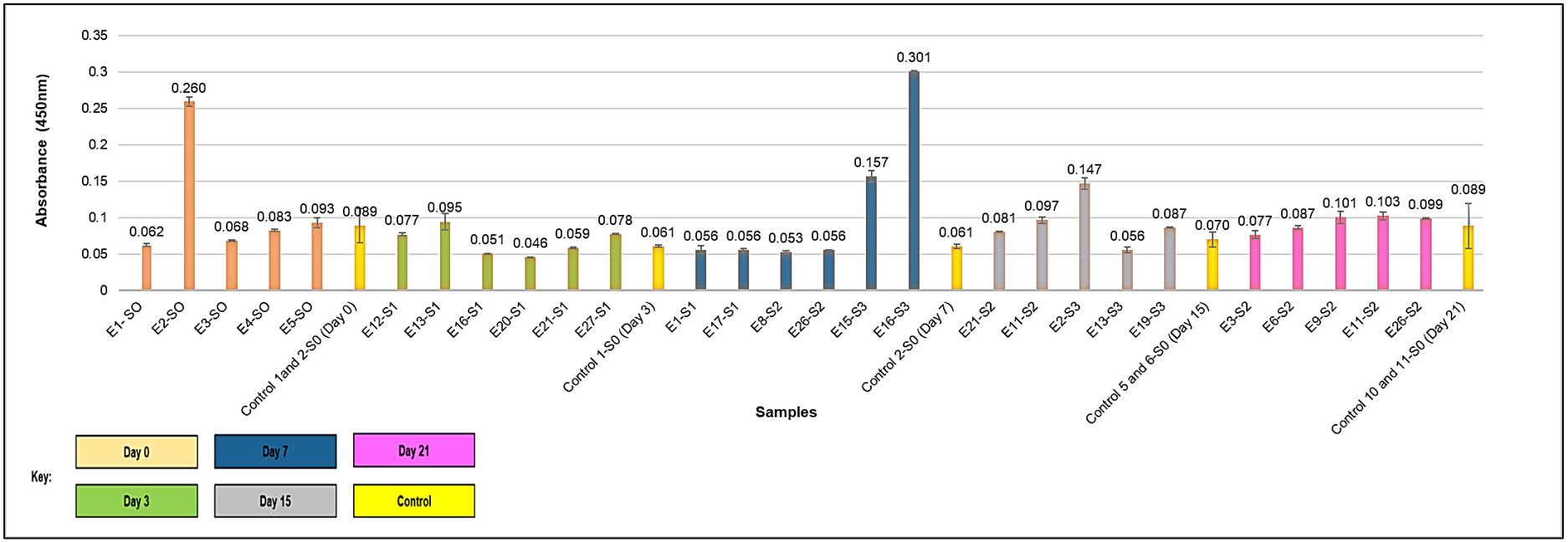


Figure 4.21: Graphical representation and statistical analysis of the sandwich ELISA assay conducted on chicken plasma samples from the experimental trial for the cytokine IL-8 ($p<0.05$). Day 0 chickens were not yet infected when they were bled at a score 0, however, moderate levels of cytokine titre for chickens E5, Control 1 and 2 were observed; with a very high cytokine titre for chicken E2. Chicken E13 with score 1 on Day 3 had a moderate cytokine titre. High cytokine titres were observed for experimental chickens presented with score 3: E15 (Day 7), E16 (Day 7) and E2 (Day 15). Moderate levels of IL-8 production, for chicken E19 with score 3 on Day 15 were observed. Only at a later stage were moderate levels of IL-8 production for chickens presented with score 2 observed: E11 (Day 15), E6, E9, E11 and E26 (Day 21); but not during the initial stages of score 2, as seen with chickens E8 and E26 having low cytokine titres. The control group had a stable and low expression of IL-8 on Days 3, 7 and 15. However there were moderate levels of cytokine titre at the beginning (Day 0) with Control 1 and 2 chickens at score 0 and at the end of the trial (Day 21) with Control 10 and 11. Although, the Day 0 and control chickens had moderate to high cytokine titres of IL-8 expressed, they did not show any IC related signs or symptoms.

4.4. Conclusion

Older birds were used in this study, as the sinuses of the birds at 25 weeks would be more developed than those at 20 weeks. This preliminary and novel study on IC infection, was successful, as disease progression was able to be monitored and each clinical score pertaining to IC related signs and symptoms observed (score 1-3), correlating the progression of the disease at different scores during different time intervals (days), thus deducing the approximate days of innate and/or adaptive mechanisms at play. Peripheral whole blood smears of chickens at different scores were also obtained, which provided us with the different cell morphologies and their respective functions in controlling disease. Furthermore, it was observed that the innate immunity was fast acting requiring less than a few hours or days to be fully functional, occurring within the first week of infection (7 days), compared to the adaptive response which took longer requiring several days (at Day 11 onwards). The leukocyte, CD4 and CD8 flow cytometry profiles of chicken blood samples at different scores were evaluated, giving us insight into the innate, humoral and cell-mediated immune responses involved in pathogen elimination. Moreover, the $CD4^+/CD8^+$ ratio was higher during the innate immune phase, indicating that Th cells have a major role to play in IC related infections, which could also be observed with the flow cytometry profile of $CD4^+$ cells percentages, however this area needs further investigation to determine whether the response was Th1 or Th2 mediated. With regards to the data obtained from egg production, highly variable egg production trends in both the control and experimental groups were observed. No statistically significant decline in egg production as stated in literature was noted. However, this anomaly could be as a result of a very small sample size in our study compared to the intensive chicken industry. Results with the expression of IL-8 with the sandwich ELISA coincide with all observed clinical scores (score 1-3) of IC in infected chickens, whereby as the disease becomes more severe the expression and cytokine titres of IL-8 increases, due to facial oedema severity as the diseases progresses, eventually leading to haemorrhage with severe facial oedema and conjunctivitis.

Strangely, the SPF chickens had been injected with *Av. paragallinarum* serovar C-3 and had developed clinical symptoms pertaining to IC whereby all scores were reached, however the disease was not as severe as observed in literature with field chickens, which is mainly because *Av. paragallinarum* infections in chickens from the field or extensive chicken industry often occur as mixed infections, hence the presence and possibility of other bacteria or viruses as secondary infections lead to complicated IC. However, from our findings only a minority of chickens reached a score of 3, whereas the rest of the chickens recovered. The control group, from the flow cytometry profiles and IL-8 assay results obtained, showed that they had an already well-established immune system suspected due to exposure to microorganisms before or during the study, however this did not lead to pathology as the chickens were asymptomatic for IC. If the entire cohort had been exposed to microorganisms before the study was conducted or during transportation of the chickens, memory cells had been formed. Therefore, the clinical signs and symptoms that was anticipated and expected of an IC infection was not fully observed during disease progression, as the chickens recovered swiftly following infection with *Av. paragallinarum* serovar C-3. In this study, after each clinical score was obtained, 2-4 chickens were sacrificed, whereby post-mortem examination and immunohistochemistry were performed (Chapter 5).

The IC infection model in this study could be used as a prognostic tool to monitor disease progression or the effect of therapeutic products (vaccines), for future studies. Failed vaccination attempts are still a major problem with IC, hence the knowledge gained from the study could help improve diagnostic testing for *Av. paragallinarum* serovar C-3 and/or other poultry diseases due to a better understanding of the avian immune system, which can also aid in the development of novel products such as an infectious coryza (IC) specific ELISA kit, avian haematology kits or CD4 and CD8 biomarkers, as well as in vaccine development.

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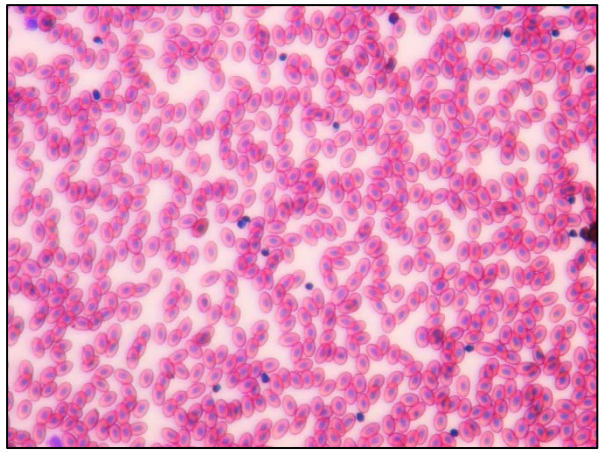
ANNEXURE C

Day 0



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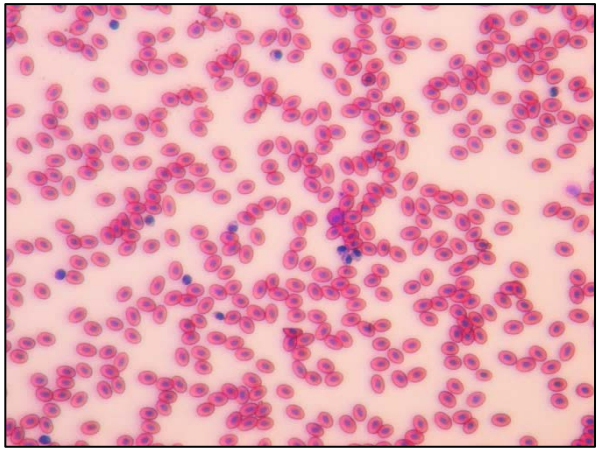

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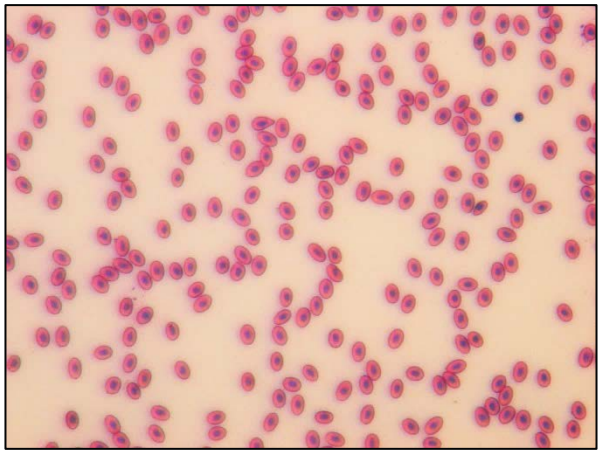

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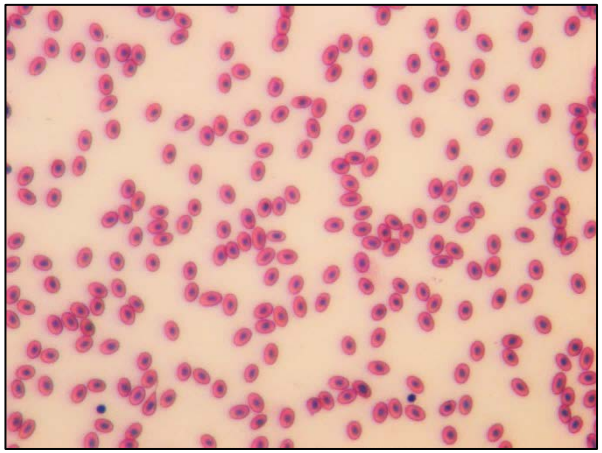

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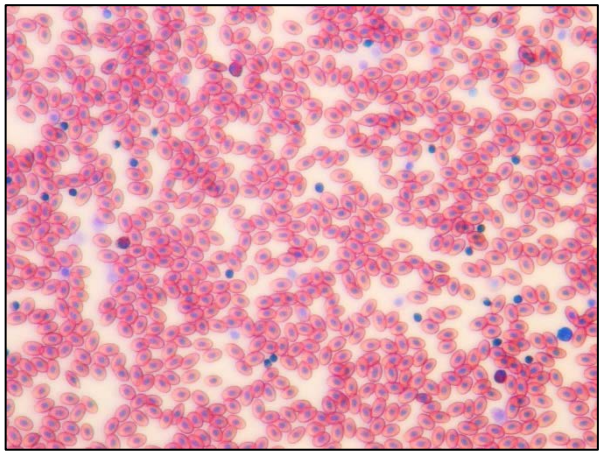

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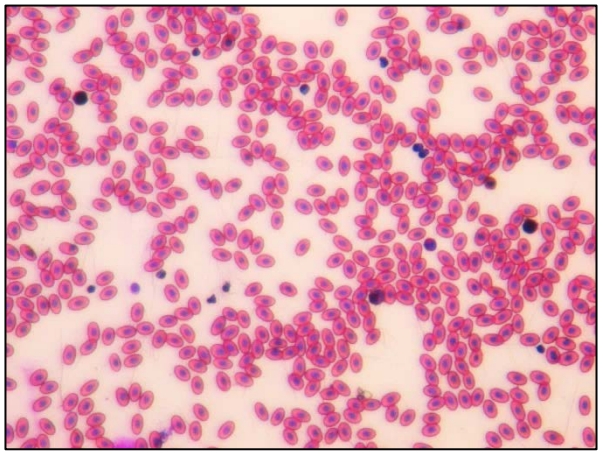

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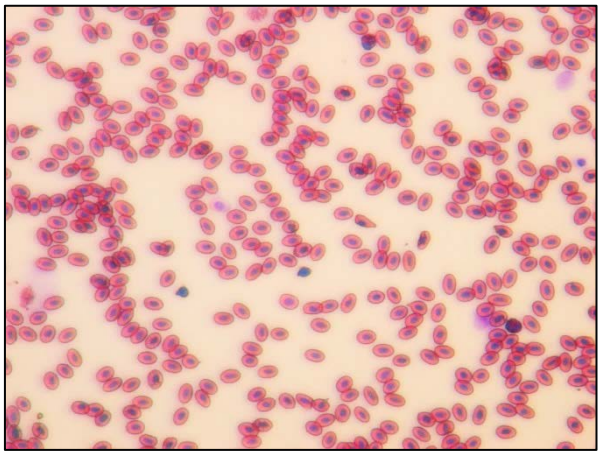

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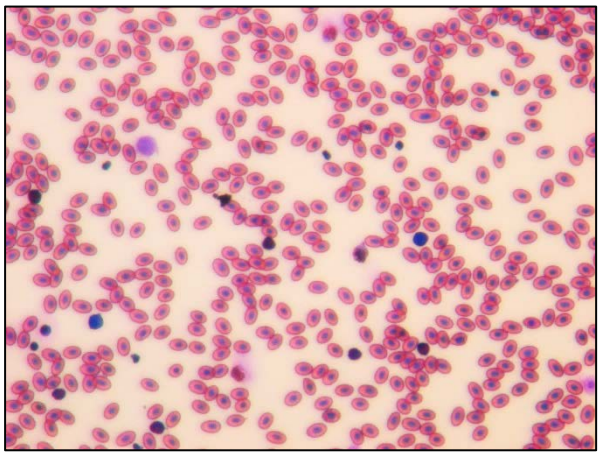

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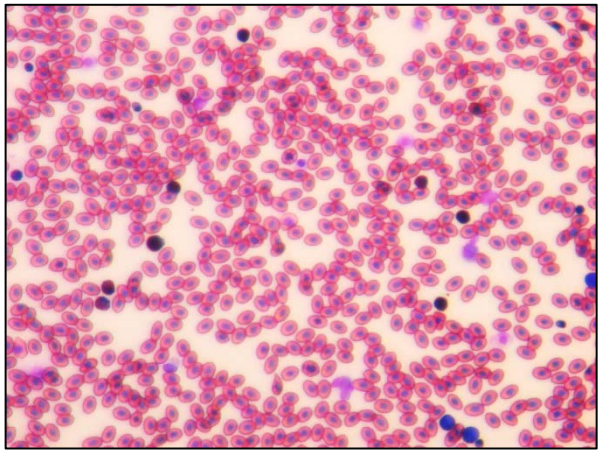

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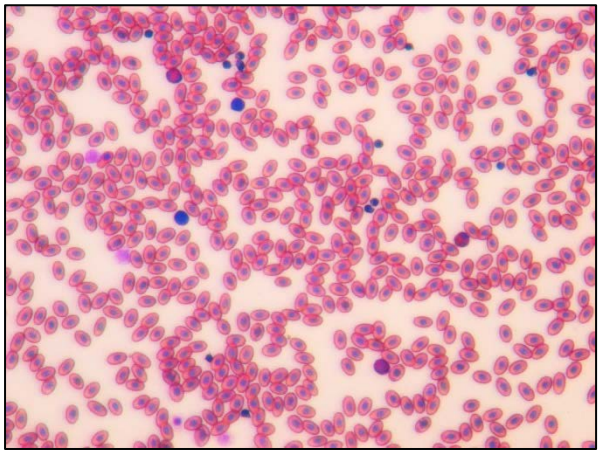

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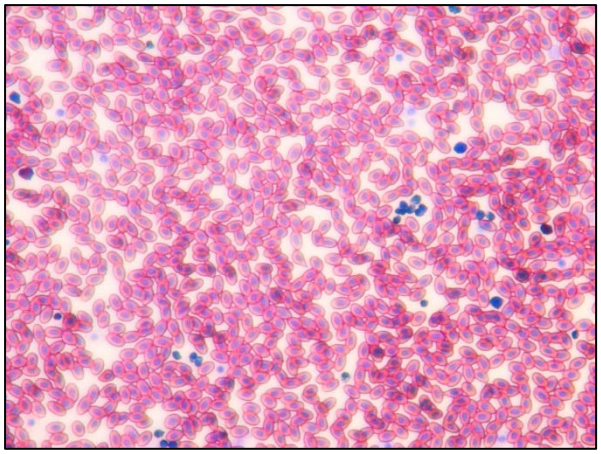

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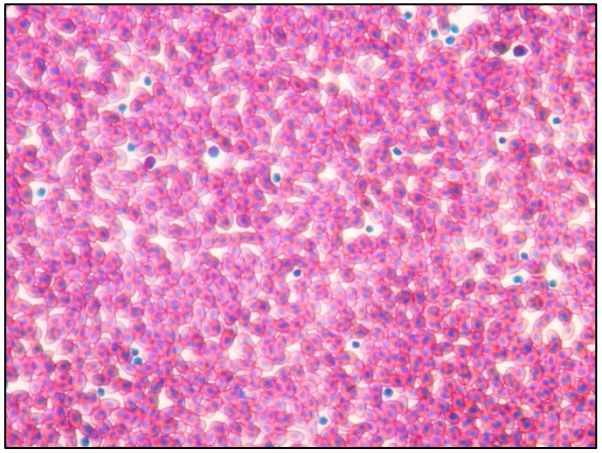

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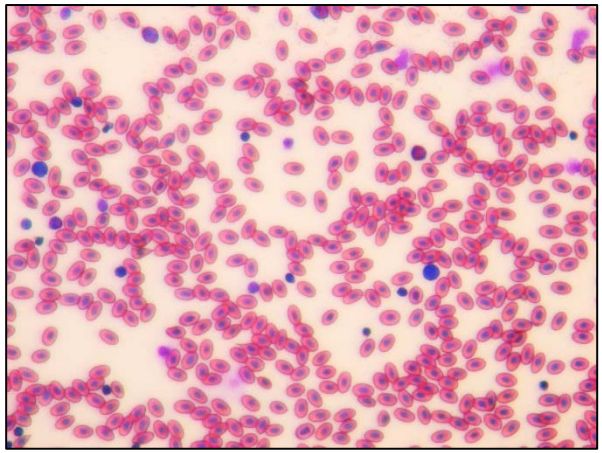

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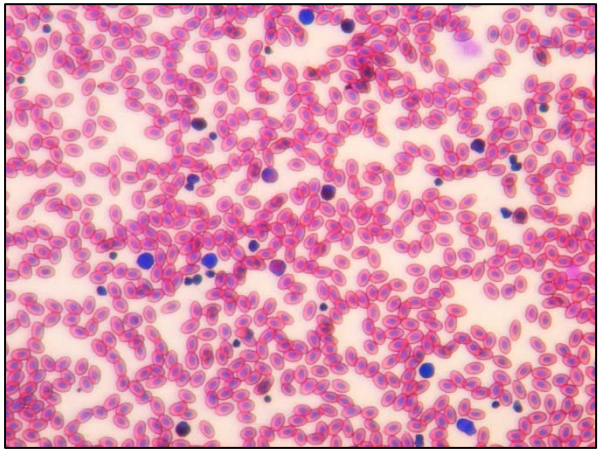

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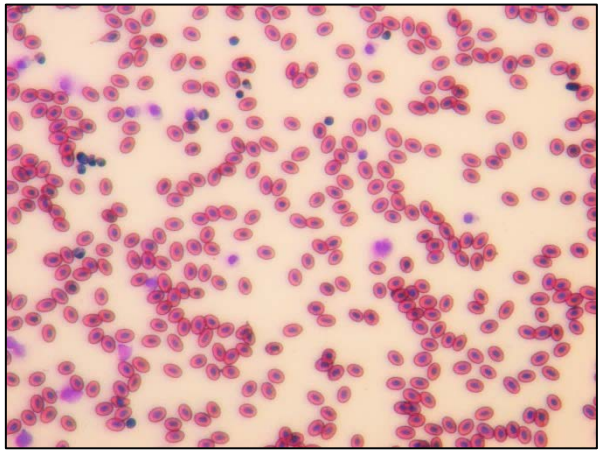
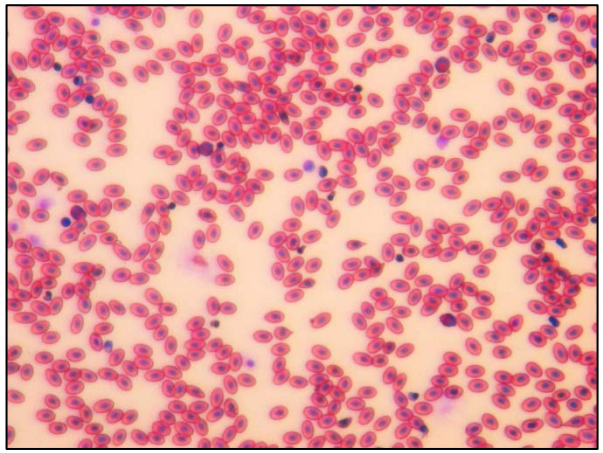
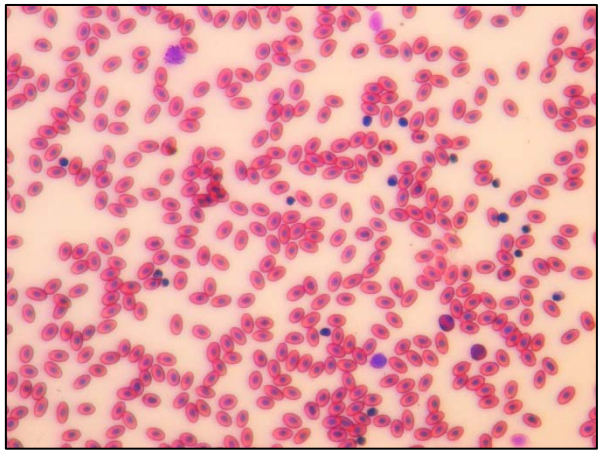


E21-Score 1

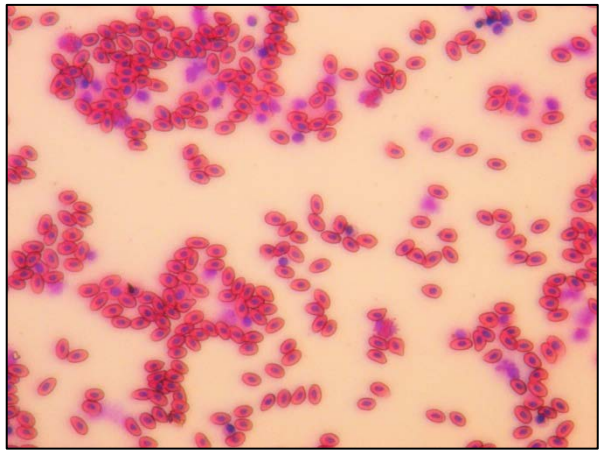
Day 4



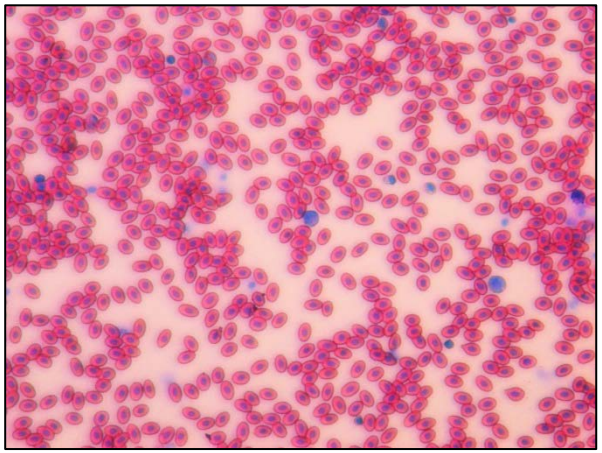
E4-Score 1



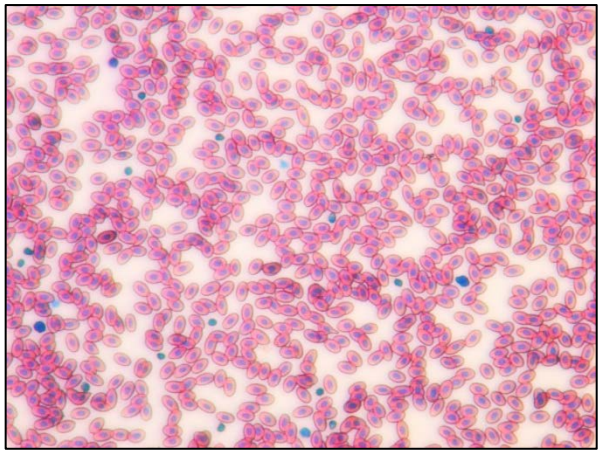
E11-Score 1



E17-Score 1

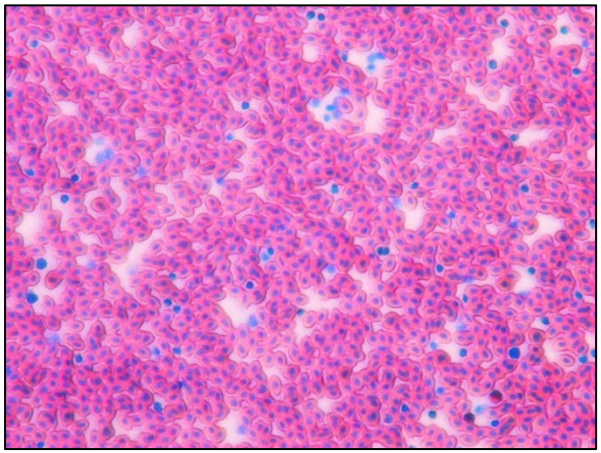


E24-Score 1

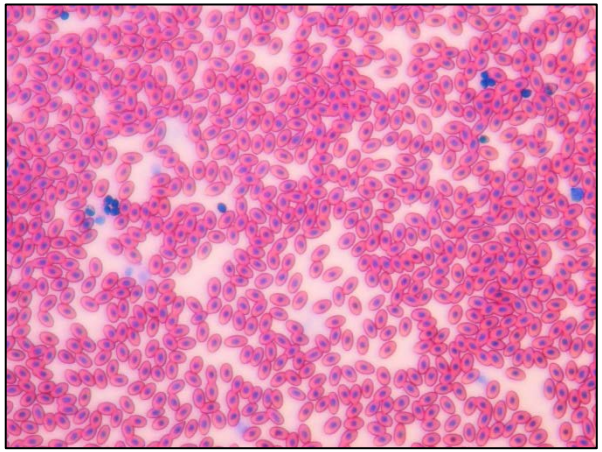


E25-Score 1

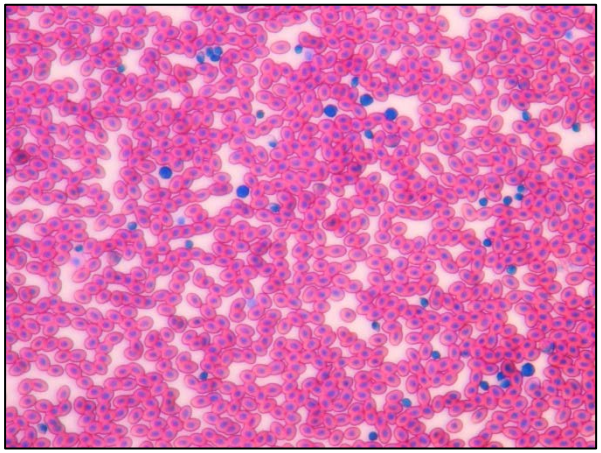
Day 7



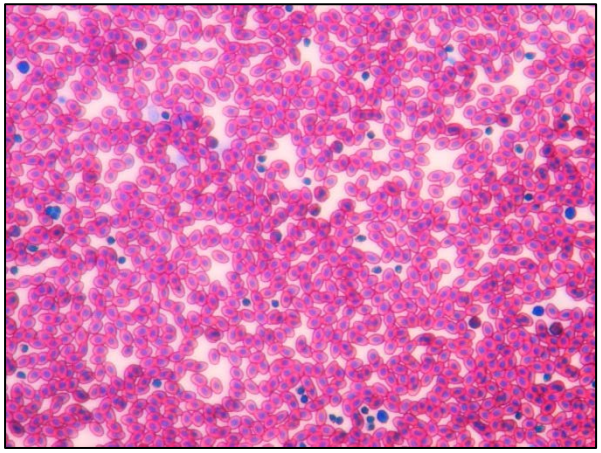
E15-Score 2



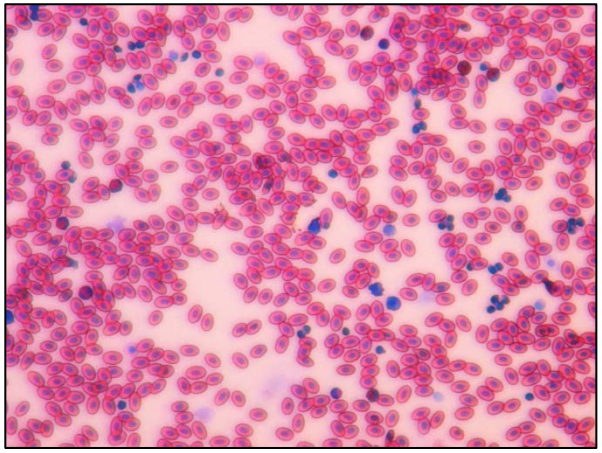
E16-Score 2



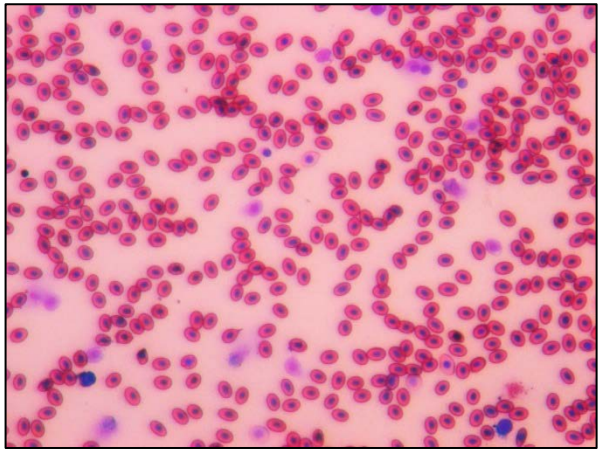
E22-Score 2



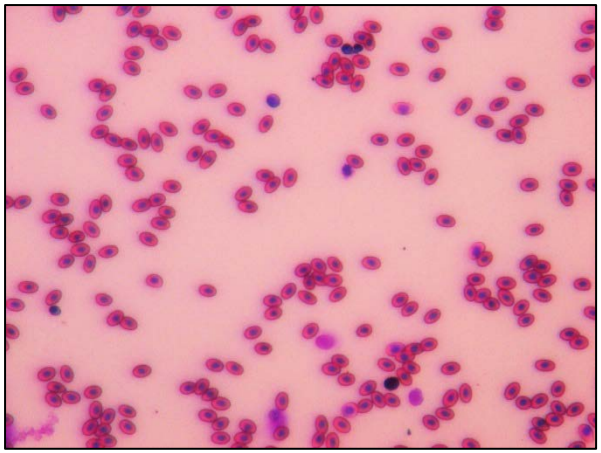
E24-Score 2



E26-Score 2

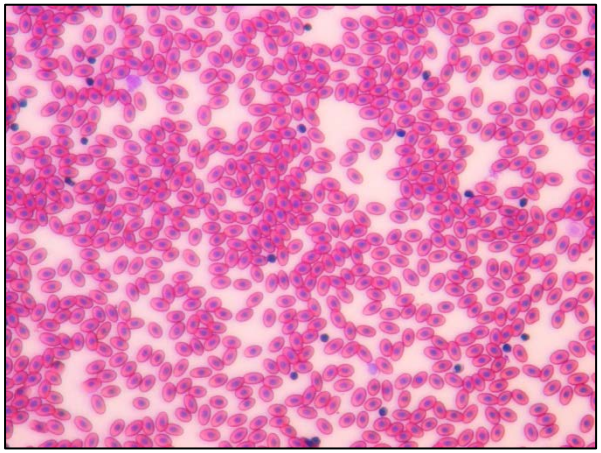


E28-Score 1

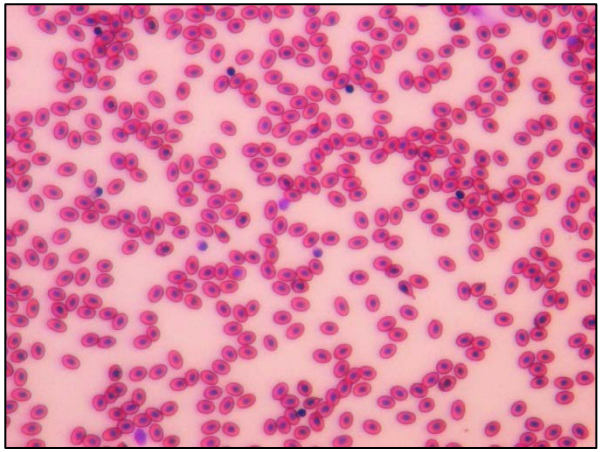


E30-Score 1

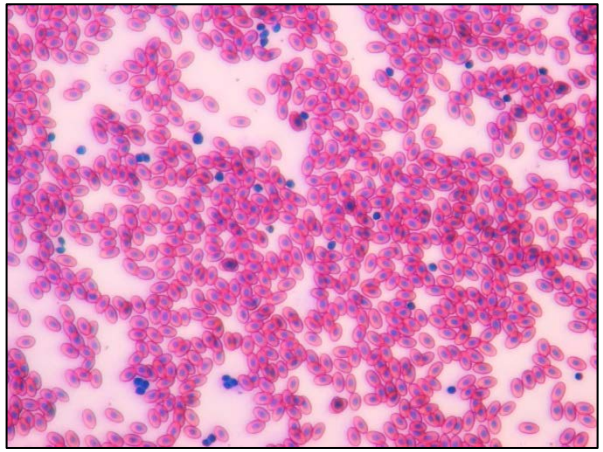
Day 10



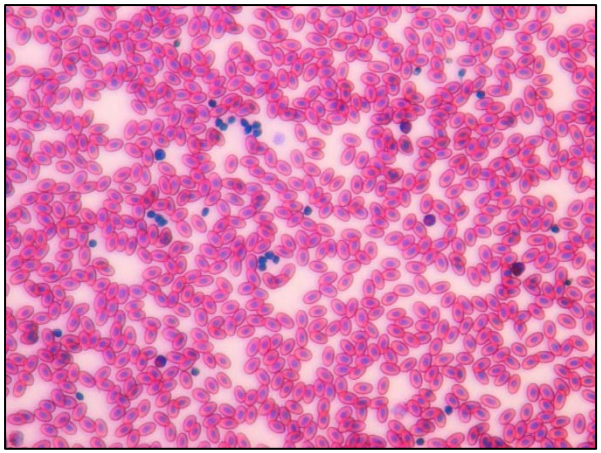
E6-Score 1



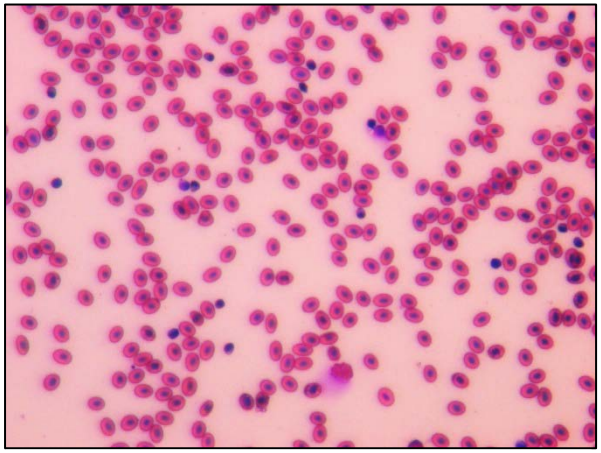
E9-Score 2



E11-Score 2

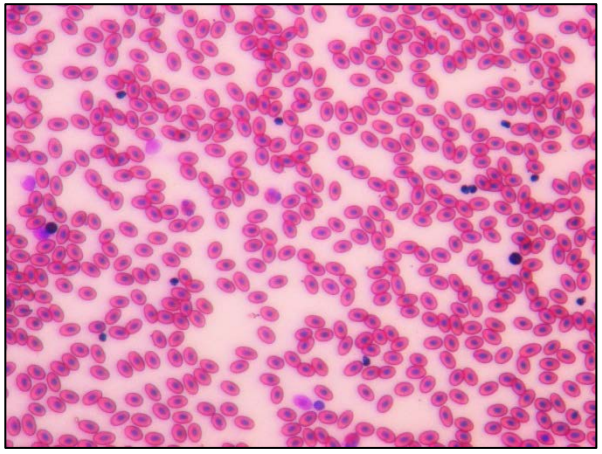


E17-Score 3

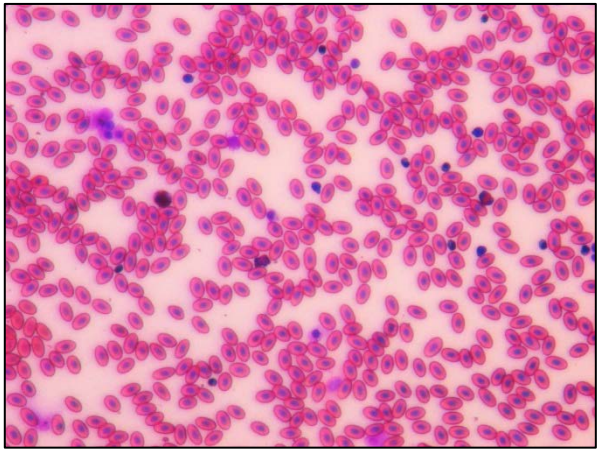


E22-Score 3

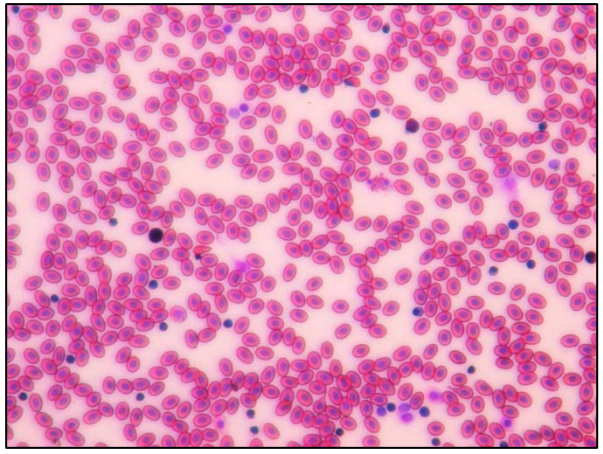
Day 11



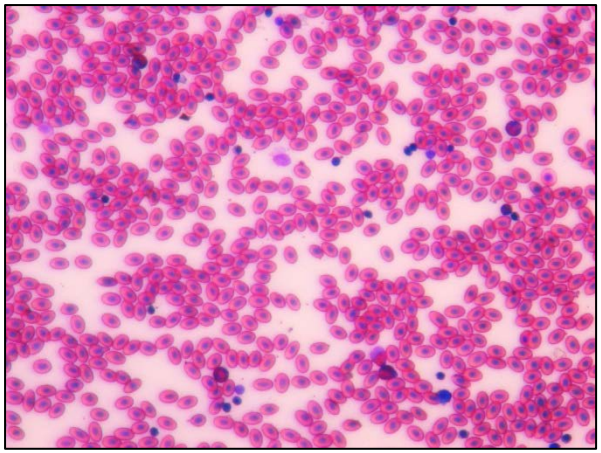
Control 1-Score 0



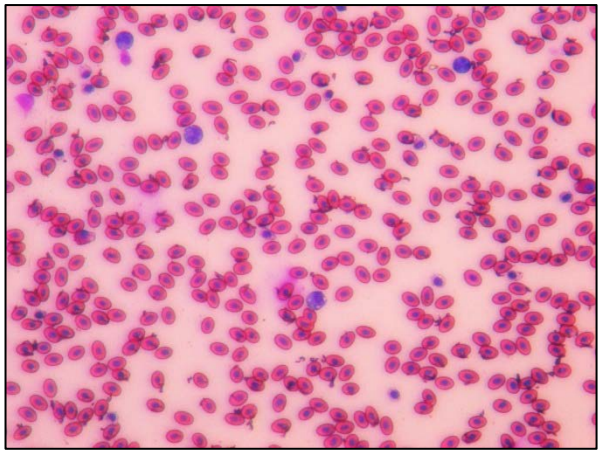
Control 2-Score 0



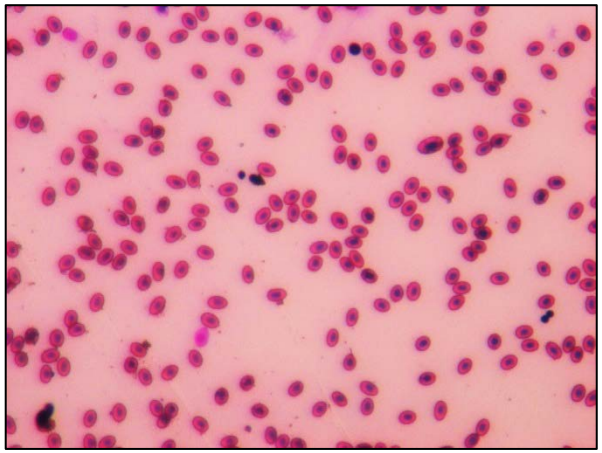
Control 3- Score 0



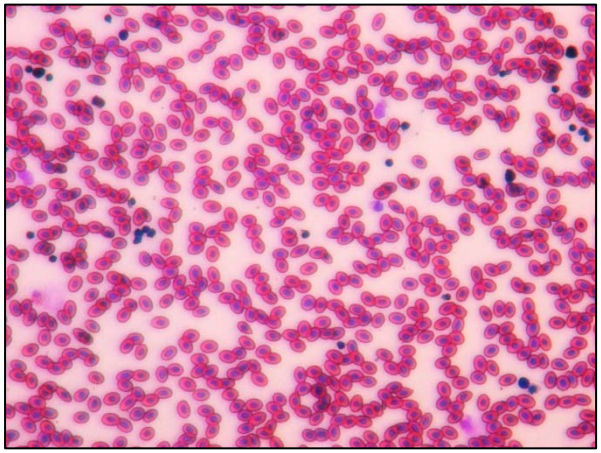
E15-Score 3



E22-Score 3

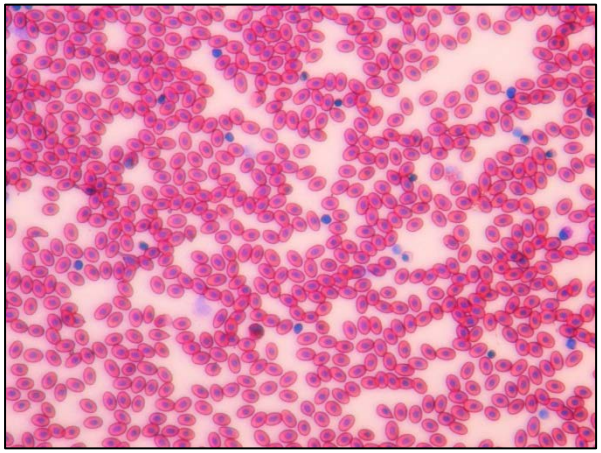


E26-Score 2

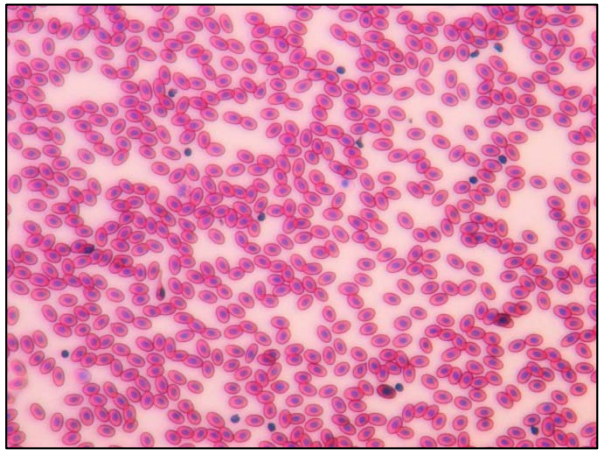


E28-Score 2

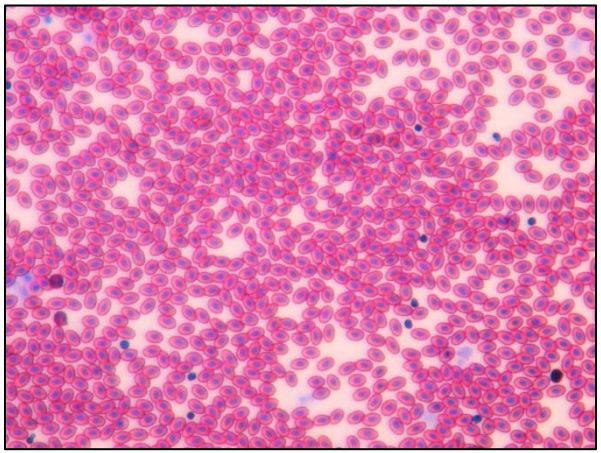
Day 15



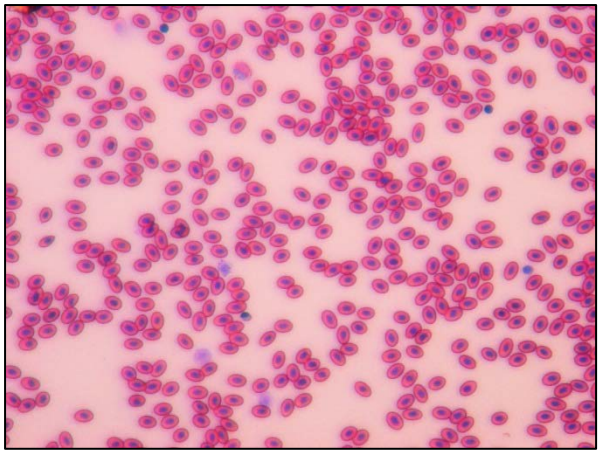
Control 4-Score 0



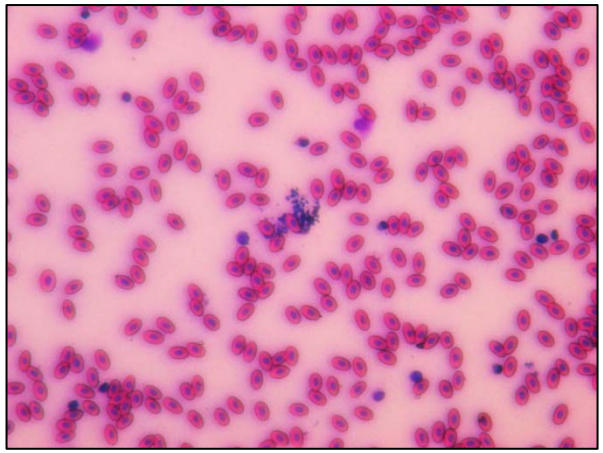
Control 5-Score 0



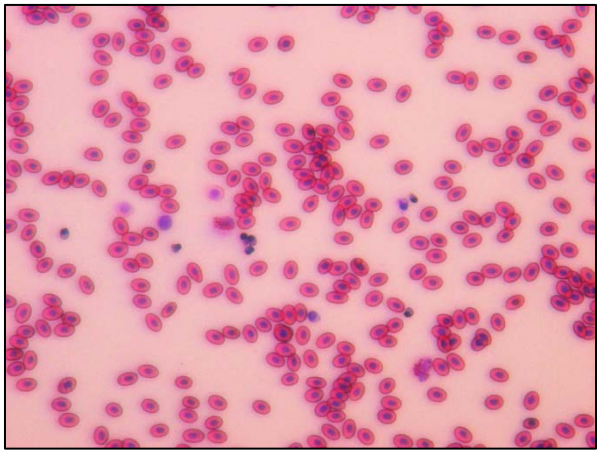
Control 6-Score 0



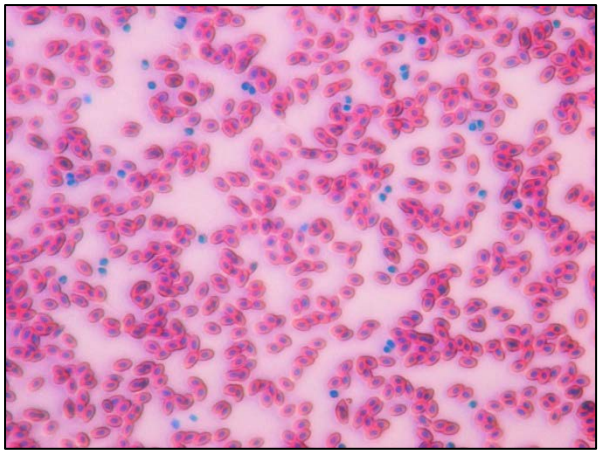
E2-Score 3



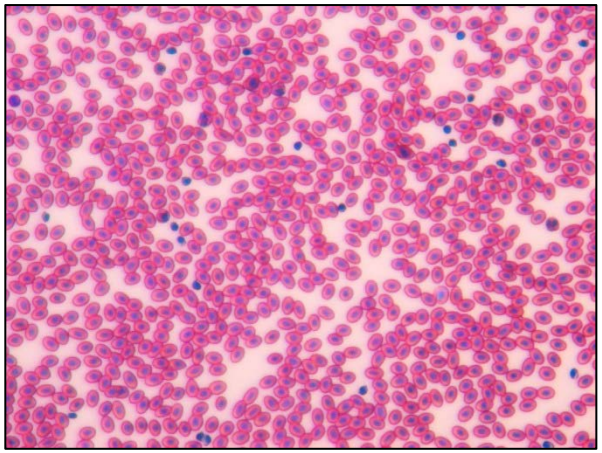
E11-Score 2



E13-Score 3

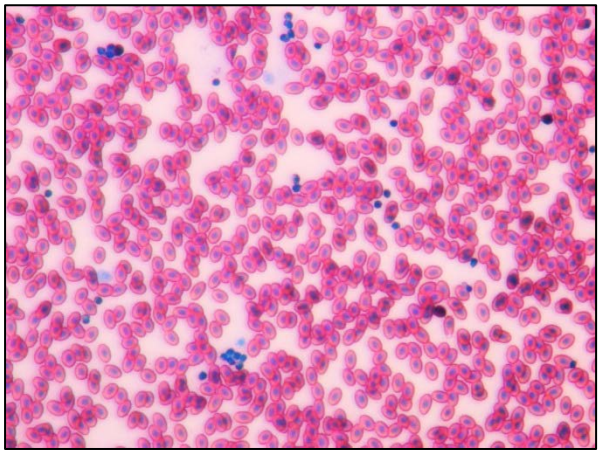


E19-Score 3

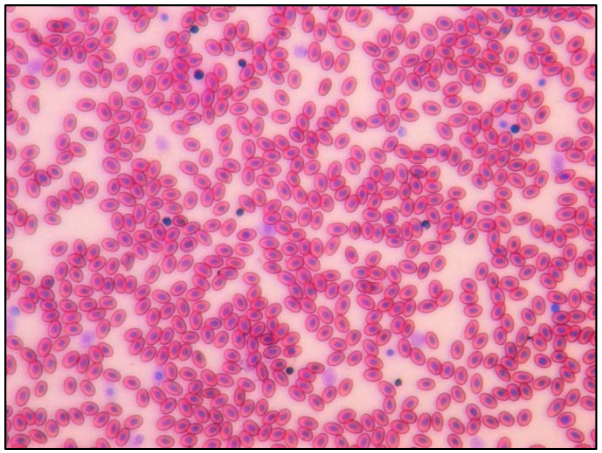


E21-Score 2

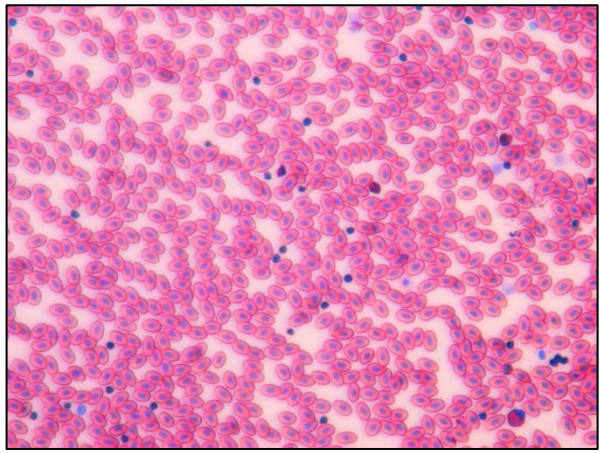
Day 18



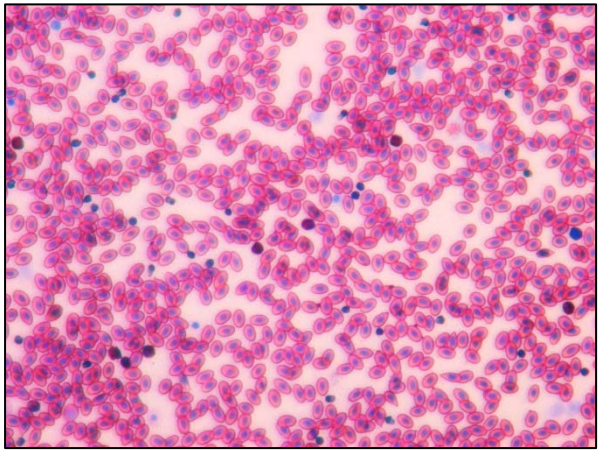
Control 7-Score 0



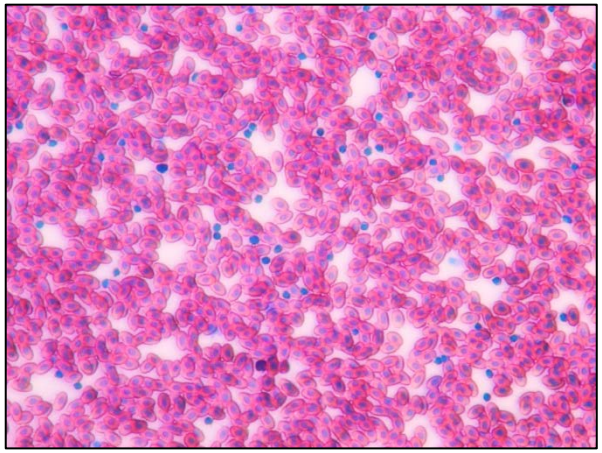
Control 8-Score 0



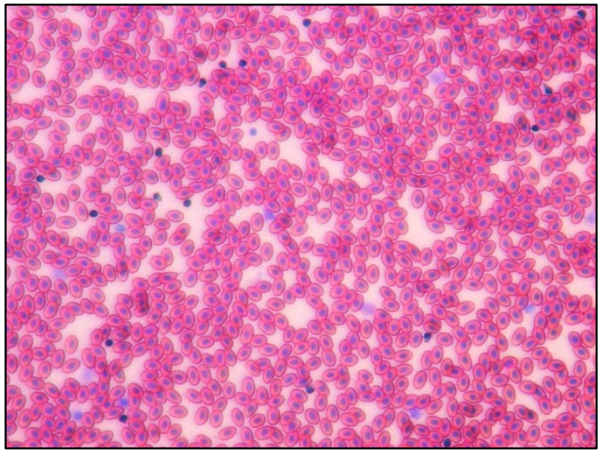
Control 9-Score 0



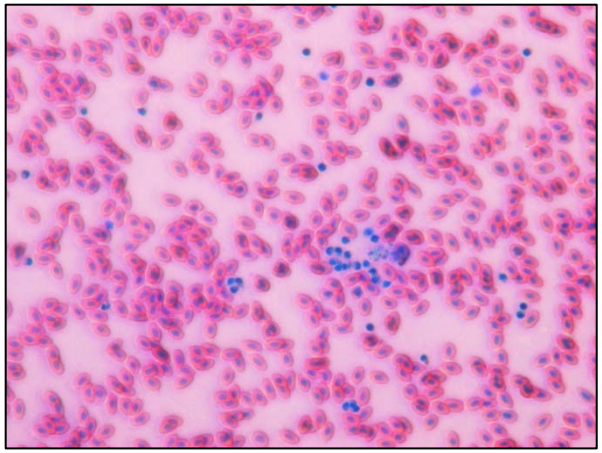
E4-Score 2



E11-Score 3



E15-Score 3



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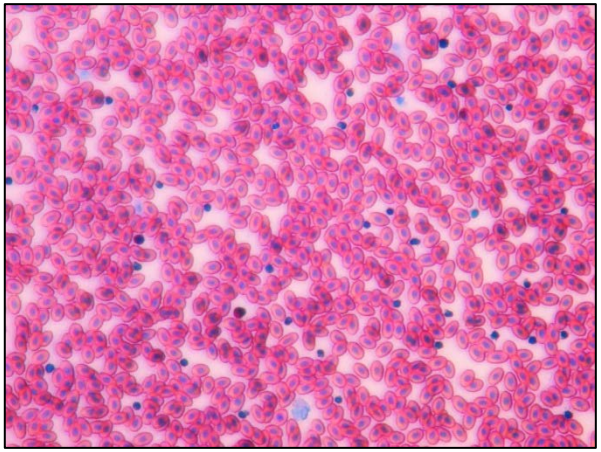
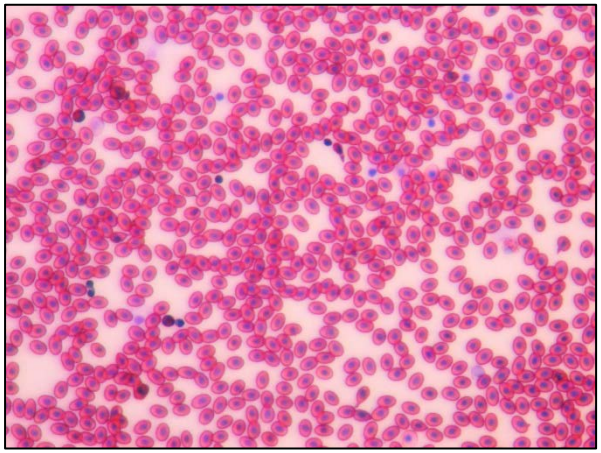
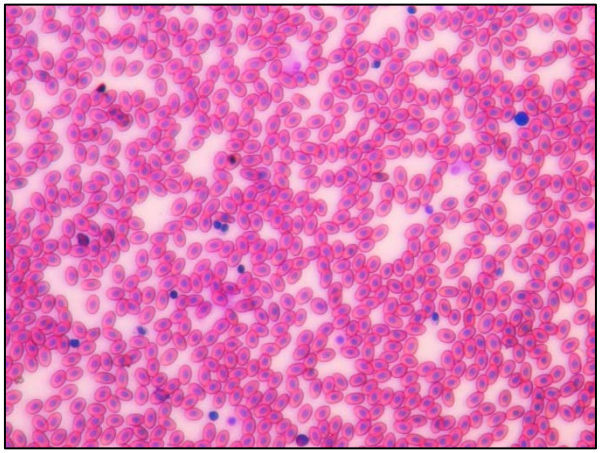


E27-Score 3

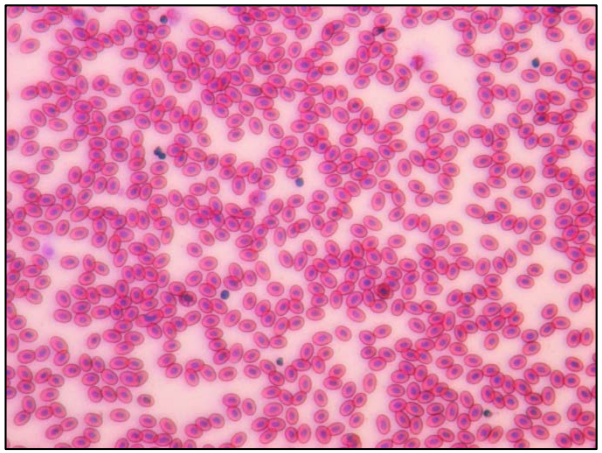
Day 21



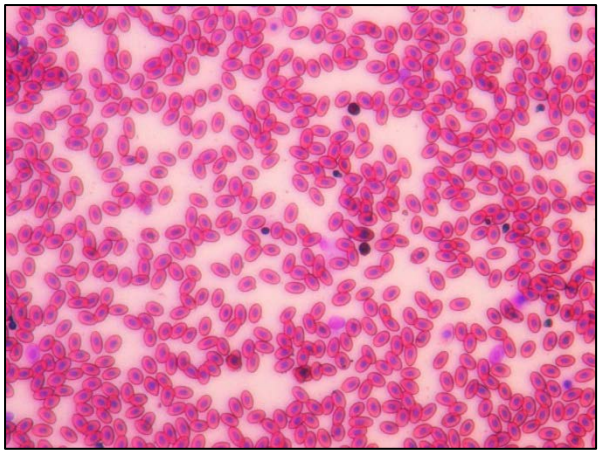
Control 10-Score 0



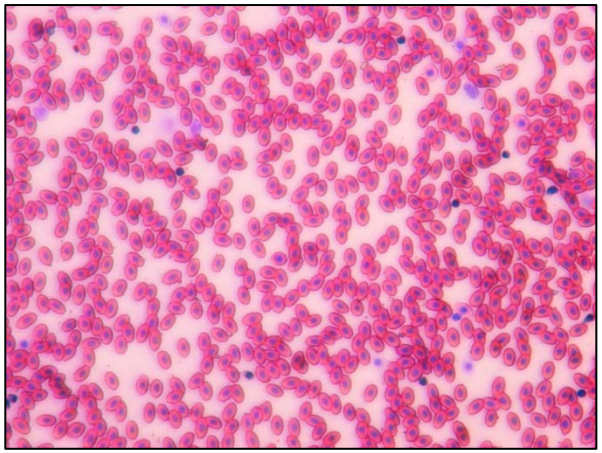
Control 11-Score 0



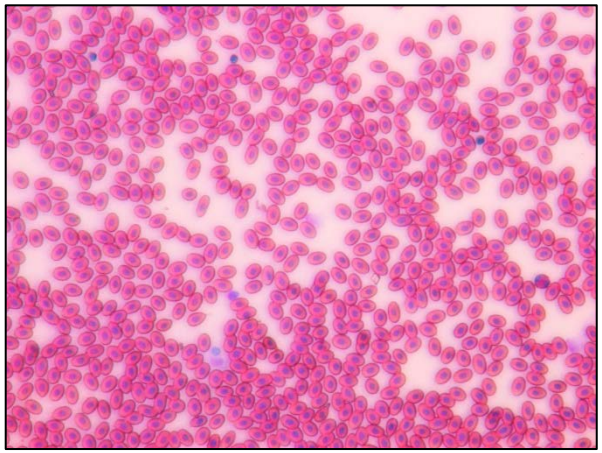
Control 12-Score 0



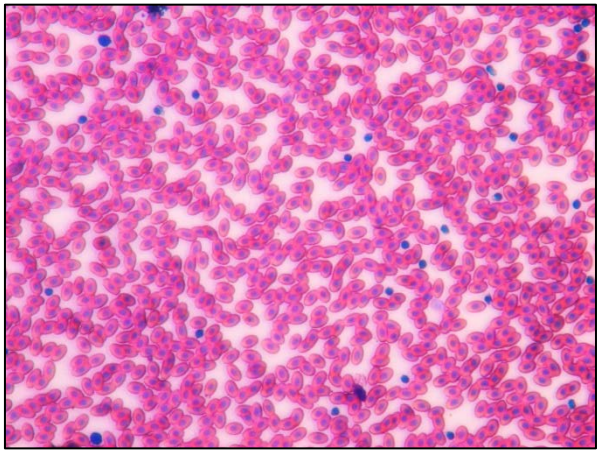
Control 13-Score 0



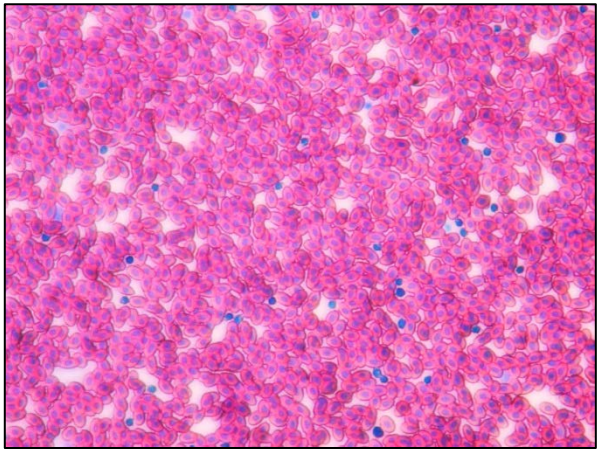
E6-Score 2



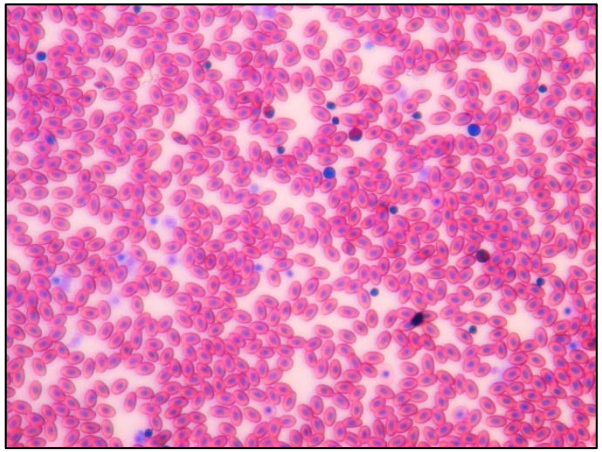
E9-Score 2



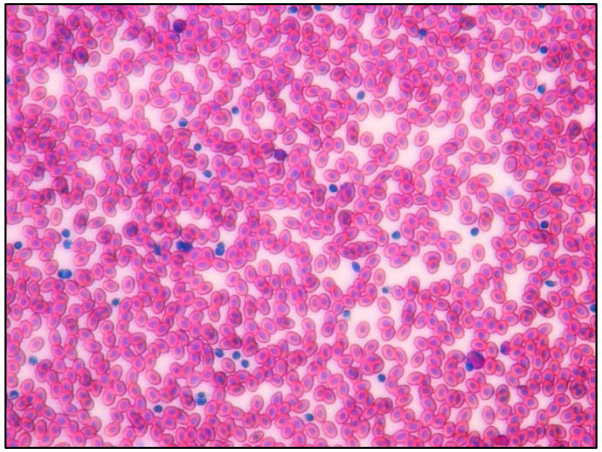
E11-Score 3



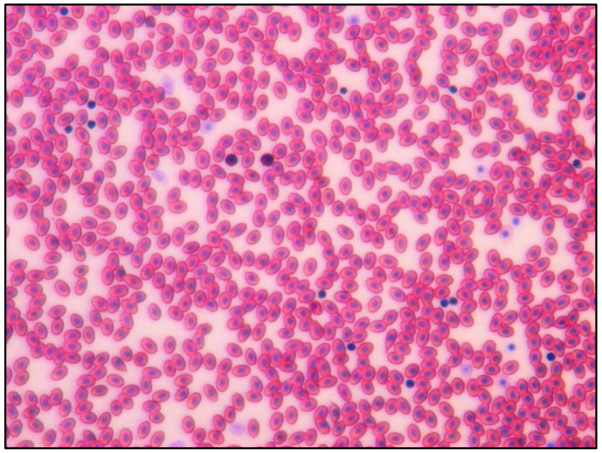
E15-Score 2



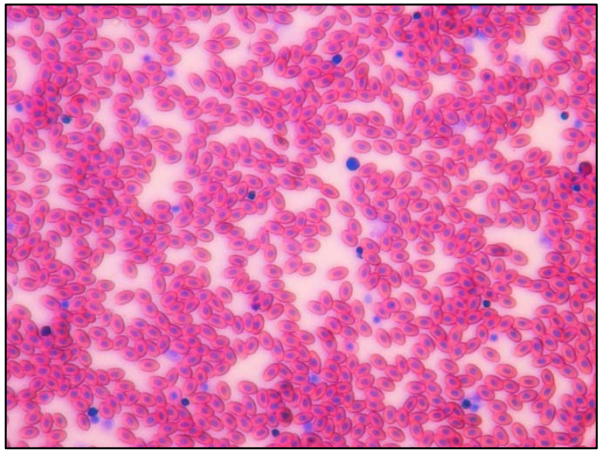
E17-Score 2



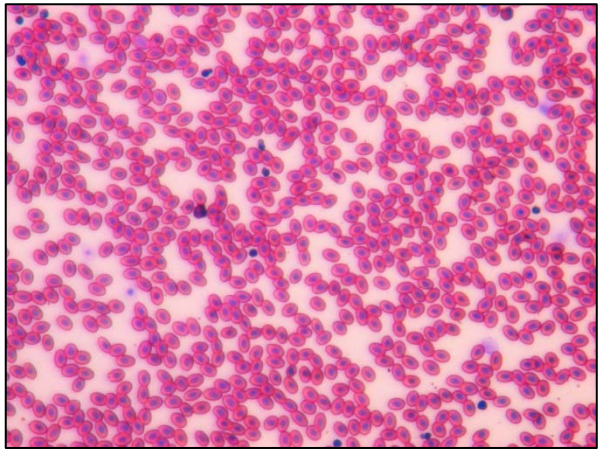
E21-Score 2



E26-Score 2



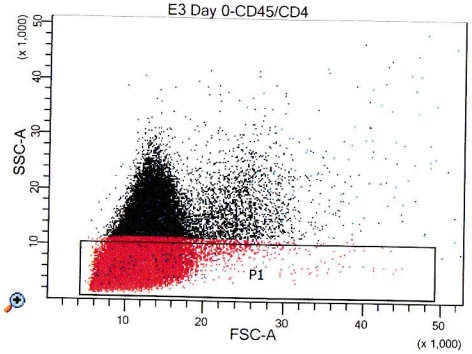
E27-Score 0

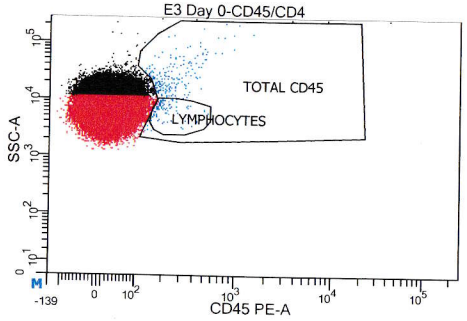


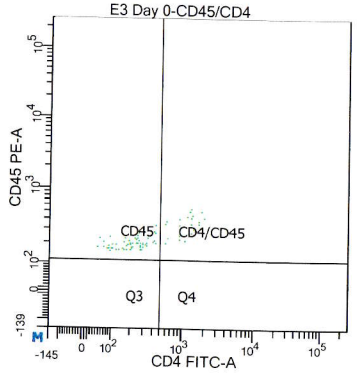
E28-Score 2

ANNEXURE D

BD FACSDiva 8.0.1





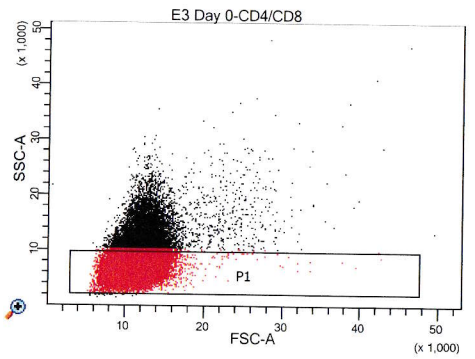


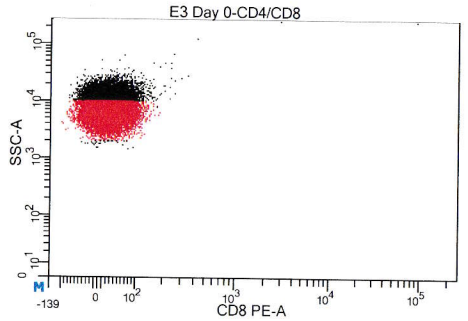
Experiment Name:	Chicken new batch
Specimen Name:	E3 Day 0
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:31:12 AM
$OP:	Administrator

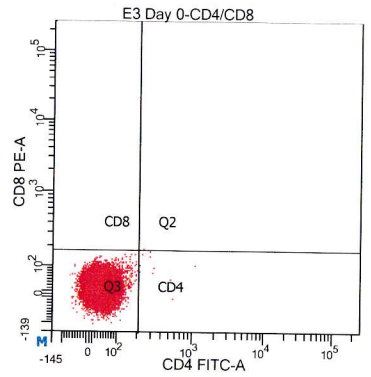
Population	#Events	%Parent
LYMPHOCYTES	90	0.0
CD45	64	71.1
CD4/CD45	26	28.9
TOTAL CD45	297	0.1

Printed by: Administrator

BD FACSDiva 8.0.1



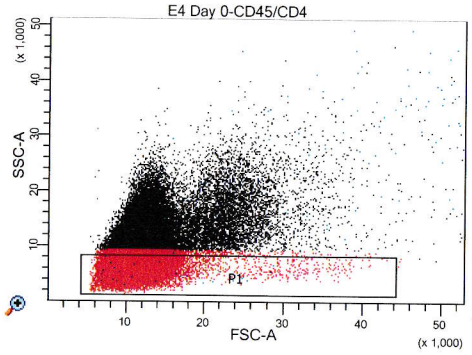


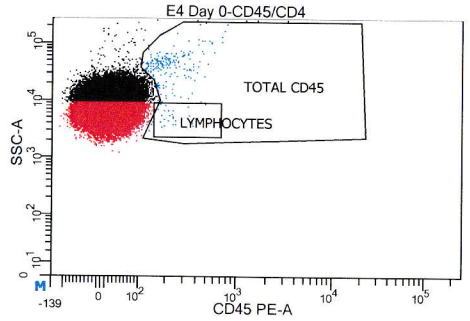


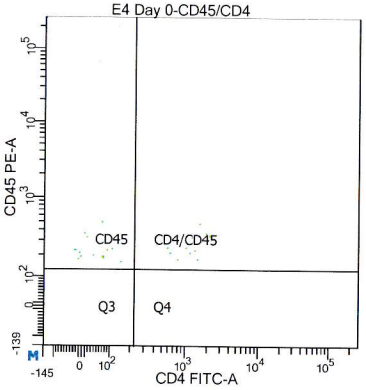
Experiment Name:	Chicken new batch
Specimen Name:	E3 Day 0
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:32:00...
$OP:	Administrator

Population	#Events
CD8	0
CD4	10

BD FACSDiva 8.0.1





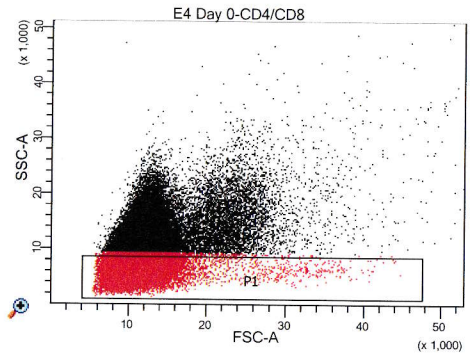


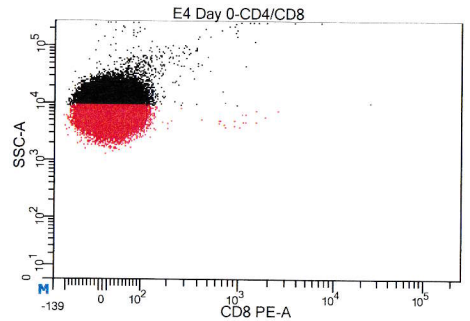
Experiment Name:	Chicken new batch
Specimen Name:	E4 Day 0
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:34:08 ...
$OP:	Administrator

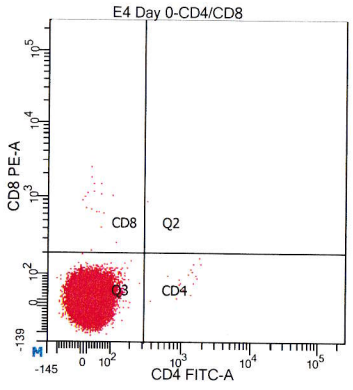
Population	#Events	%Parent
LYMPHOCYTES	27	0.0
CD45	15	55.6
CD4/CD45	12	44.4
TOTAL CD45	232	0.1

Printed by: Administrator

BD FACSDiva 8.0.1



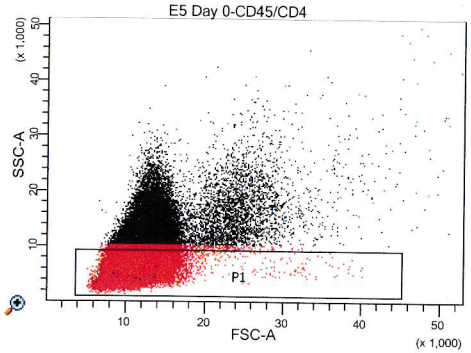


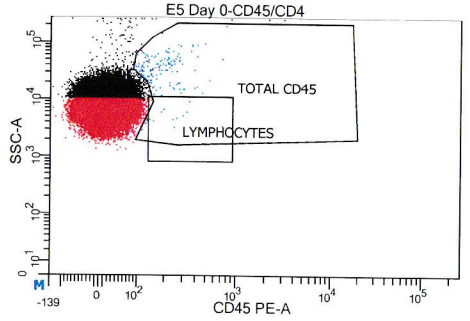


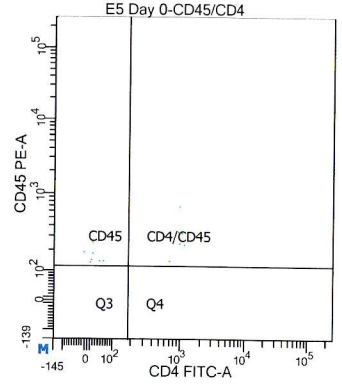
Experiment Name:	Chicken new batch
Specimen Name:	E4 Day 0
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:35:13 ...
$OP:	Administrator

Population	#Events
CD8	18
CD4	19

BD FACSDiva 8.0.1





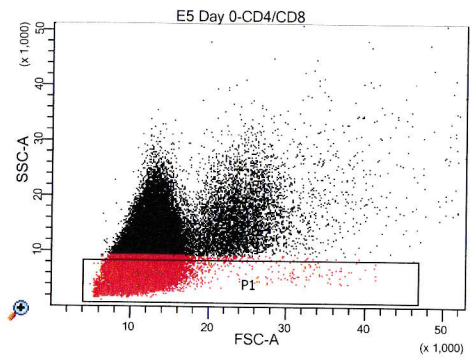


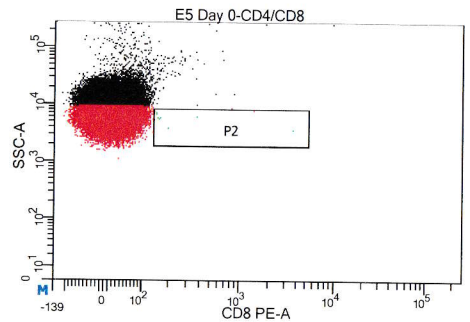
Experiment Name:	Chicken new batch
Specimen Name:	E5 Day 0
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:40:34...
$OP:	Administrator

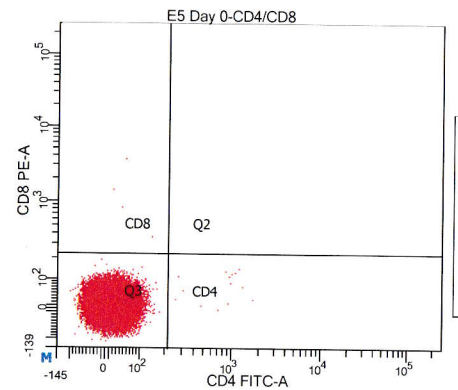
Population	#Events	%Parent
LYMPHOCYTES	14	0.0
CD45	7	50.0
CD4/CD45	7	50.0
TOTAL CD45	158	0.1

Printed by: Administrator

BD FACSDiva 8.0.1



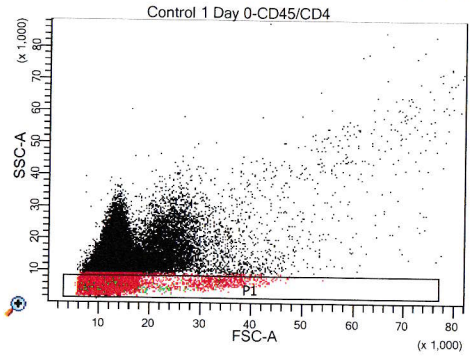


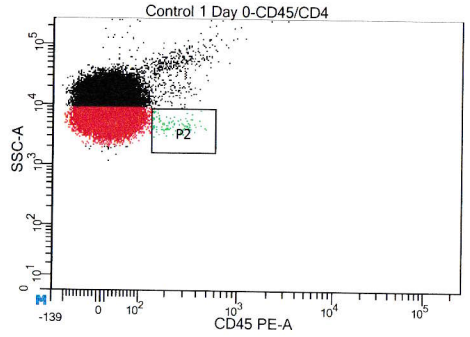


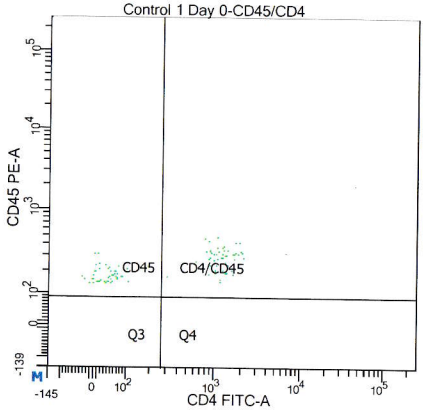
Experiment Name:	Chicken new batch
Specimen Name:	E5 Day 0
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:41:3...
$OP:	Administrator

Population	#Events
CD8	4
CD4	14

BD FACSDiva 8.0.1





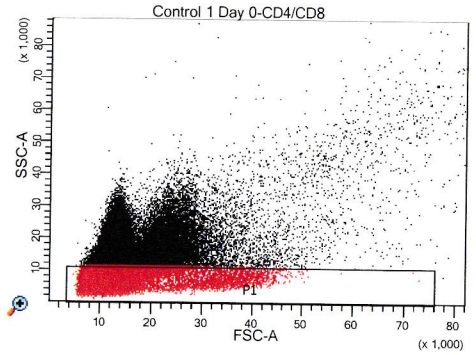


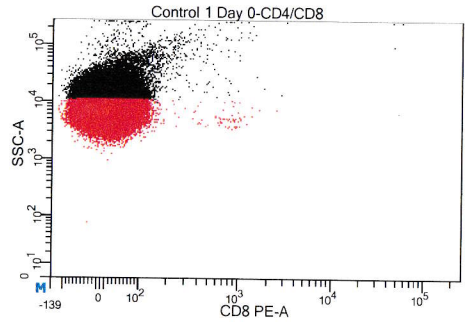
Experiment Name:	Chicken new batch
Specimen Name:	Control 1 Day 0
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 10:56:3...
$OP:	Administrator

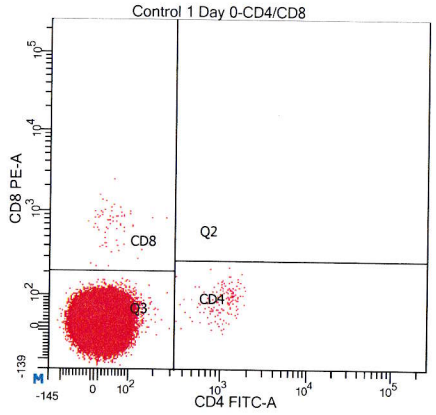
Population	#Events	%Parent
P2	102	0.1
CD45	49	48.0
CD4/CD45	53	52.0

Printed by: Administrator

BD FACSDiva 8.0.1



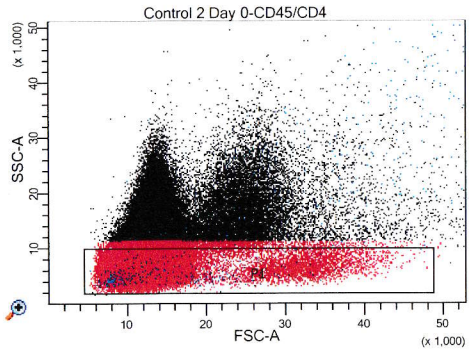


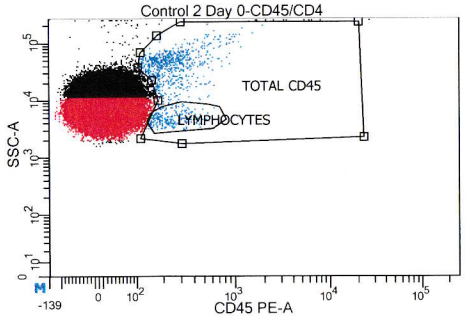


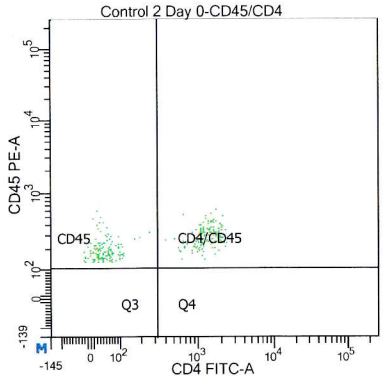
Experiment Name:	Chicken new batch
Specimen Name:	Control 1 Day 0
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 10:58:23 ...
$OP:	Administrator

Population	#Events
CD8	66
CD4	155

BD FACSDiva 8.0.1





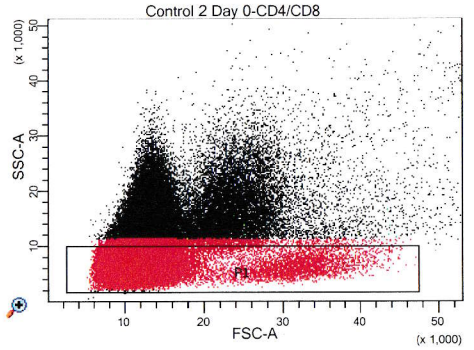


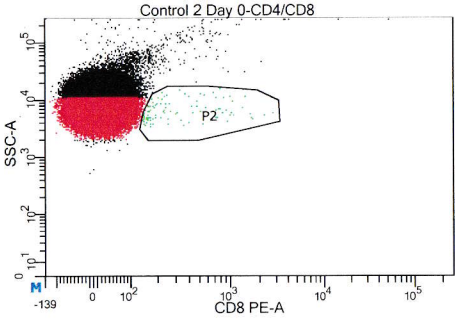
Experiment Name:	Chicken new batch
Specimen Name:	Control 2 Day 0
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:26:00 ...
$OP:	Administrator

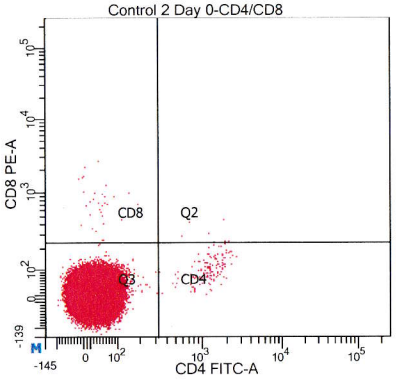
Population	#Events	%Parent
LYMPHOCYTES	261	0.1
CD45	132	50.6
CD4/CD45	129	49.4
TOTAL CD45	1,006	0.2

Printed by: Administrator

BD FACSDiva 8.0.1



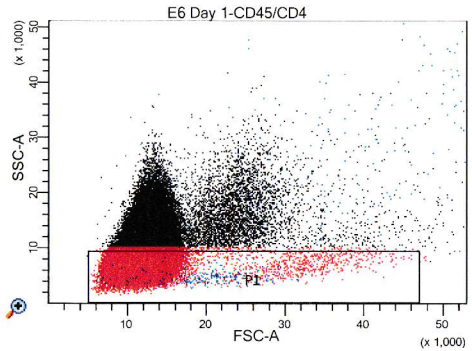


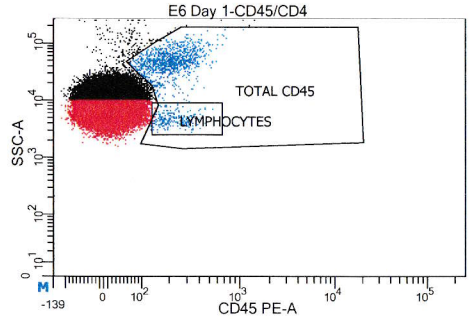


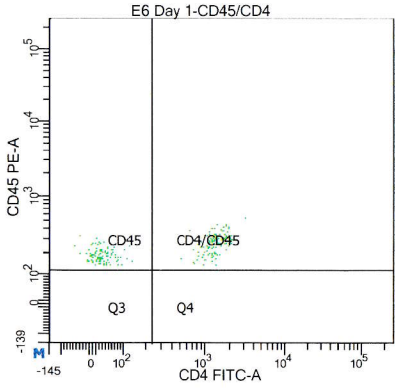
Experiment Name:	Chicken new batch
Specimen Name:	Control 2 Day 0
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:27:4...
$OP:	Administrator

Population	#Events
CD8	31
CD4	139

BD FACSDiva 8.0.1





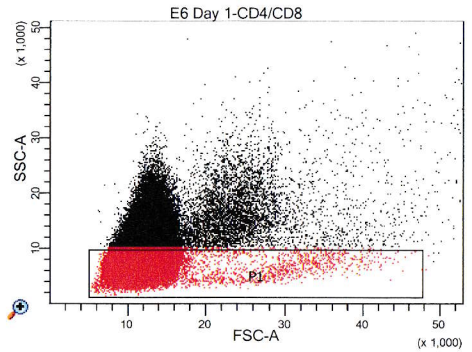


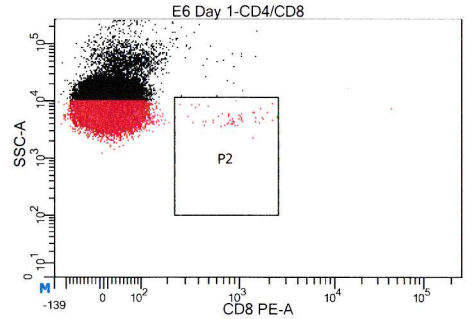
Experiment Name:	Chicken new batch
Specimen Name:	E6 Day 1
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:42:5...
$OP:	Administrator

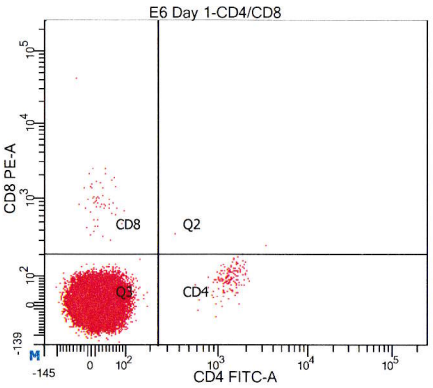
Population	#Events	%Parent
LYMPHOCYTES	246	0.1
CD45	112	45.5
CD4/CD45	134	54.5
TOTAL CD45	1,315	0.7

Printed by: Administrator

BD FACSDiva 8.0.1



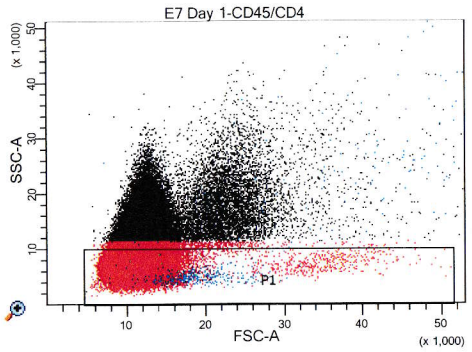


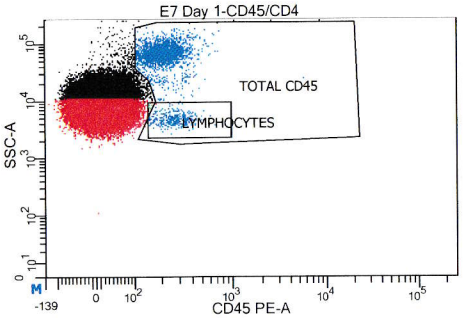


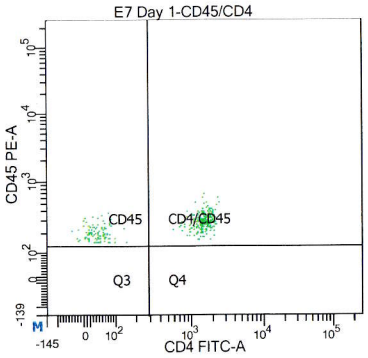
Experiment Name:	Chicken new batch
Specimen Name:	E6 Day 1
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:44...
$OP:	Administrator

Population	#Events
CD8	53
CD4	149

BD FACSDiva 8.0.1





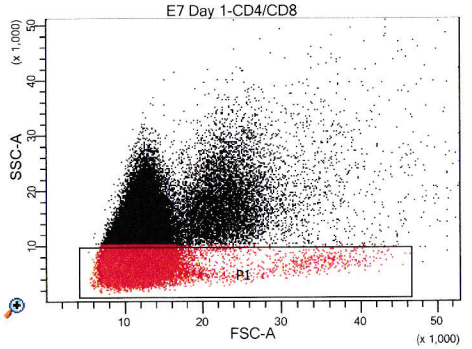


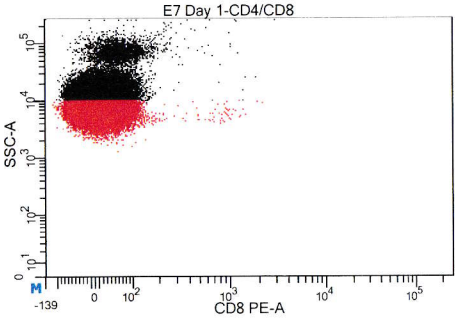
Experiment Name:	Chicken new batch
Specimen Name:	E7 Day 1
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:45:26 AM
$OP:	Administrator

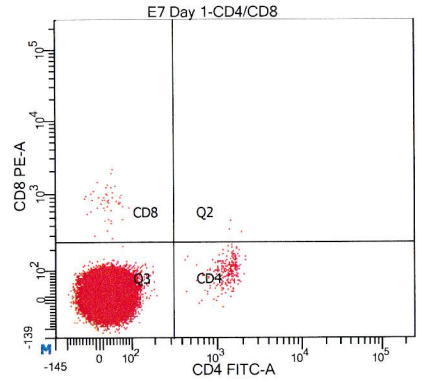
Population	#Events	%Parent
LYMPHOCYTES	359	0.2
CD45	105	29.2
CD4/CD45	254	70.8
TOTAL CD45	2,840	1.3

Printed by: Administrator

BD FACSDiva 8.0.1



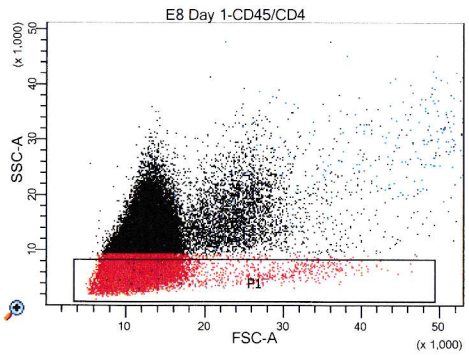


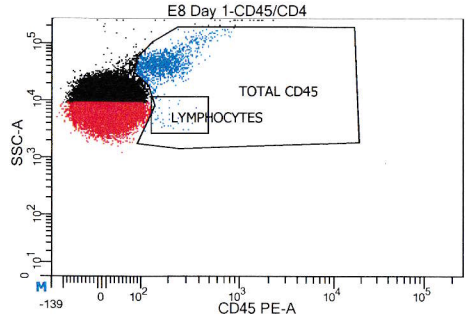


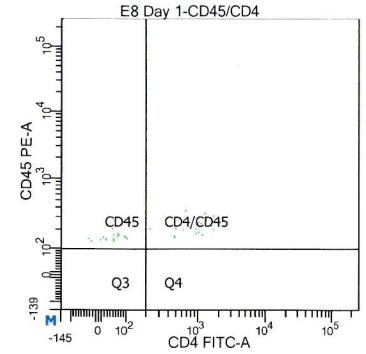
Experiment Name:	Chicken new batch
Specimen Name:	E7 Day 1
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:46:2...
$OP:	Administrator

Population	#Events
CD8	48
CD4	247

BD FACSDiva 8.0.1





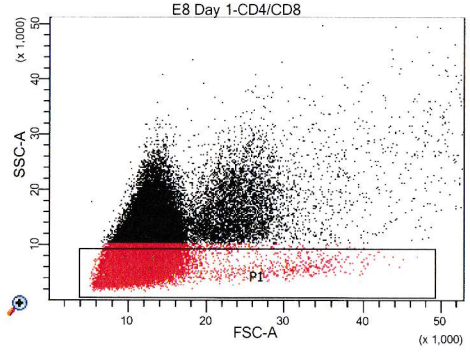


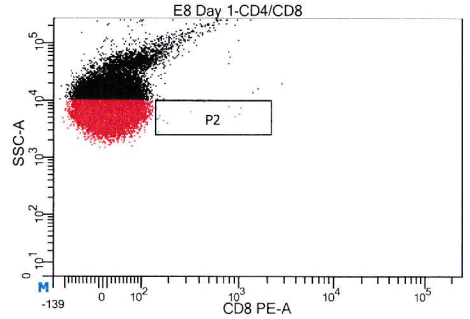
Experiment Name:	Chicken new batch
Specimen Name:	E8 Day 1
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:47:4...
$OP:	Administrator

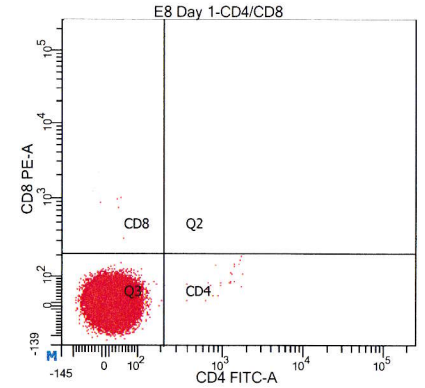
Population	#Events	%Parent
LYMPHOCYTES	37	0.0
CD45	18	48.6
CD4/CD45	19	51.4
TOTAL CD45	1,578	1.0

Printed by: Administrator

BD FACSDiva 8.0.1



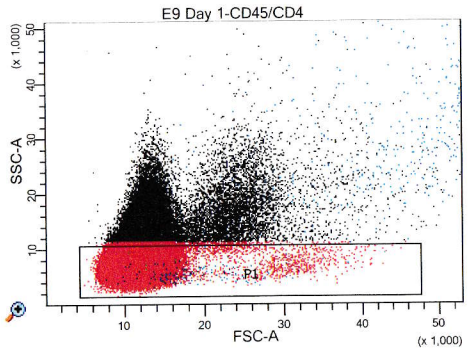


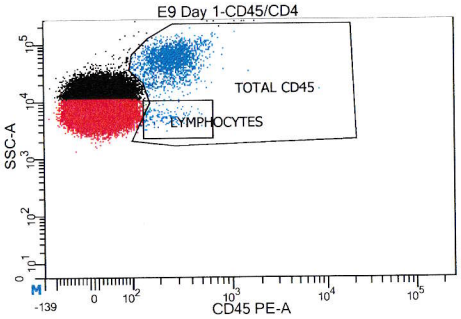


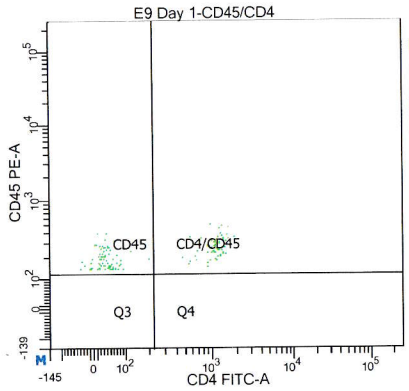
Experiment Name:	Chicken new batch
Specimen Name:	E8 Day 1
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:49:...
$OP:	Administrator

Population	#Events
CD8	5
CD4	5

BD FACSDiva 8.0.1



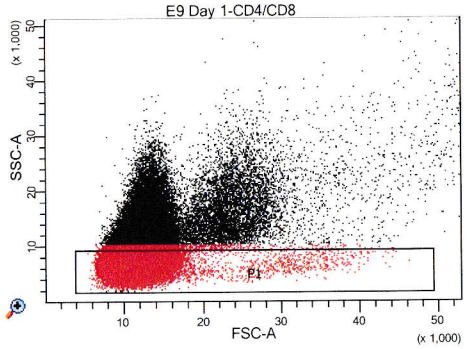


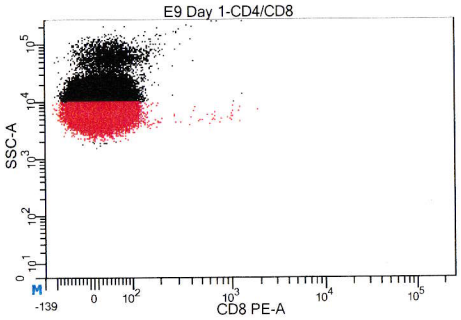


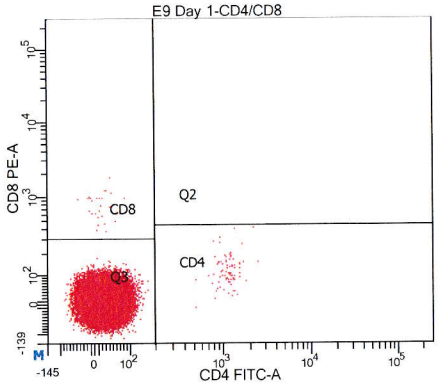
Experiment Name:	Chicken new batch
Specimen Name:	E9 Day 1
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:50:18 ...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	151	0.1
CD45	84	55.6
CD4/CD45	67	44.4
TOTAL CD45	2,339	1.2

BD FACSDiva 8.0.1



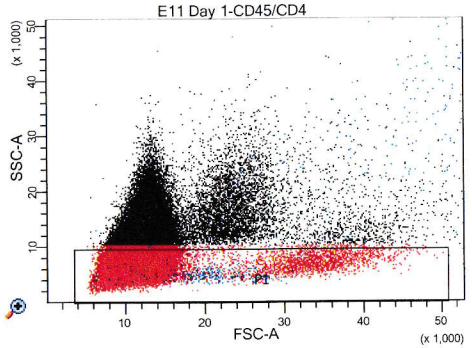


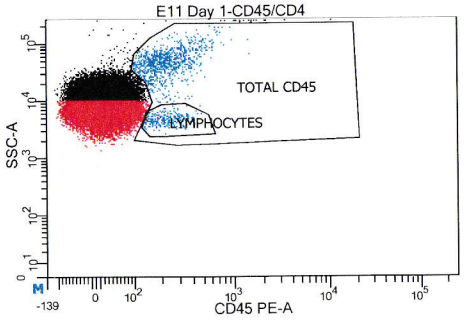


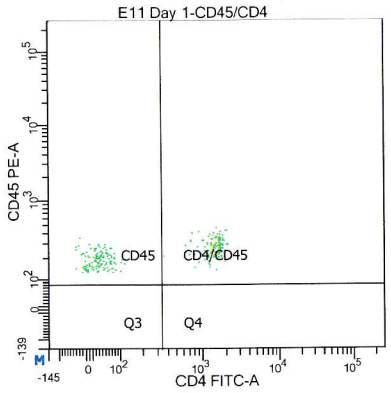
Experiment Name:	Chicken new batch
Specimen Name:	E9 Day 1
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:51:...
$OP:	Administrator

Population	#Events
CD8	28
CD4	80

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 1
Tube Name:	CD45/CD4
Record Date:	Aug 2, 2017 11:52:48 ...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	281	0.1
CD45	147	52.3
CD4/CD45	134	47.7
TOTAL CD45	1,200	0.5

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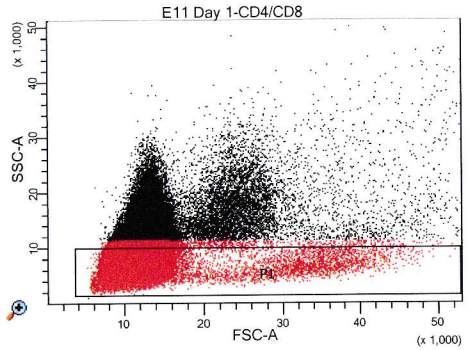
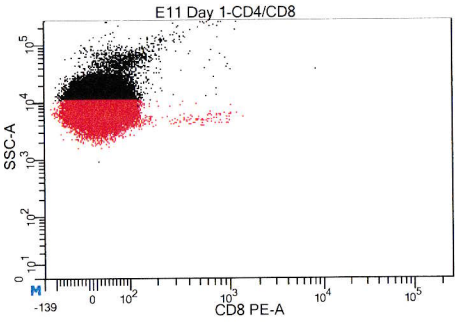
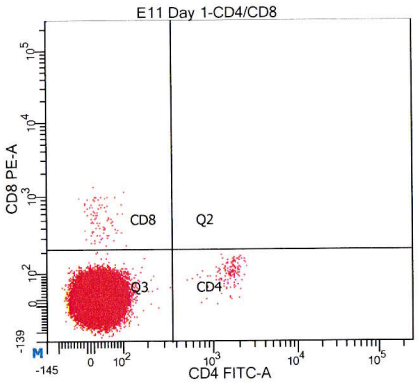
BD FACSDiva 8.0.1



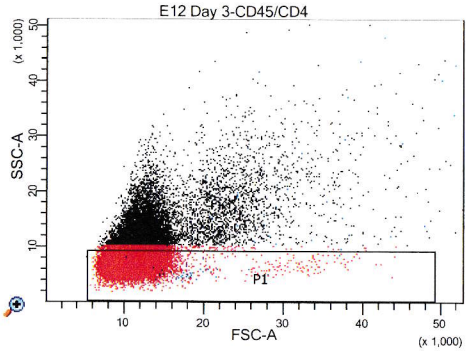


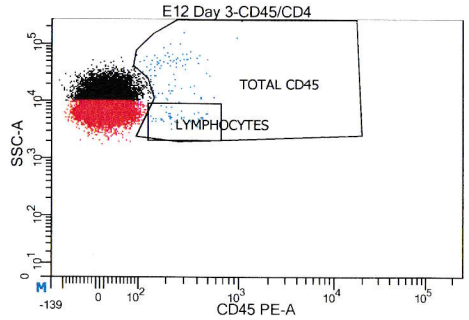


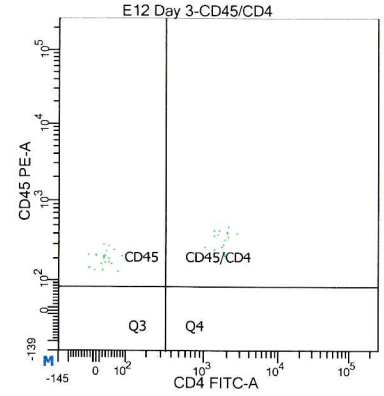
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 1
Tube Name:	CD4/CD8
Record Date:	Aug 2, 2017 11:54:0...
$OP:	Administrator

Population	#Events
CD8	89
CD4	143

BD FACSDiva 8.0.1





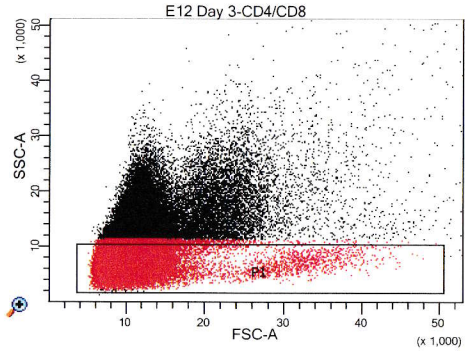


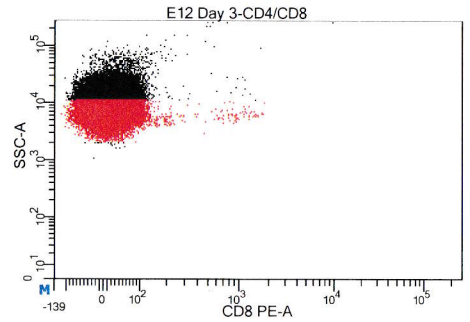
Experiment Name:	Chicken new batch
Specimen Name:	E12 Day 3
Tube Name:	CD45/CD4
Record Date:	Aug 4, 2017 10:48:5...
$OP:	Administrator

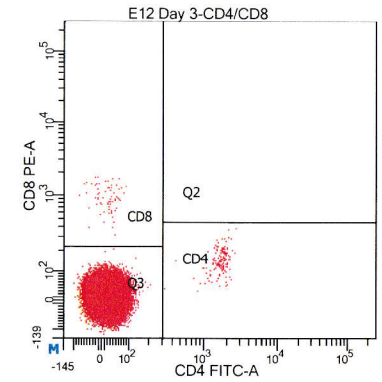
Population	#Events	%Parent
LYMPHOCYTES	42	0.2
CD45	23	54.8
CD45/CD4	19	45.2
TOTAL CD45	131	0.5

Printed by: Administrator

BD FACSDiva 8.0.1



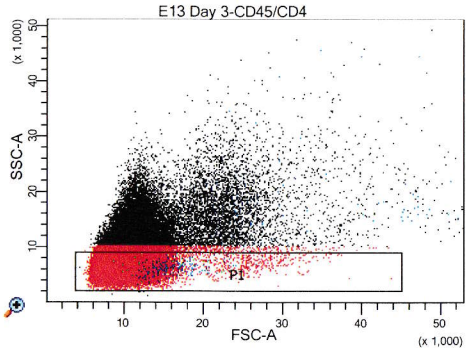


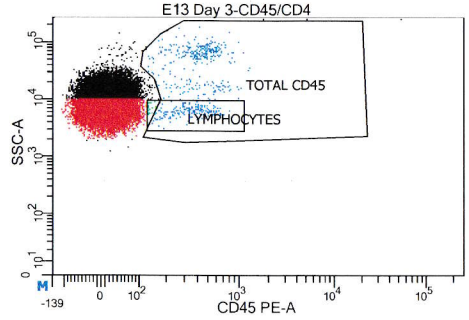


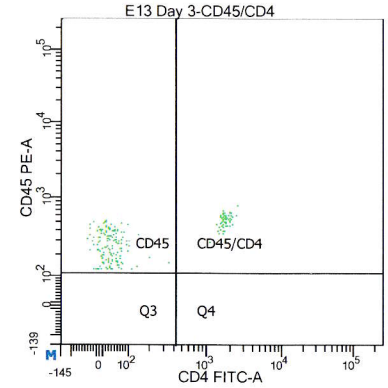
Experiment Name:	Chicken new batch
Specimen Name:	E12 Day 3
Tube Name:	CD4/CD8
Record Date:	Aug 4, 2017 10:49:49...
$OP:	Administrator

Population	#Events
CD8	71
CD4	146

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E13 Day 3
Tube Name:	CD45/CD4
Record Date:	Aug 4, 2017 10:50:37 AM
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	212	0.3
CD45	153	72.2
CD45/CD4	59	27.8
TOTAL CD45	502	0.7

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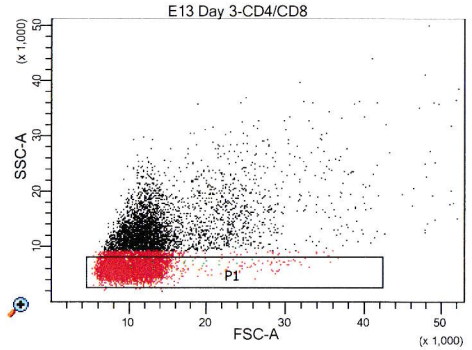
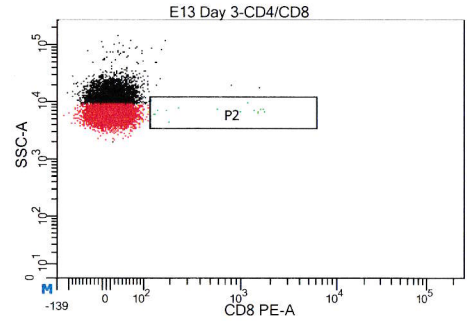
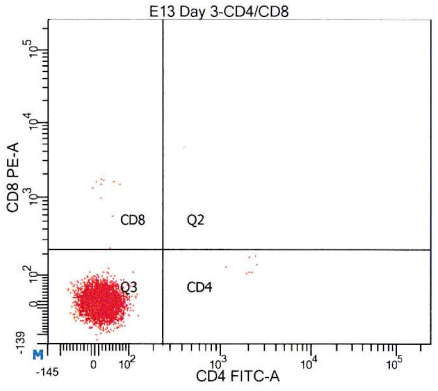
BD FACSDiva 8.0.1





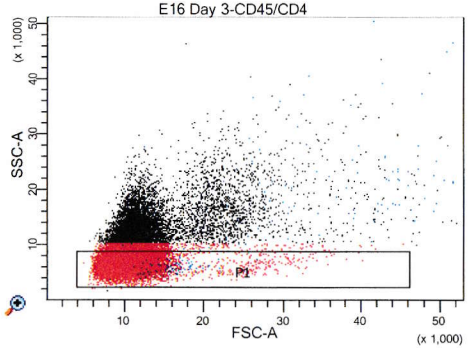


Experiment Name:	Chicken new batch
Specimen Name:	E13 Day 3
Tube Name:	CD4/CD8
Record Date:	Aug 4, 2017 10:51:...
$OP:	Administrator

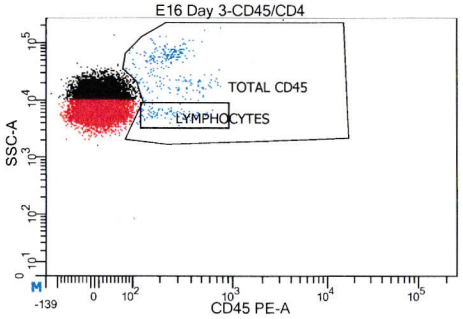
Population	#Events
CD8	10
CD4	7

BD FACSDiva 8.0.1

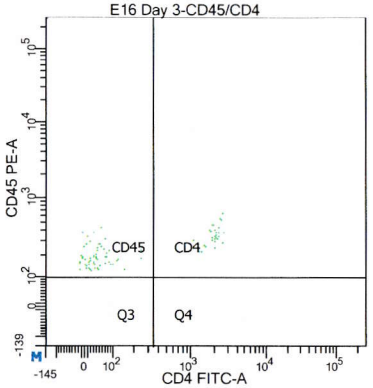
E16 Day 3-CD45/CD4



E16 Day 3-CD45/CD4



E16 Day 3-CD45/CD4



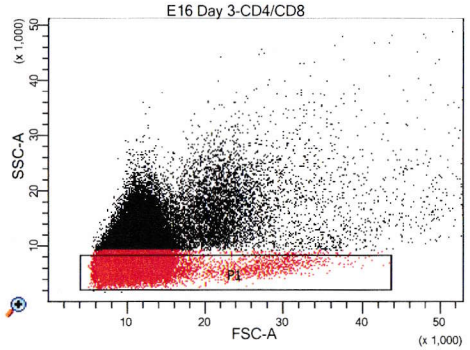
Experiment Name:	Chicken new batch
Specimen Name:	E16 Day 3
Tube Name:	CD45/CD4
Record Date:	Aug 4, 2017 10:52:19 AM
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	90	0.4
CD45	59	65.6
CD4	31	34.4
TOTAL CD45	360	1.5

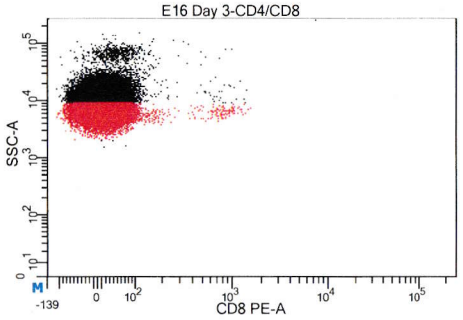
Printed by: Administrator

BD FACSDiva 8.0.1

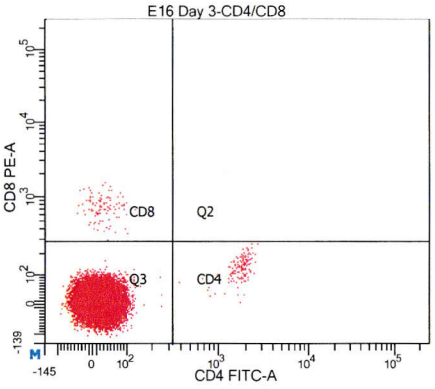
E16 Day 3-CD4/CD8



E16 Day 3-CD4/CD8



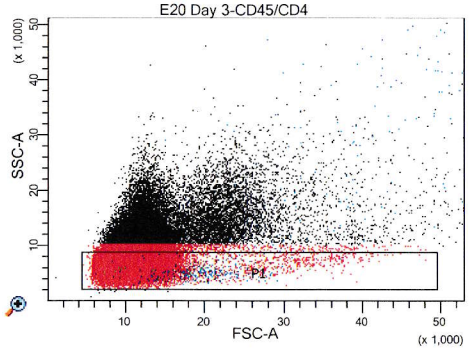
E16 Day 3-CD4/CD8

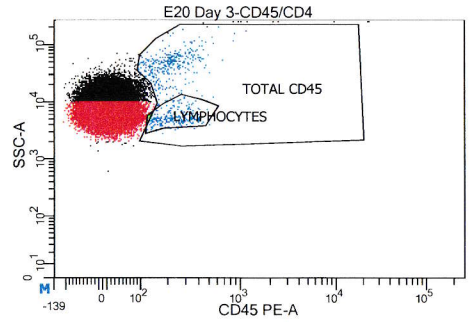


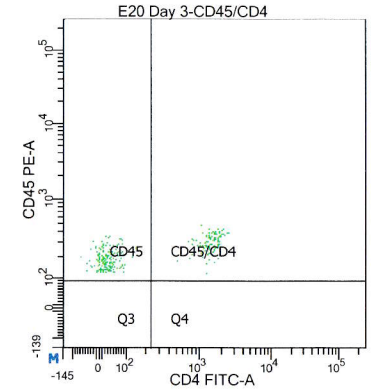
Experiment Name:	Chicken new batch
Specimen Name:	E16 Day 3
Tube Name:	CD4/CD8
Record Date:	Aug 4, 2017 10:53:...
$OP:	Administrator

Population	#Events
CD8	110
CD4	133

BD FACSDiva 8.0.1





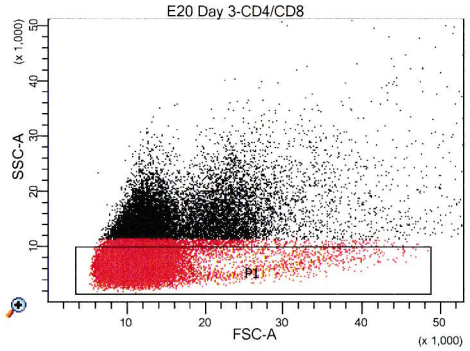


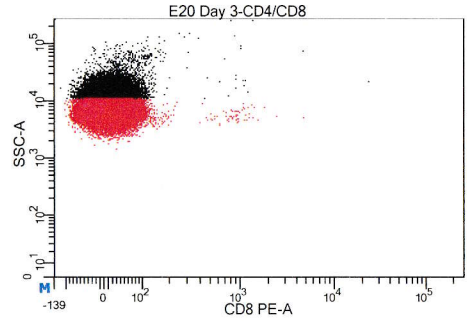
Experiment Name:	Chicken new batch
Specimen Name:	E20 Day 3
Tube Name:	CD45/CD4
Record Date:	Aug 4, 2017 10:53:55 AM
$OP:	Administrator

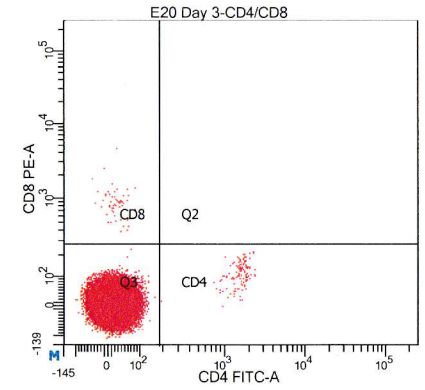
Population	#Events	%Parent
LYMPHOCYTES	272	0.3
CD45	167	61.4
CD45/CD4	105	38.6
TOTAL CD45	625	0.7

Printed by: Administrator

BD FACSDiva 8.0.1



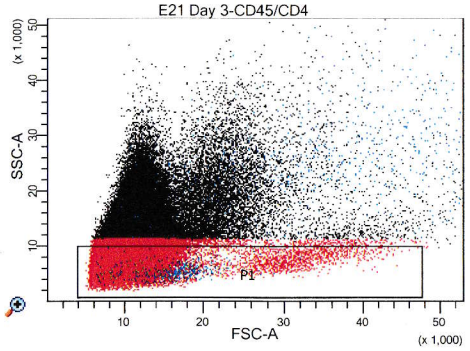


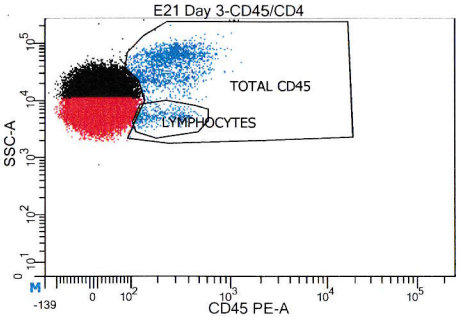


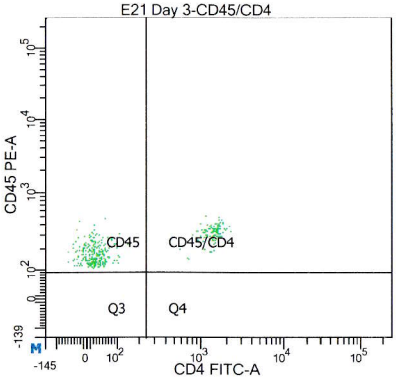
Experiment Name:	Chicken new batch
Specimen Name:	E20 Day 3
Tube Name:	CD4/CD8
Record Date:	Aug 4, 2017 10:54:45...
$OP:	Administrator

Population	#Events
CD8	57
CD4	125

BD FACSDiva 8.0.1



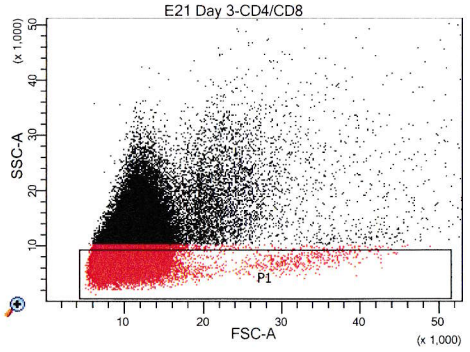


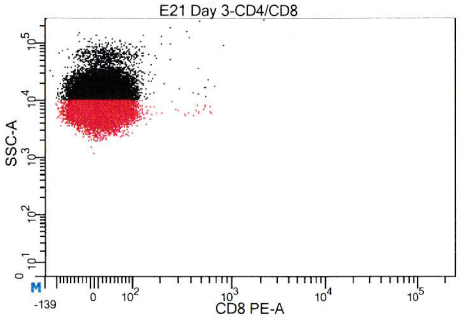


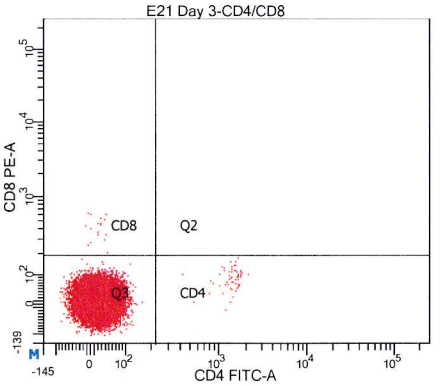
Experiment Name:	Chicken new batch
Specimen Name:	E21 Day 3
Tube Name:	CD45/CD4
Record Date:	Aug 4, 2017 10:55:3...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	387	0.2
CD45	263	68.0
CD45/CD4	124	32.0
TOTAL CD45	2,308	1.3

BD FACSDiva 8.0.1



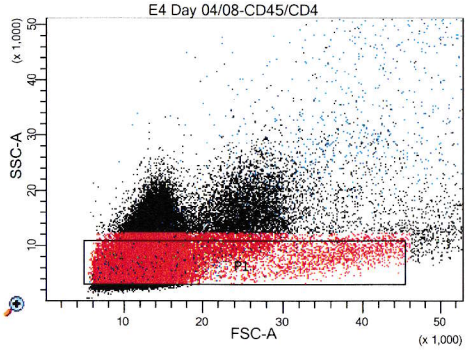


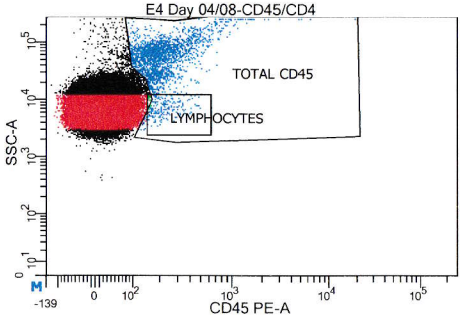


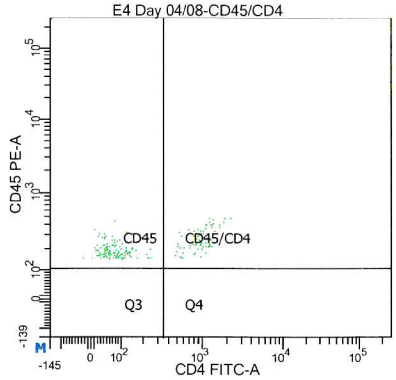
Experiment Name:	Chicken new batch
Specimen Name:	E21 Day 3
Tube Name:	CD4/CD8
Record Date:	Aug 4, 2017 10:56:...
$OP:	Administrator

Population	#Events
CD8	18
CD4	61

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E4 Day 04/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 9:28:16 AM
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	200	0.1
CD45	121	60.5
CD45/CD4	79	39.5
TOTAL CD45	2,526	0.9

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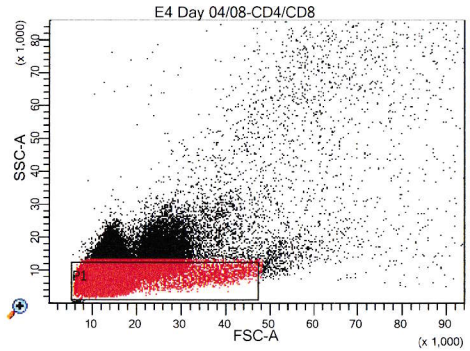
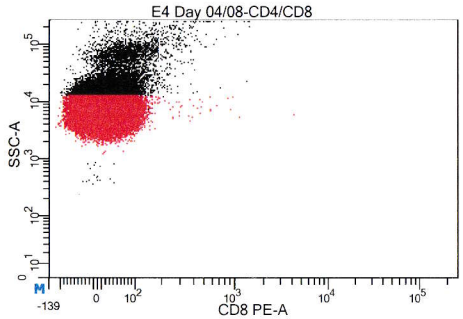
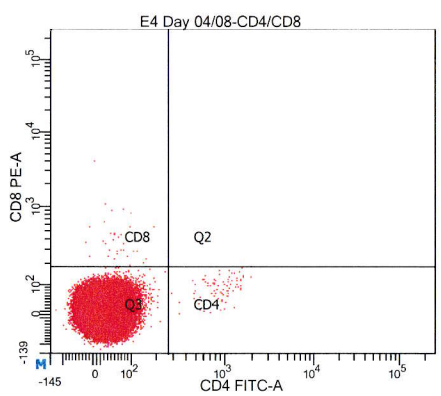
BD FACSDiva 8.0.1



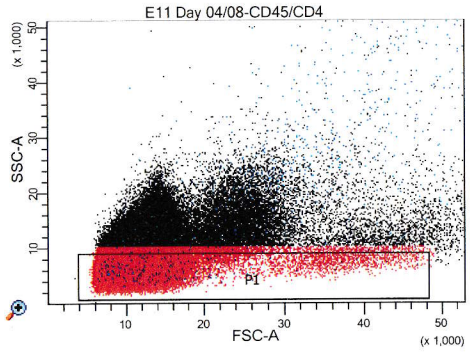


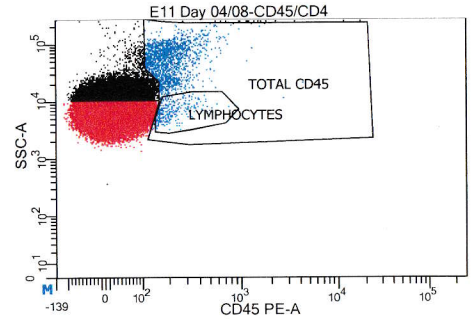


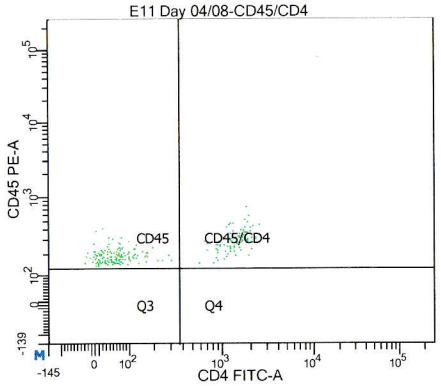
Experiment Name:	Chicken new batch
Specimen Name:	E4 Day 04/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 9:29:19...
$OP:	Administrator

Population	#Events
CD8	36
CD4	79

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 04/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 9:31:2...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	241	0.1
CD45	149	61.8
CD45/CD4	92	38.2
TOTAL CD45	1,986	0.4

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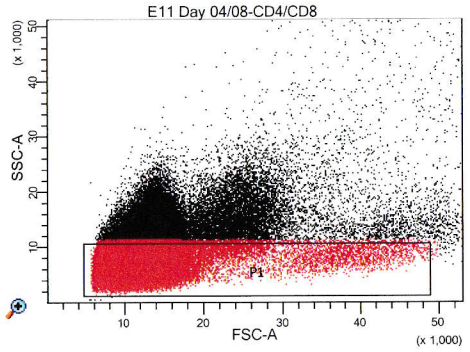
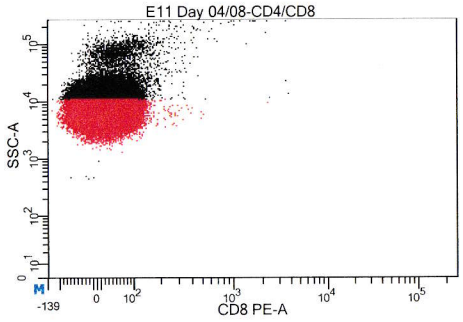
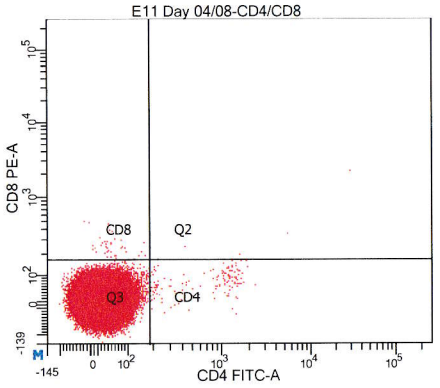
BD FACSDiva 8.0.1



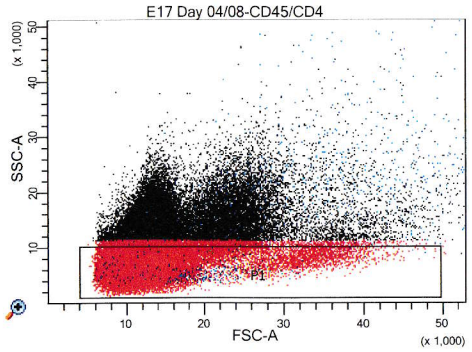


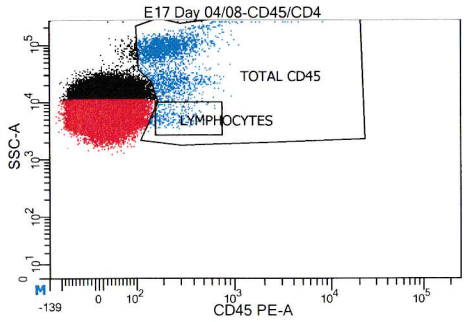


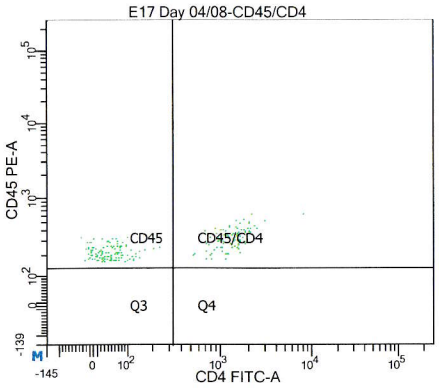
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 04/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 9:33:2...
$OP:	Administrator

Population	#Events
CD8	43
CD4	135

BD FACSDiva 8.0.1





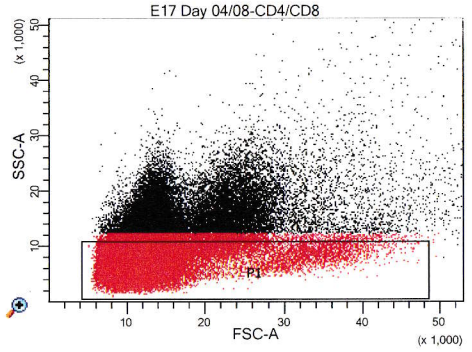


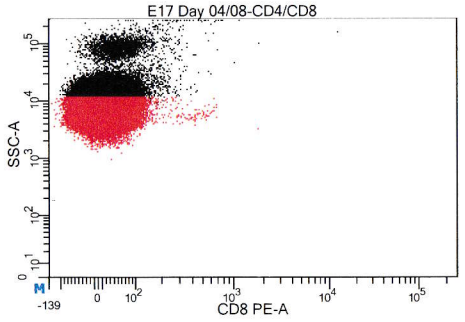
Experiment Name:	Chicken new batch
Specimen Name:	E17 Day 04/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 9:34:5...
$OP:	Administrator

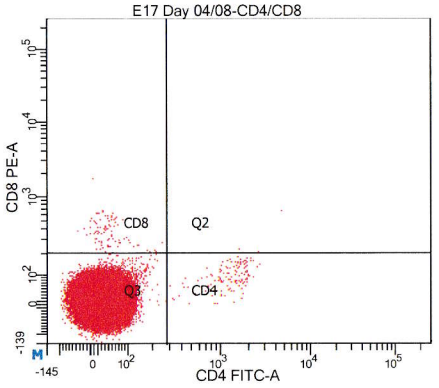
Population	#Events	%Parent
LYMPHOCYTES	221	0.1
CD45	125	56.6
CD45/CD4	96	43.4
TOTAL CD45	2,887	0.7

Printed by: Administrator

BD FACSDiva 8.0.1



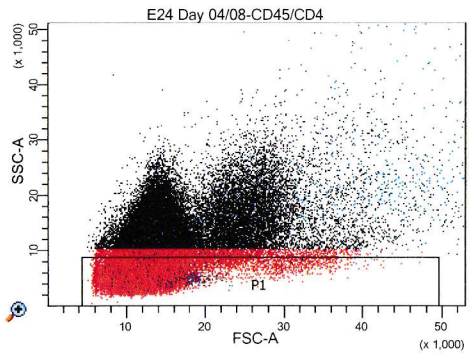


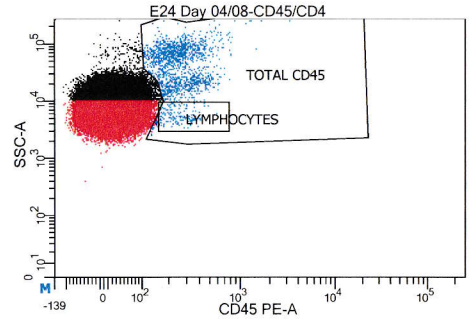


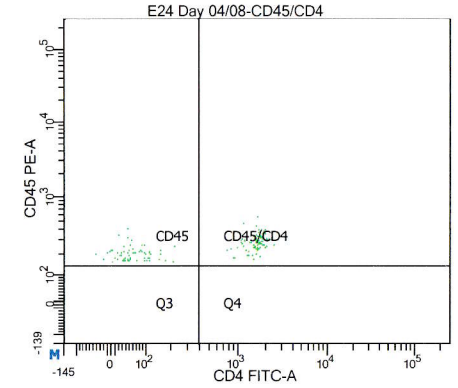
Experiment Name:	Chicken new batch
Specimen Name:	E17 Day 04/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 9:36:1...
$OP:	Administrator

Population	#Events
CD8	93
CD4	134

BD FACSDiva 8.0.1





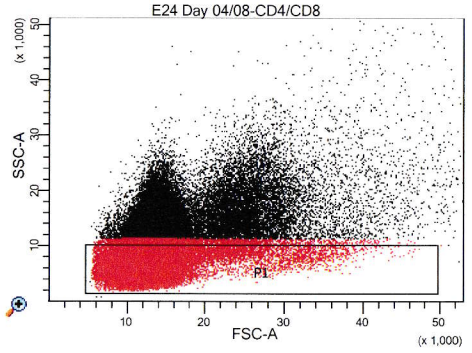


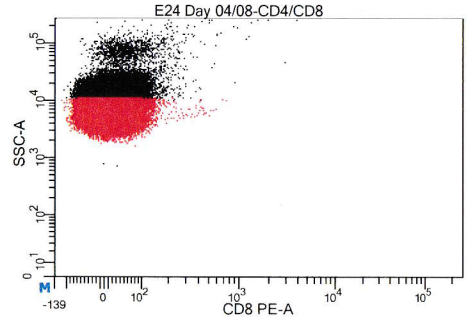
Experiment Name:	Chicken new batch
Specimen Name:	E24 Day 04/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 9:37:2...
$OP:	Administrator

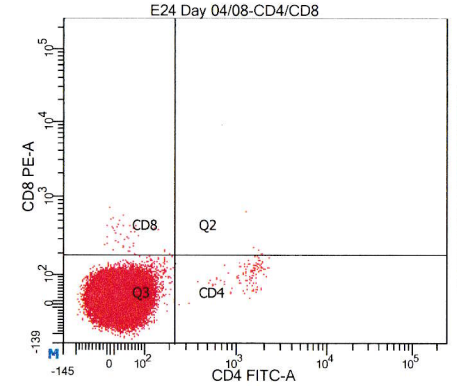
Population	#Events	%Parent
LYMPHOCYTES	147	0.1
CD45	62	42.2
CD45/CD4	85	57.8
TOTAL CD45	1,637	0.6

Printed by: Administrator

BD FACSDiva 8.0.1



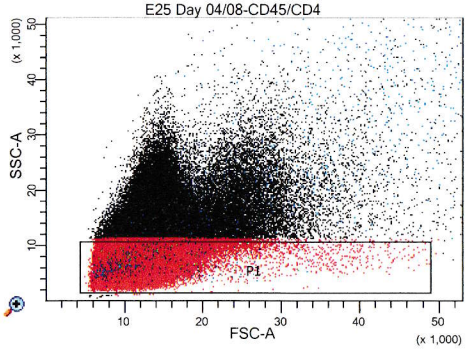


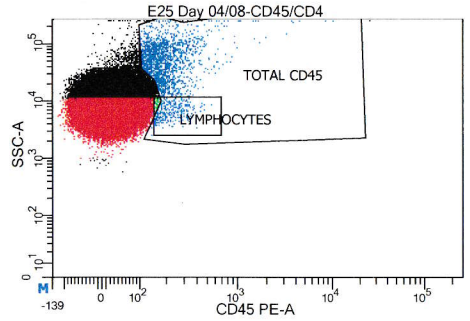


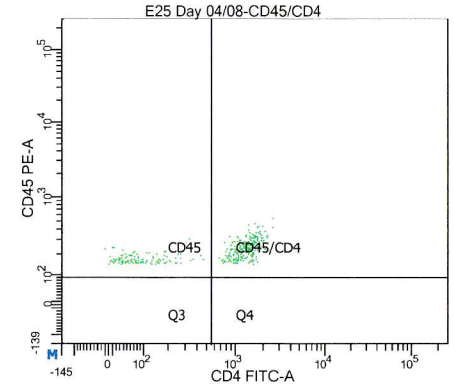
Experiment Name:	Chicken new batch
Specimen Name:	E24 Day 04/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 9:38:30...
$OP:	Administrator

Population	#Events
CD8	43
CD4	117

BD FACSDiva 8.0.1





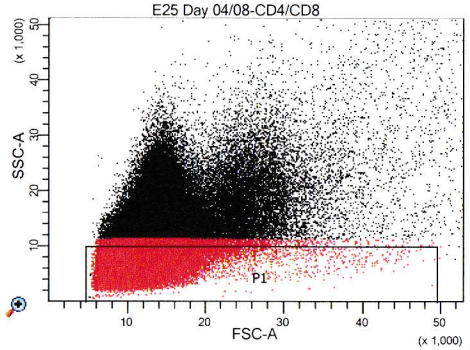


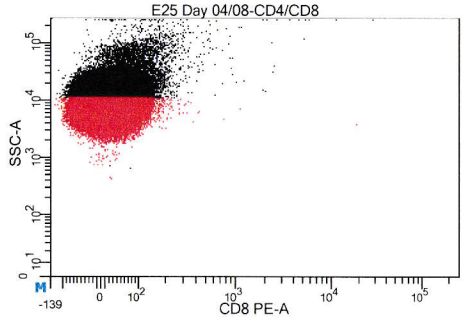
Experiment Name:	Chicken new batch
Specimen Name:	E25 Day 04/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 9:40:5...
$OP:	Administrator

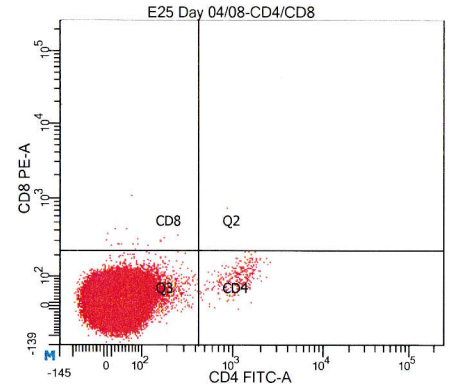
Population	#Events	%Parent
LYMPHOCYTES	346	0.1
CD45	117	33.8
CD45/CD4	229	66.2
TOTAL CD45	2,223	0.4

Printed by: Administrator

BD FACSDiva 8.0.1



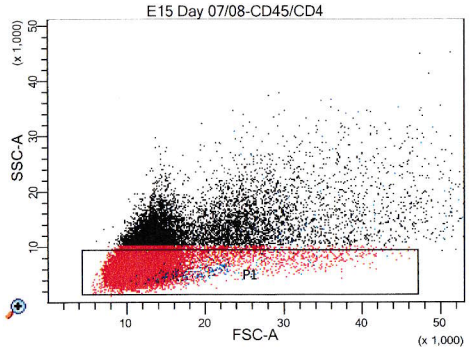


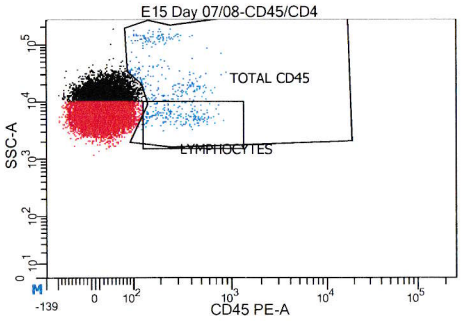


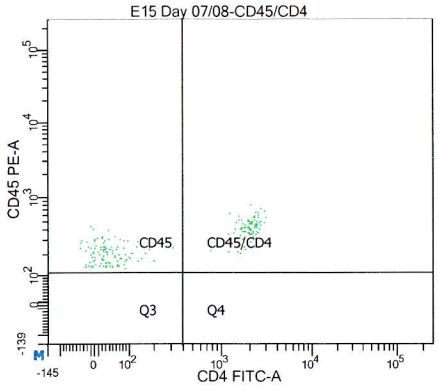
Experiment Name:	Chicken new batch
Specimen Name:	E25 Day 04/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 9:43:3...
$OP:	Administrator

Population	#Events
CD8	22
CD4	290

BD FACSDiva 8.0.1





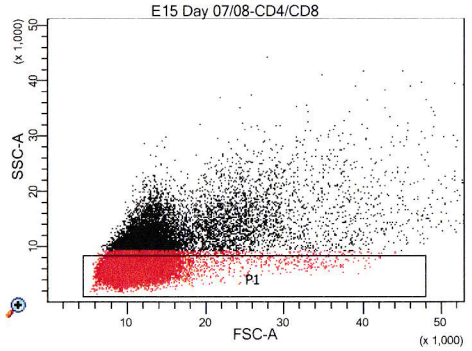


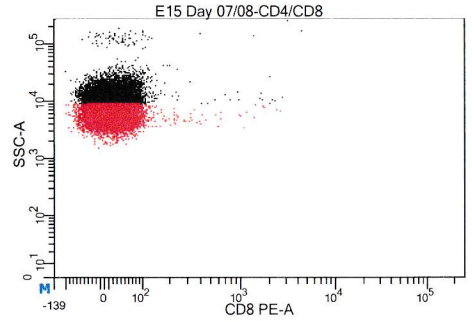
Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 07/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 4:02:5...
$OP:	Administrator

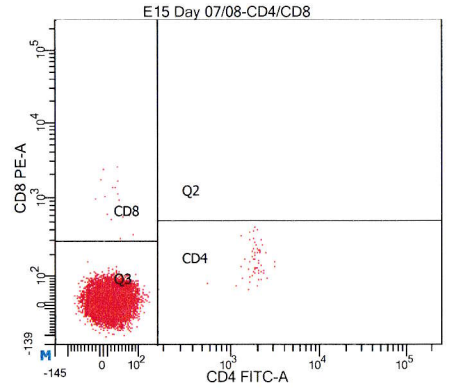
Population	#Events	%Parent
LYMPHOCYTES	199	0.5
CD45	105	52.8
CD45/CD4	94	47.2
TOTAL CD45	470	1.3

Printed by: Administrator

BD FACSDiva 8.0.1



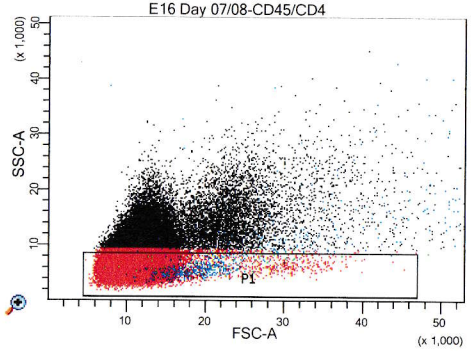


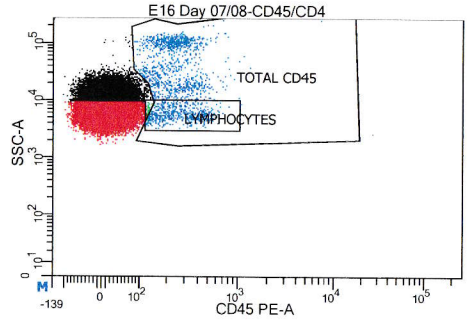


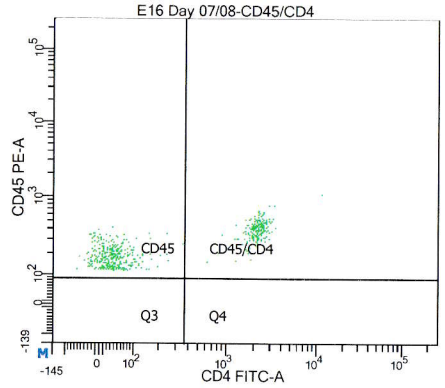
Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 07/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 4:03:42...
$OP:	Administrator

Population	#Events
CD8	16
CD4	58

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E16 Day 07/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 4:04:30...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	572	0.7
CD45	361	63.1
CD45/CD4	211	36.9
TOTAL CD45	1,645	2.0

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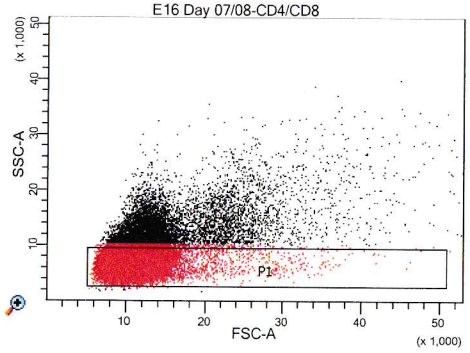
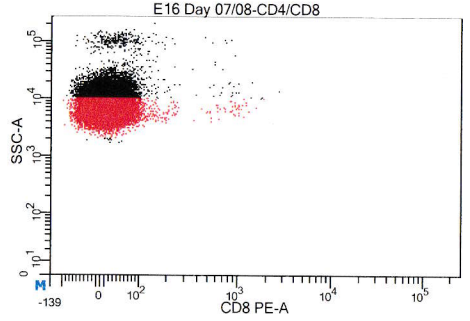
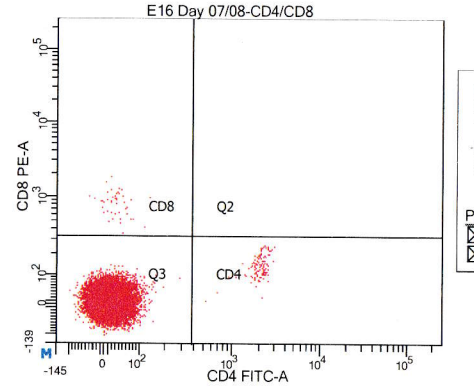
BD FACSDiva 8.0.1



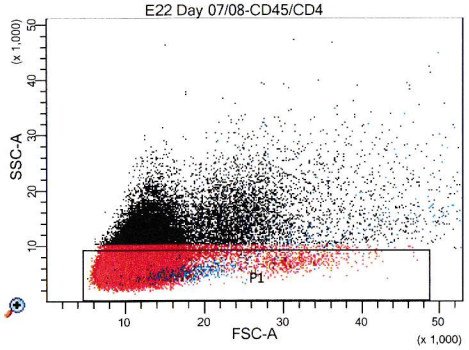


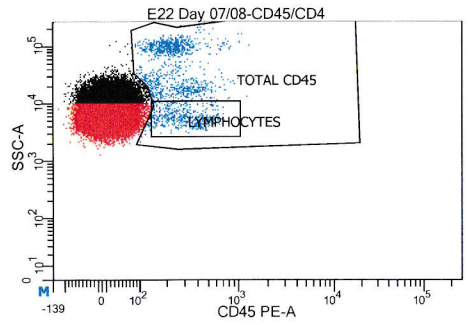


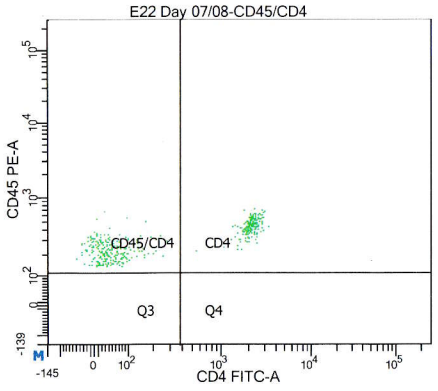
Experiment Name:	Chicken new batch
Specimen Name:	E16 Day 07/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 4:05:1...
$OP:	Administrator

Population	#Events
CD8	45
CD4	116

BD FACSDiva 8.0.1





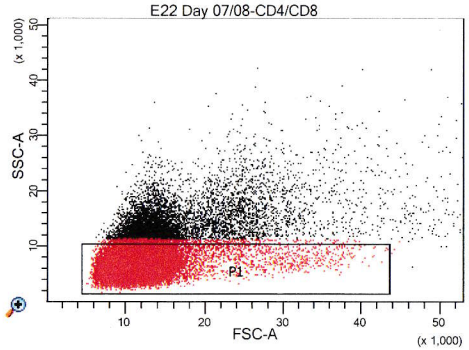


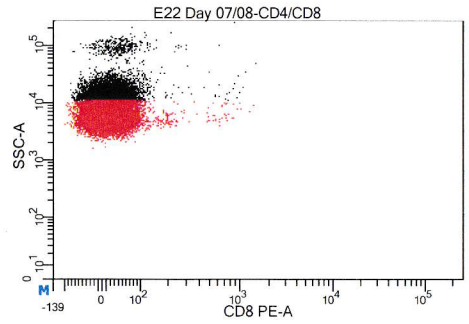
Experiment Name:	Chicken new batch
Specimen Name:	E22 Day 07/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 4:06:10...
$OP:	Administrator

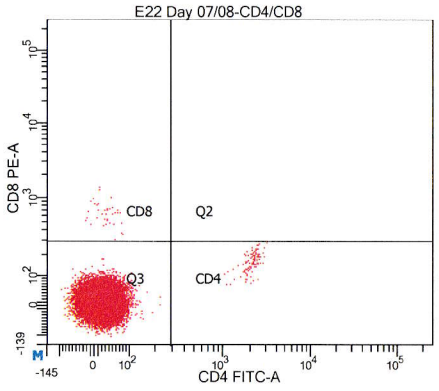
Population	#Events	%Parent
LYMPHOCYTES	445	0.6
CD45/CD4	251	56.4
CD4	194	43.6
TOTAL CD45	1,541	2.1

Printed by: Administrator

BD FACSDiva 8.0.1





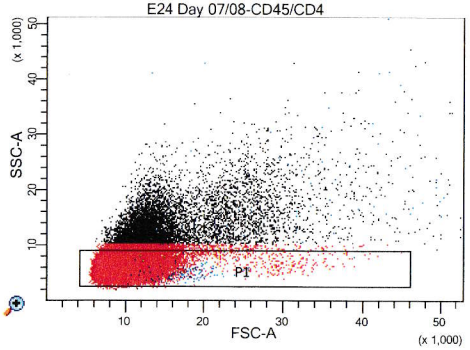


Experiment Name:	Chicken new batch
Specimen Name:	E22 Day 07/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 4:06:56...
$OP:	Administrator

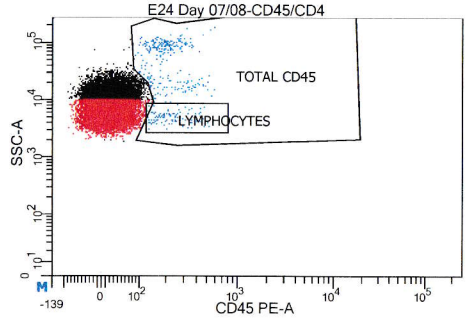
Population	#Events
CD8	38
CD4	102

BD FACSDiva 8.0.1

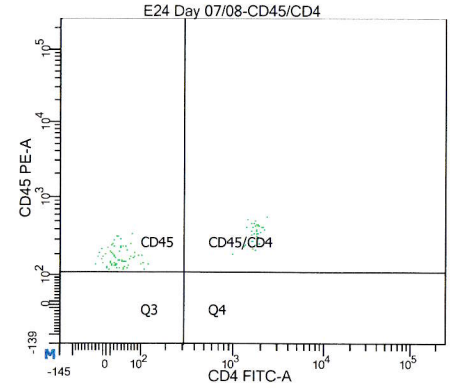
E24 Day 07/08-CD45/CD4



E24 Day 07/08-CD45/CD4



E24 Day 07/08-CD45/CD4



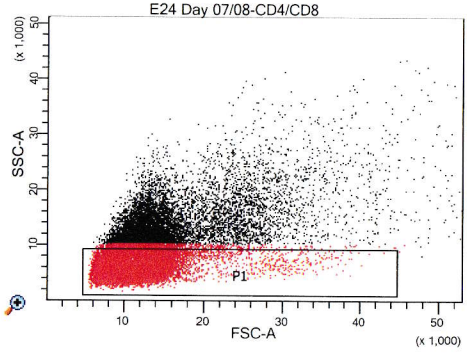
Experiment Name:	Chicken new batch
Specimen Name:	E24 Day 07/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 4:07:46...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	110	0.4
CD45	73	66.4
CD45/CD4	37	33.6
TOTAL CD45	360	1.2

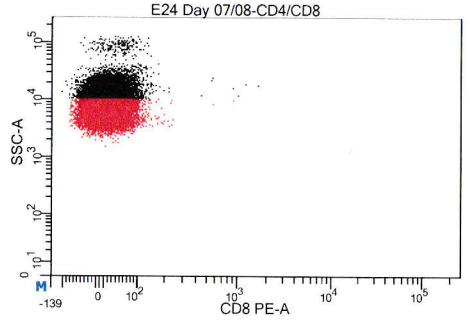
Printed by: Administrator

BD FACSDiva 8.0.1

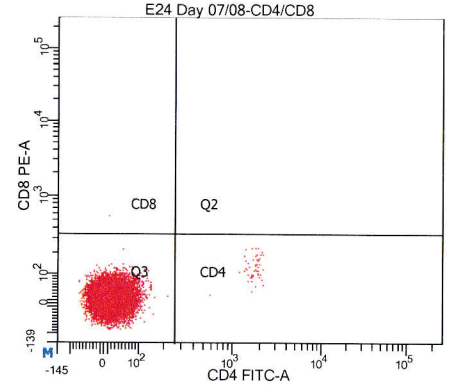
E24 Day 07/08-CD4/CD8



E24 Day 07/08-CD4/CD8



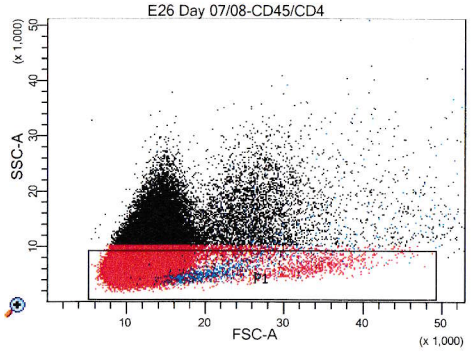
E24 Day 07/08-CD4/CD8

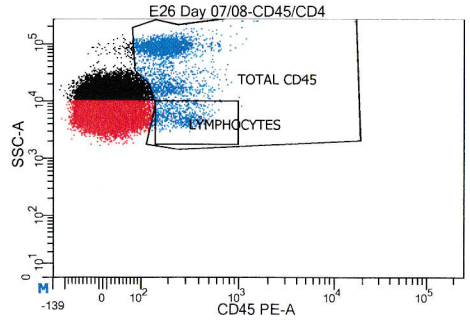


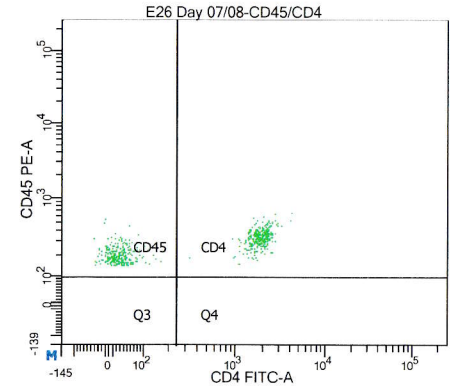
Experiment Name:	Chicken new batch
Specimen Name:	E24 Day 07/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 4:08:3...
$OP:	Administrator

Population	#Events
CD8	2
CD4	52

BD FACSDiva 8.0.1





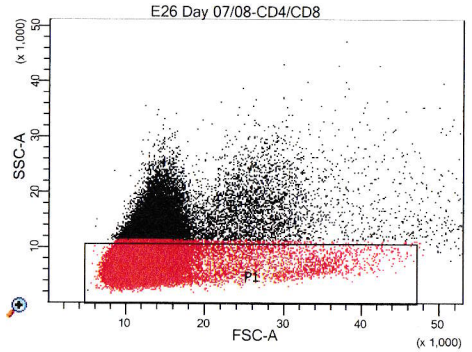


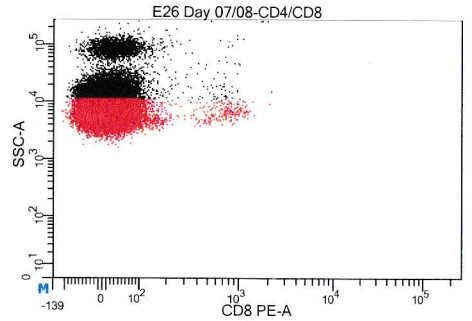
Experiment Name:	Chicken new batch
Specimen Name:	E26 Day 07/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 4:09:4...
$OP:	Administrator

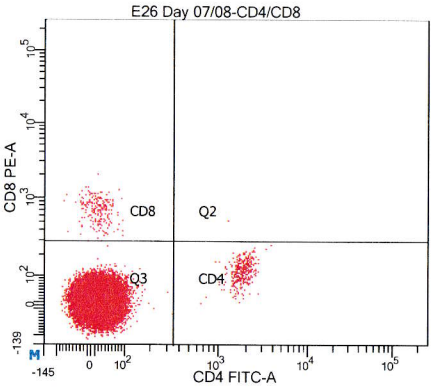
Population	#Events	%Parent
LYMPHOCYTES	633	0.5
CD45	277	43.8
CD4	356	56.2
TOTAL CD45	3,781	2.8

Printed by: Administrator

BD FACSDiva 8.0.1



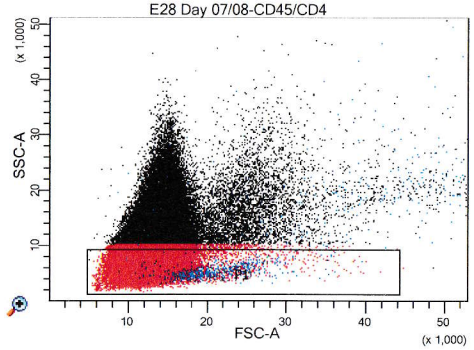


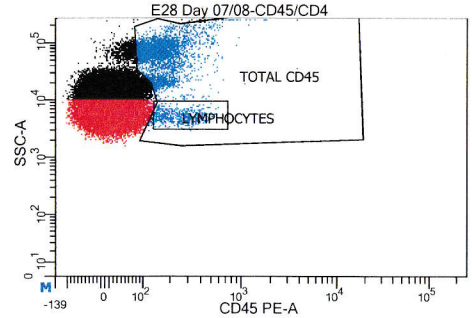


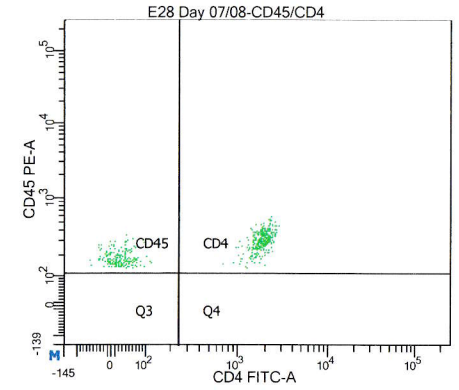
Experiment Name:	Chicken new batch
Specimen Name:	E26 Day 07/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 4:10:4...
$OP:	Administrator

Population	#Events
CD8	219
CD4	366

BD FACSDiva 8.0.1





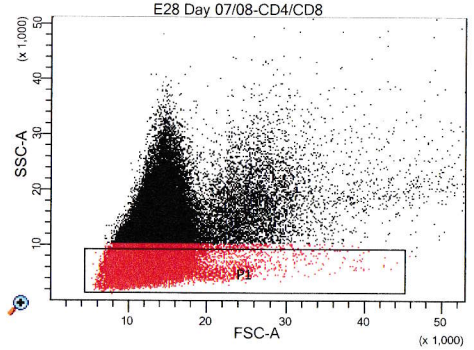


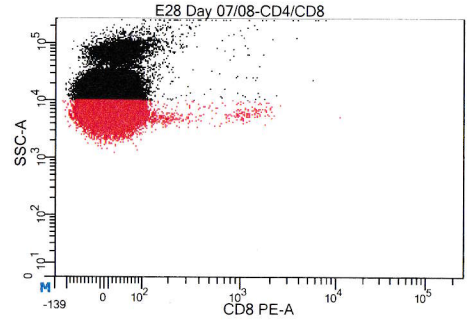
Experiment Name:	Chicken new batch
Specimen Name:	E28 Day 07/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 4:12:3...
$OP:	Administrator

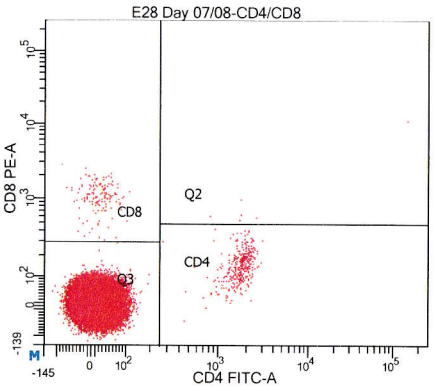
Population	#Events	%Parent
LYMPHOCYTES	524	0.2
CD45	212	40.5
CD4	312	59.5
TOTAL CD45	4,481	1.7

Printed by: Administrator

BD FACSDiva 8.0.1



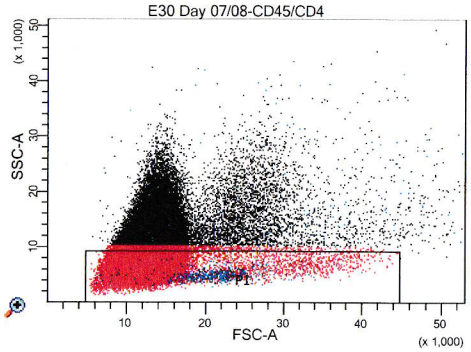


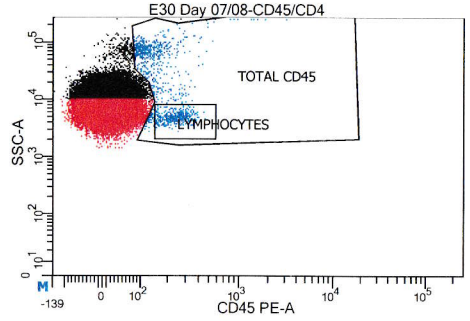


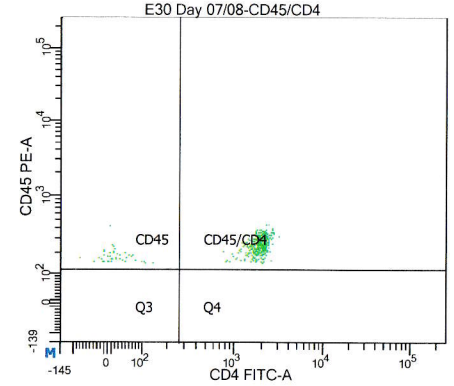
Experiment Name:	Chicken new batch
Specimen Name:	E28 Day 07/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 4:14:0...
$OP:	Administrator

Population	#Events
CD8	149
CD4	359

BD FACSDiva 8.0.1





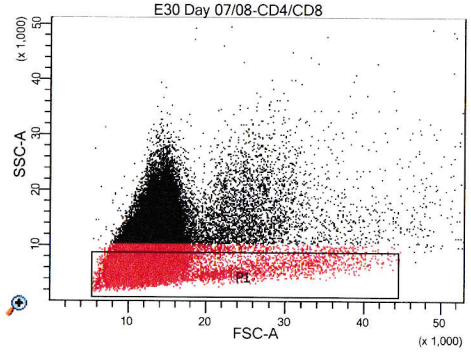


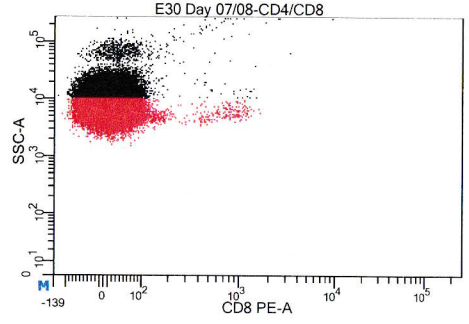
Experiment Name:	Chicken new batch
Specimen Name:	E30 Day 07/08
Tube Name:	CD45/CD4
Record Date:	Aug 7, 2017 4:15:2...
$OP:	Administrator

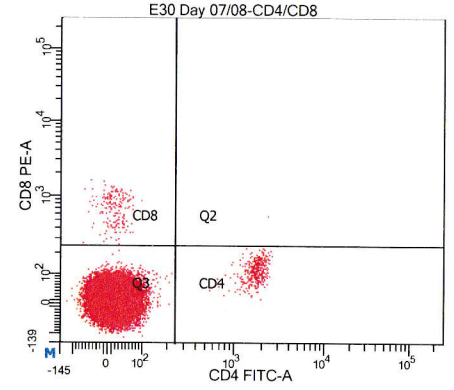
Population	#Events	%Parent
LYMPHOCYTES	447	0.3
CD45	43	9.6
CD45/CD4	404	90.4
TOTAL CD45	1,062	0.6

Printed by: Administrator

BD FACSDiva 8.0.1



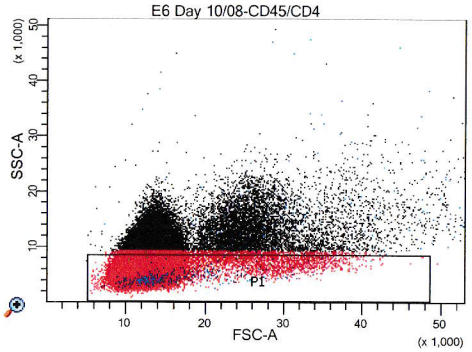


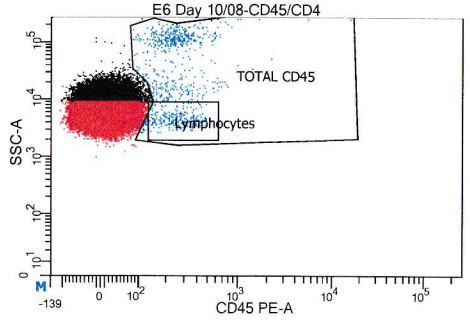


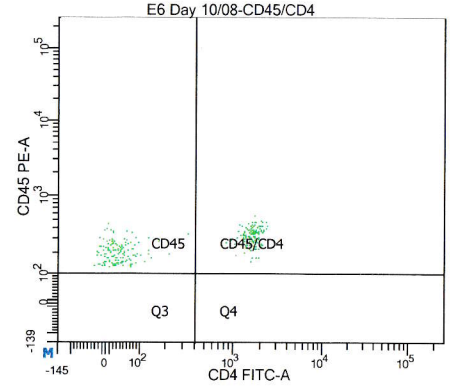
Experiment Name:	Chicken new batch
Specimen Name:	E30 Day 07/08
Tube Name:	CD4/CD8
Record Date:	Aug 7, 2017 4:16:37...
$OP:	Administrator

Population	#Events
CD8	186
CD4	405

BD FACSDiva 8.0.1





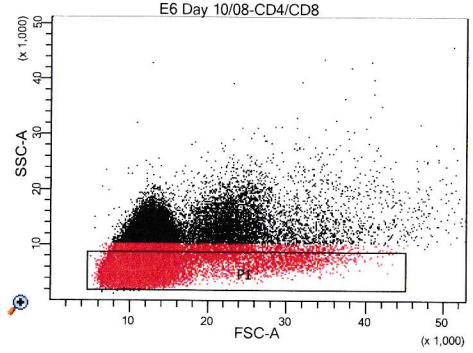


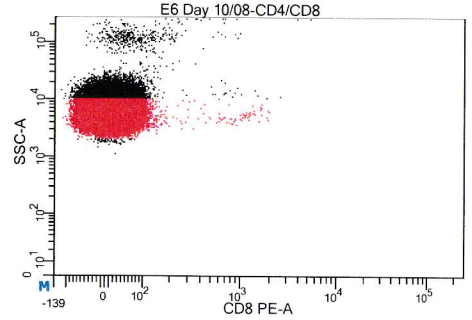
Experiment Name:	Chicken new batch
Specimen Name:	E6 Day 10/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:26:...
$OP:	Administrator

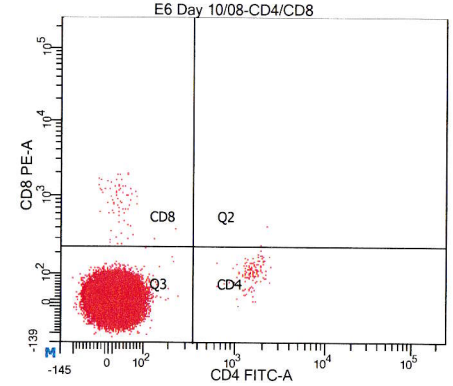
Population	#Events	%Parent
Lymphocytes	282	0.1
CD45	150	53.2
CD45/CD4	132	46.8
TOTAL CD45	874	0.4

Printed by: Administrator

BD FACSDiva 8.0.1



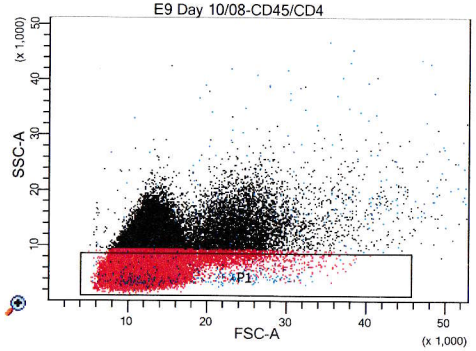


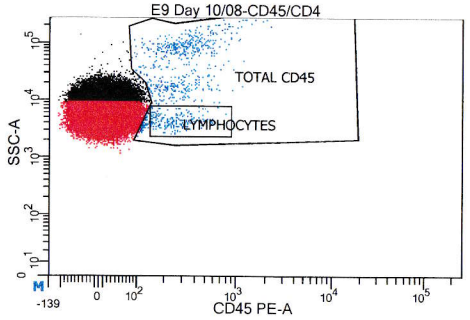


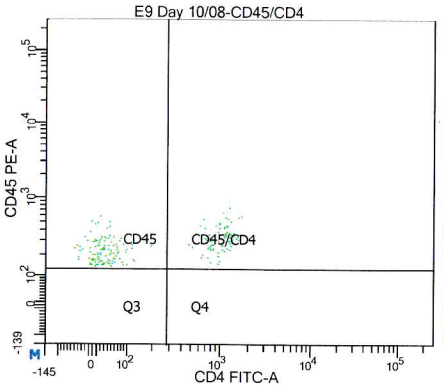
Experiment Name:	Chicken new batch
Specimen Name:	E6 Day 10/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:27:...
$OP:	Administrator

Population	#Events
CD8	71
CD4	133

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E9 Day 10/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:29:1...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	208	0.1
CD45	123	59.1
CD45/CD4	85	40.9
TOTAL CD45	818	0.2

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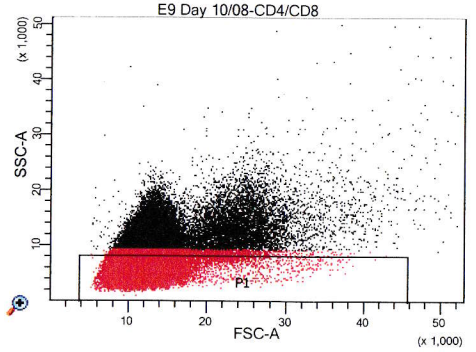
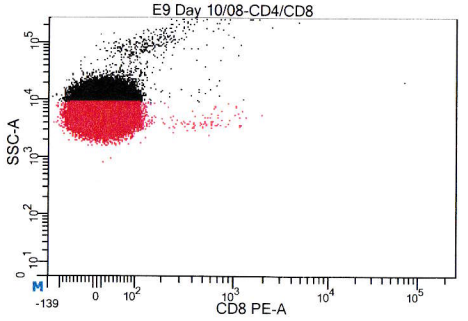
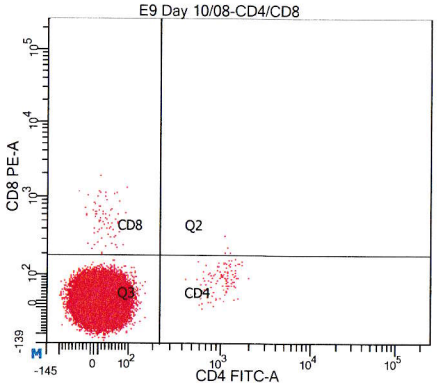
BD FACSDiva 8.0.1





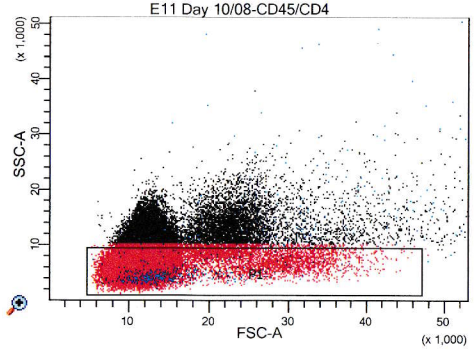


Experiment Name:	Chicken new batch
Specimen Name:	E9 Day 10/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:30:5...
$OP:	Administrator

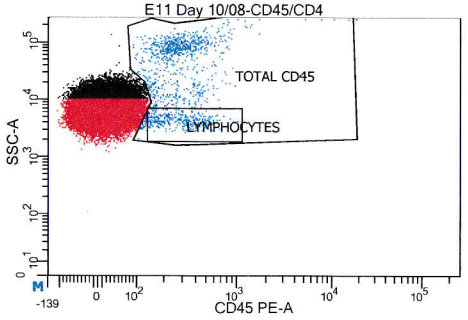
Population	#Events
CD8	79
CD4	109

BD FACSDiva 8.0.1

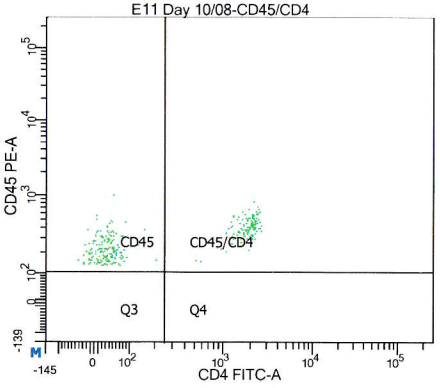
E11 Day 10/08-CD45/CD4



E11 Day 10/08-CD45/CD4



E11 Day 10/08-CD45/CD4



Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 10/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:31:5...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	321	0.2
CD45	186	57.9
CD45/CD4	135	42.1
TOTAL CD45	1,159	0.6

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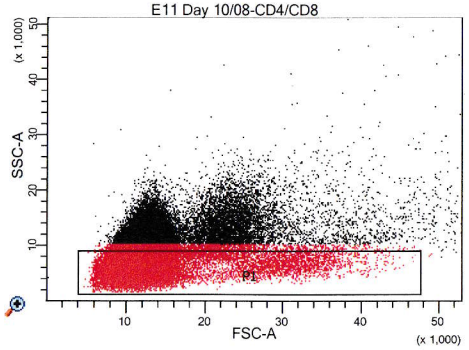
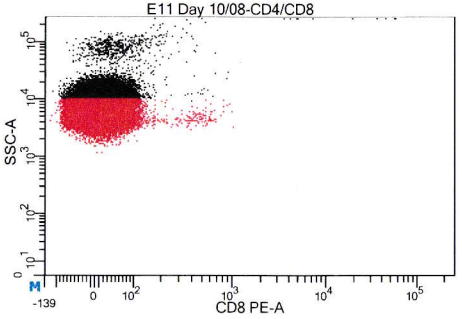
BD FACSDiva 8.0.1

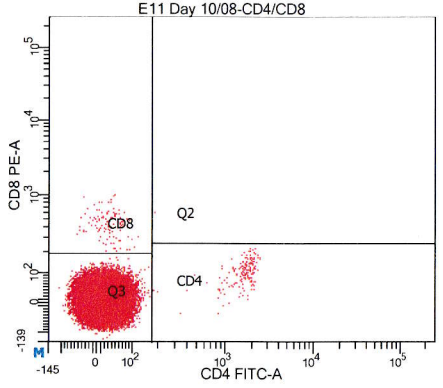
E11 Day 10/08-CD4/CD8



E11 Day 10/08-CD4/CD8



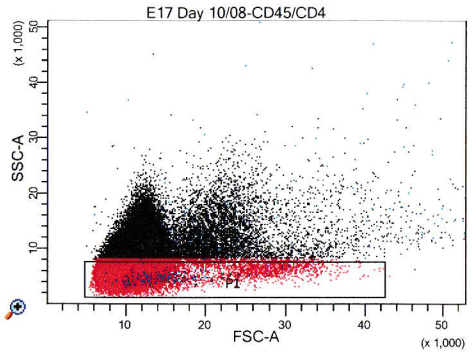
E11 Day 10/08-CD4/CD8

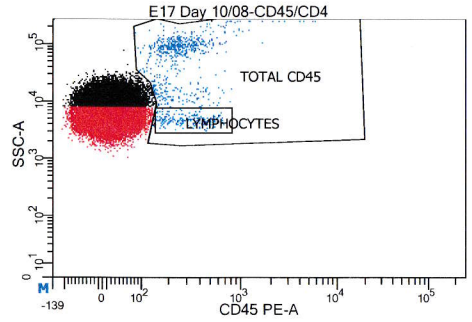


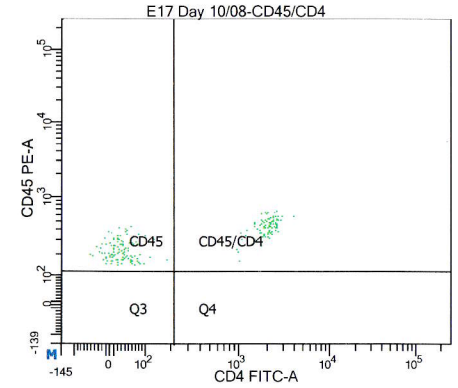
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 10/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:33:1...
$OP:	Administrator

Population	#Events
CD8	117
CD4	174

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E17 Day 10/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:34:1...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	202	0.1
CD45	112	55.4
CD45/CD4	90	44.6
TOTAL CD45	855	0.5

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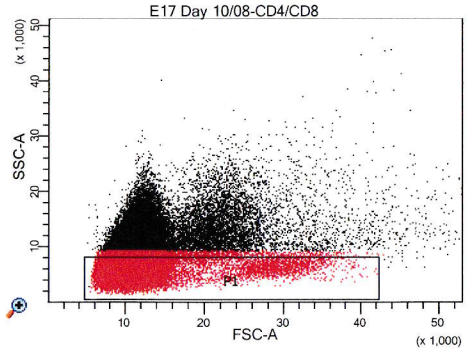
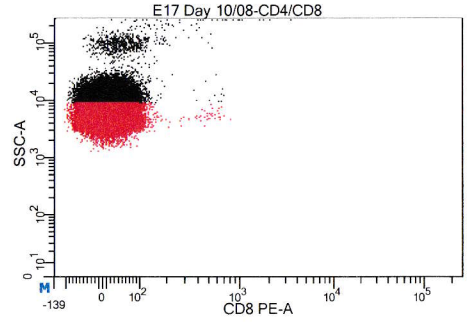
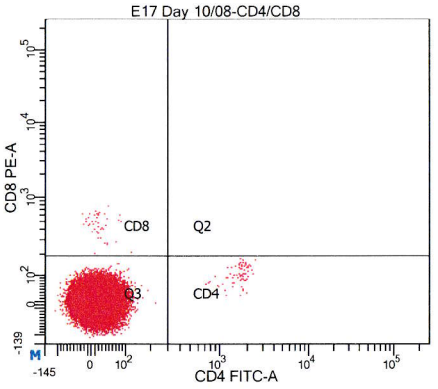
BD FACSDiva 8.0.1



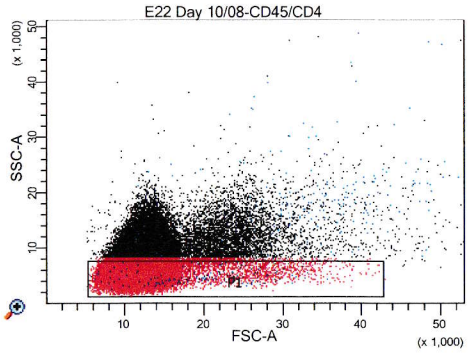


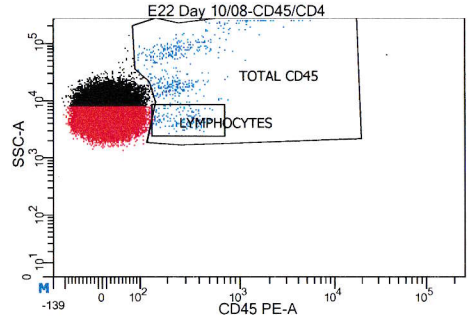


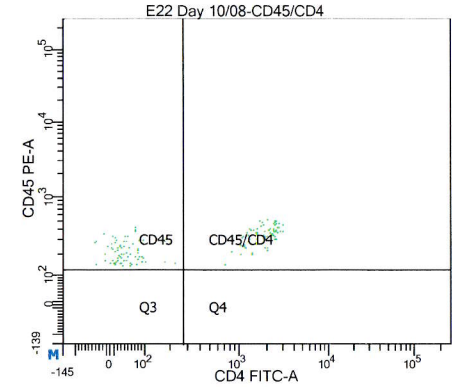
Experiment Name:	Chicken new batch
Specimen Name:	E17 Day 10/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:35:1...
$OP:	Administrator

Population	#Events
CD8	43
CD4	70

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E22 Day 10/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:36:1...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	155	0.1
CD45	75	48.4
CD45/CD4	80	51.6
TOTAL CD45	593	0.3

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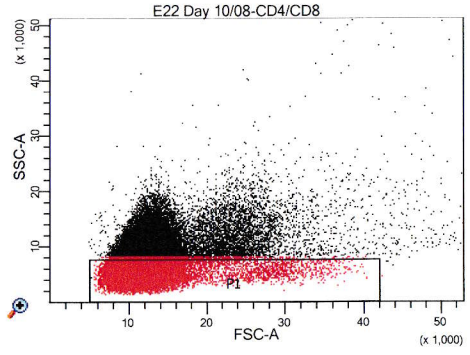
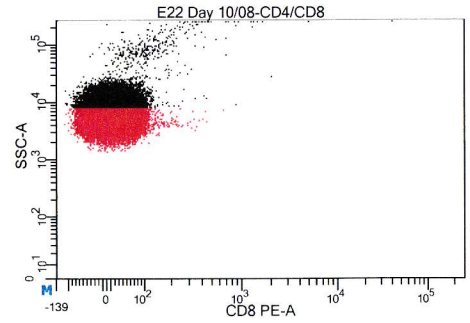
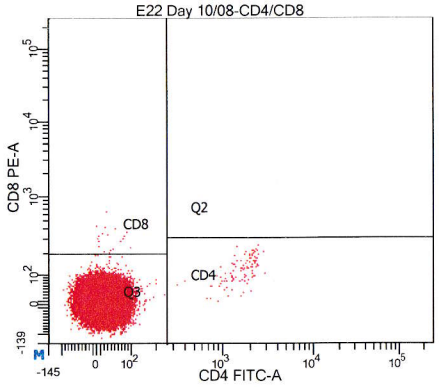
BD FACSDiva 8.0.1



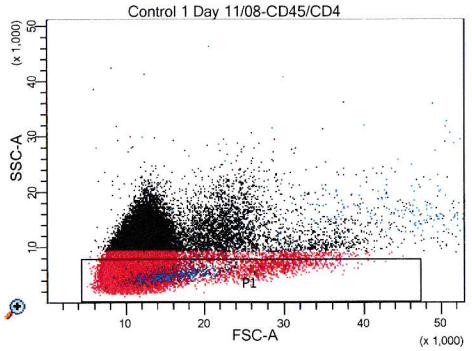


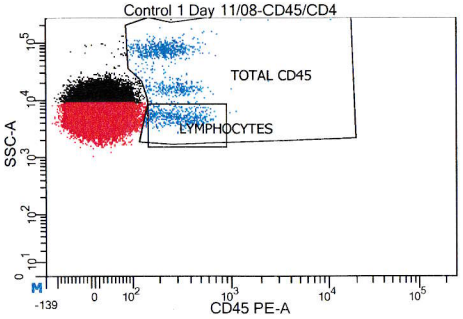


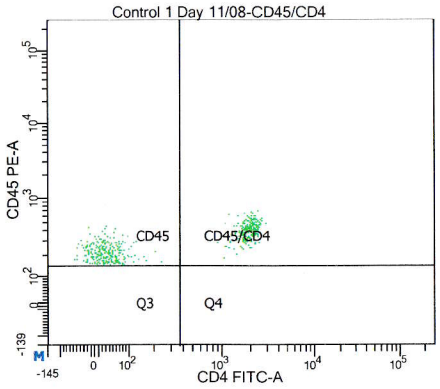
Experiment Name:	Chicken new batch
Specimen Name:	E22 Day 10/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:37:3...
$OP:	Administrator

Population	#Events
CD8	20
CD4	103

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	Control 1 Day 11/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:39:1...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	507	0.2
CD45	269	53.1
CD45/CD4	238	46.9
TOTAL CD45	1,489	0.6

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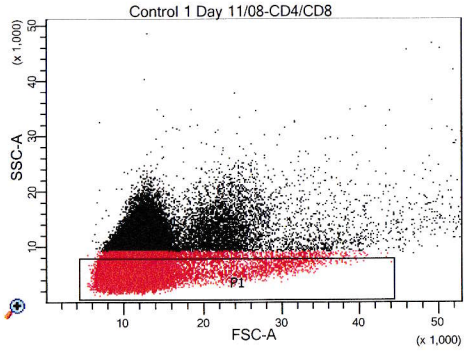
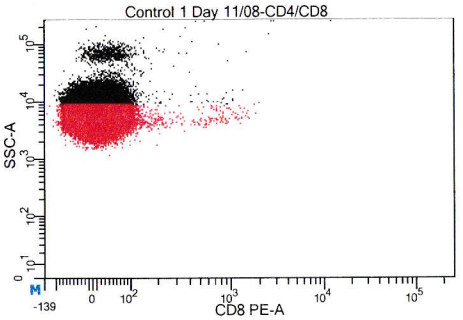
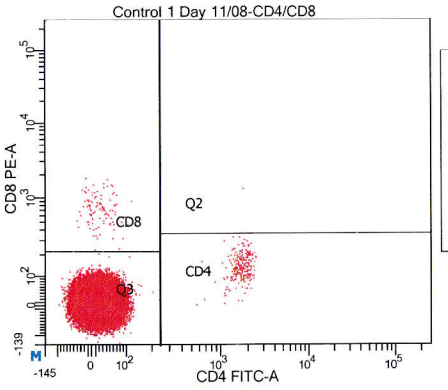
BD FACSDiva 8.0.1



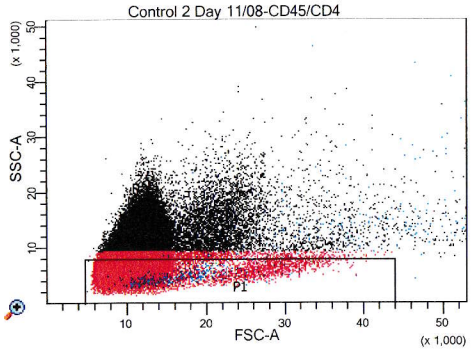


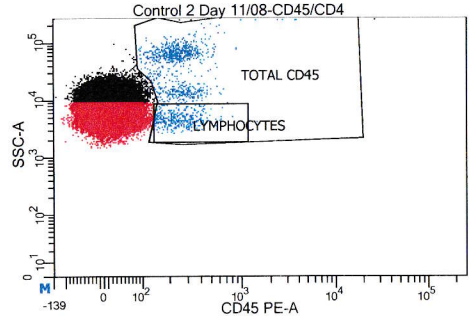


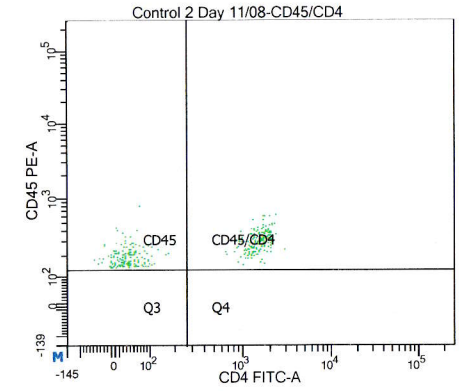
Experiment Name:	Chicken new batch
Specimen Name:	Control 1 Day 11/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:40:4...
$OP:	Administrator

Population	#Events
CD8	106
CD4	243

BD FACSDiva 8.0.1





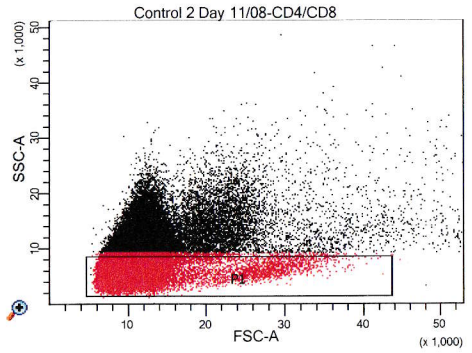


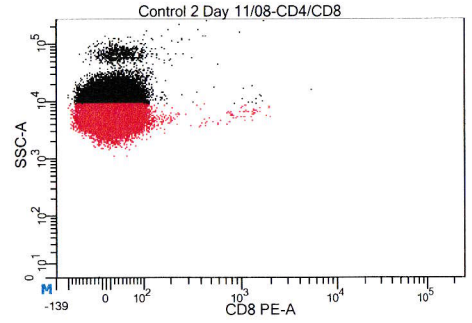
Experiment Name:	Chicken new batch
Specimen Name:	Control 2 Day 11/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:41:...
$OP:	Administrator

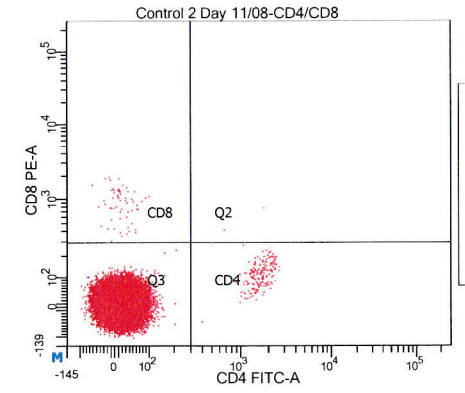
Population	#Events	%Parent
LYMPHOCYTES	353	0.2
CD45	161	45.6
CD45/CD4	192	54.4
TOTAL CD45	1,179	0.7

Printed by: Administrator

BD FACSDiva 8.0.1



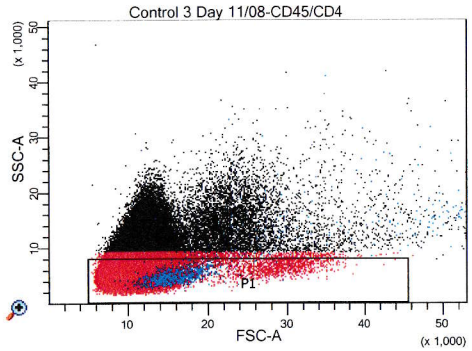


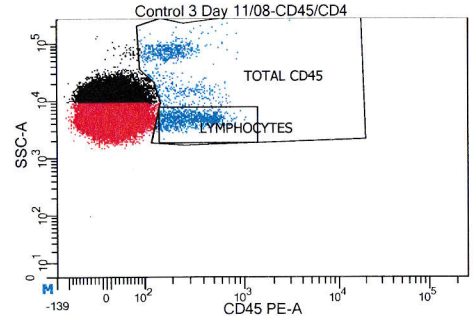


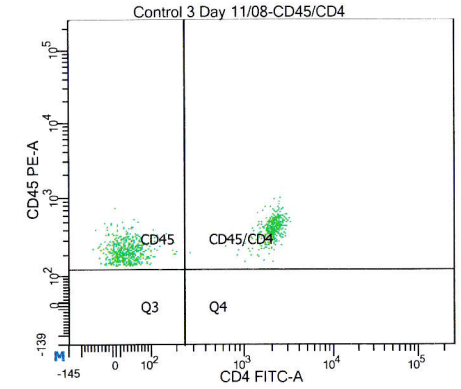
Experiment Name:	Chicken new batch
Specimen Name:	Control 2 Day 11/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:42:4...
$OP:	Administrator

Population	#Events
CD8	63
CD4	178

BD FACSDiva 8.0.1





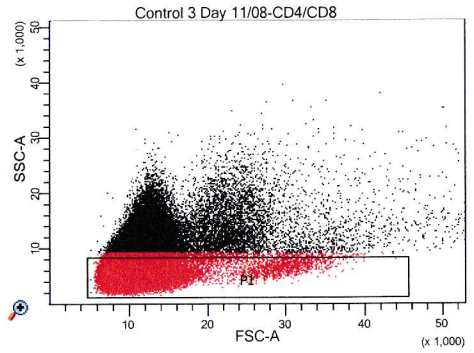


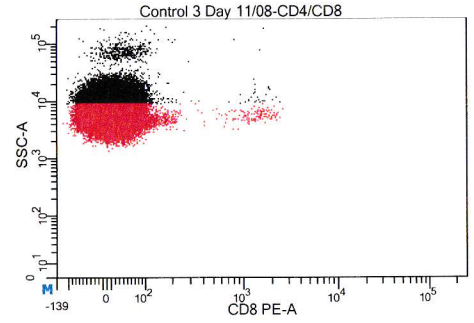
Experiment Name:	Chicken new batch
Specimen Name:	Control 3 Day 11/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:43:4...
$OP:	Administrator

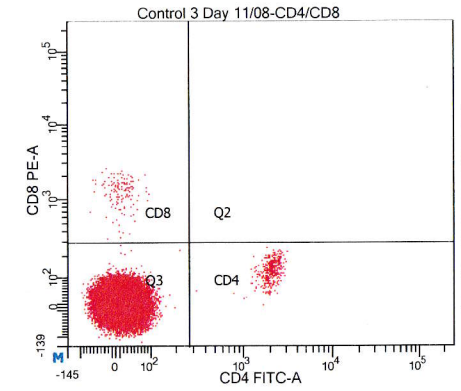
Population	#Events	%Parent
LYMPHOCYTES	973	0.5
CD45	566	58.2
CD45/CD4	407	41.8
TOTAL CD45	1,792	0.8

Printed by: Administrator

BD FACSDiva 8.0.1



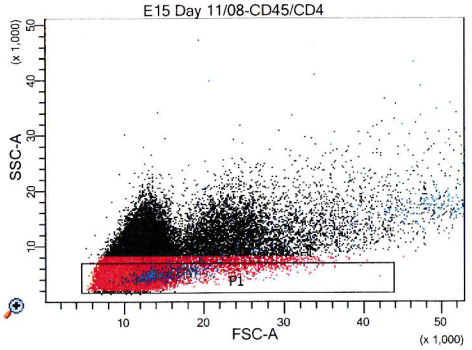


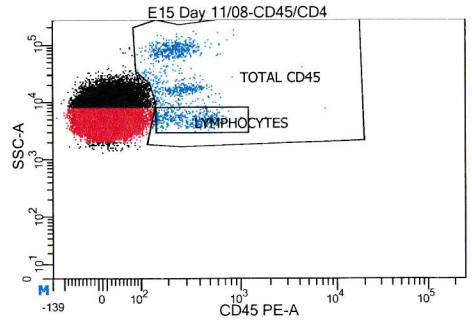


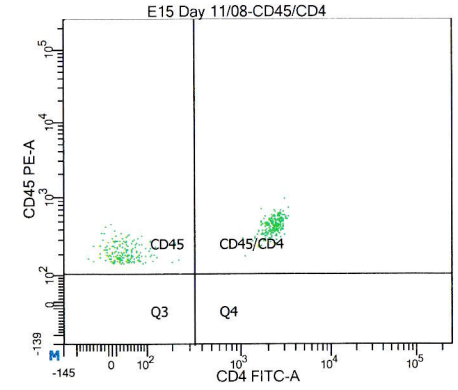
Experiment Name:	Chicken new batch
Specimen Name:	Control 3 Day 11/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:44:5...
$OP:	Administrator

Population	#Events
CD8	143
CD4	386

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 11/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:46:0...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	453	0.3
CD45	194	42.8
CD45/CD4	259	57.2
TOTAL CD45	1,374	0.9

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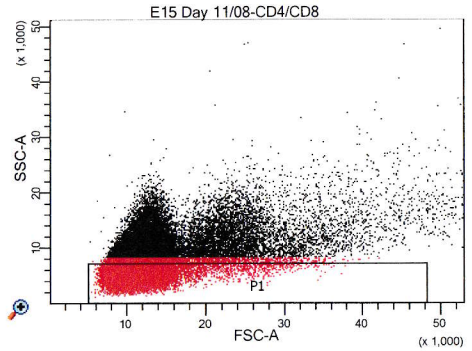
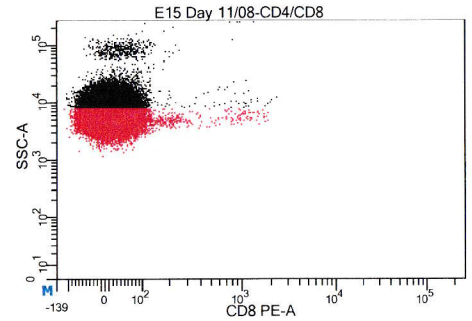
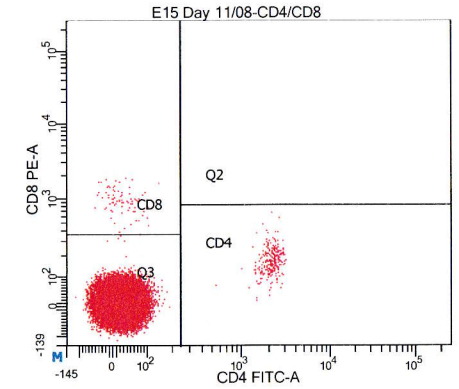
BD FACSDiva 8.0.1



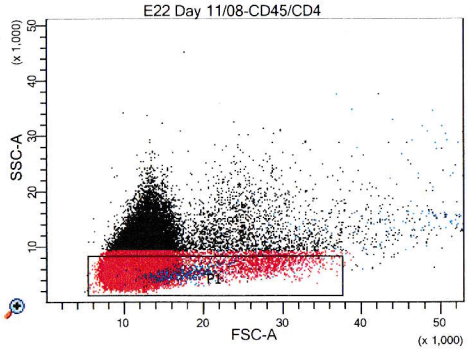


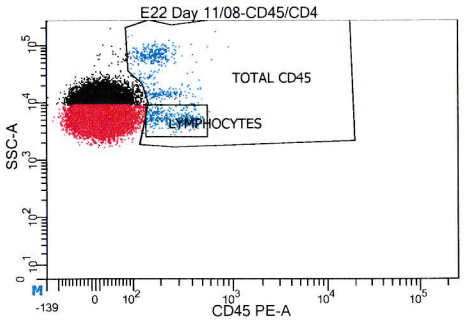


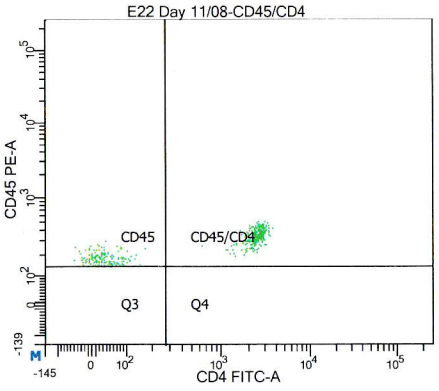
Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 11/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:47:0...
$OP:	Administrator

Population	#Events	CD4 FIT... Mean	CD8 PE... Mean
CD8	84	26	1,001
CD4	251	2,141	182

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E22 Day 11/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:48:1...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	465	0.6
CD45	172	37.0
CD45/CD4	293	63.0
TOTAL CD45	945	1.1

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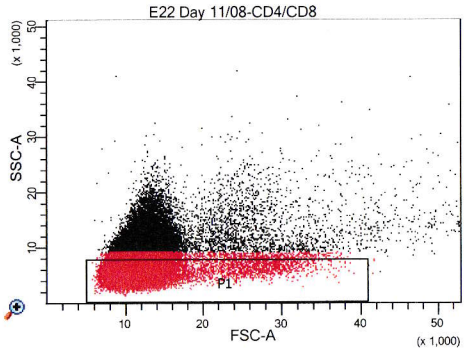
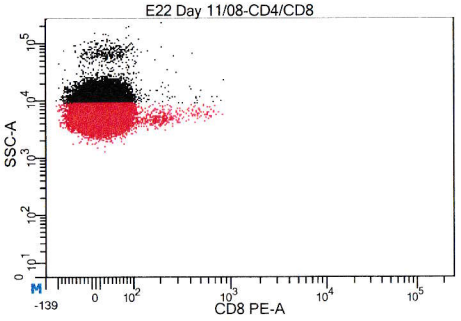
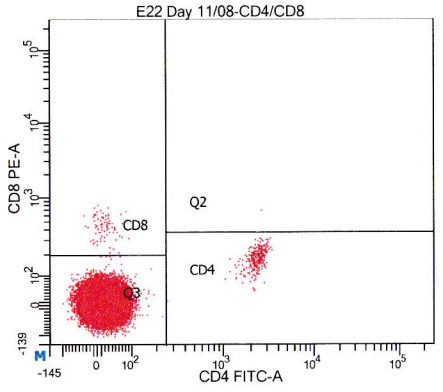
BD FACSDiva 8.0.1



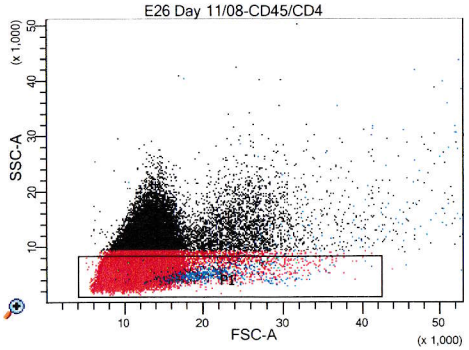


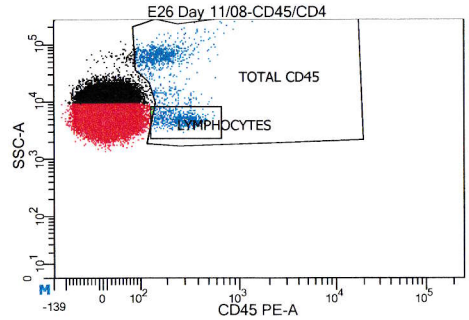


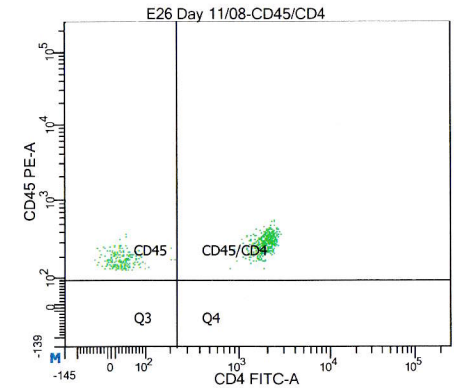
Experiment Name:	Chicken new batch
Specimen Name:	E22 Day 11/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:49:1...
$OP:	Administrator

Population	#Events
CD8	83
CD4	308

BD FACSDiva 8.0.1





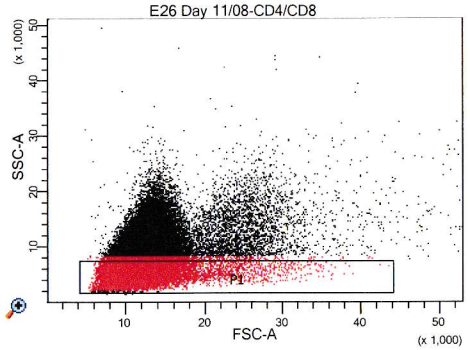


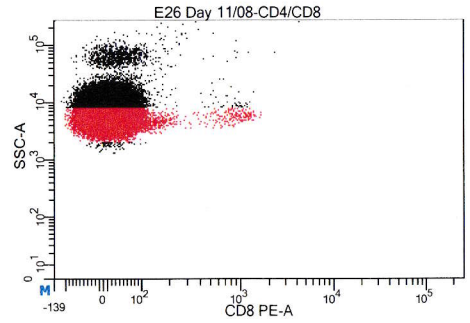
Experiment Name:	Chicken new batch
Specimen Name:	E26 Day 11/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:50:...
$OP:	Administrator

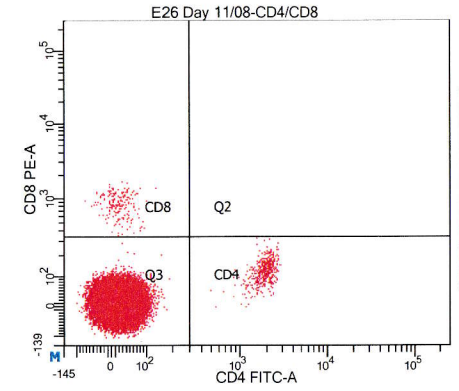
Population	#Events	%Parent
LYMPHOCYTES	636	0.4
CD45	210	33.0
CD45/CD4	426	67.0
TOTAL CD45	1,569	1.0

Printed by: Administrator

BD FACSDiva 8.0.1



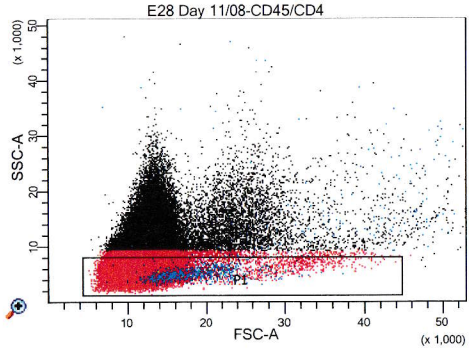


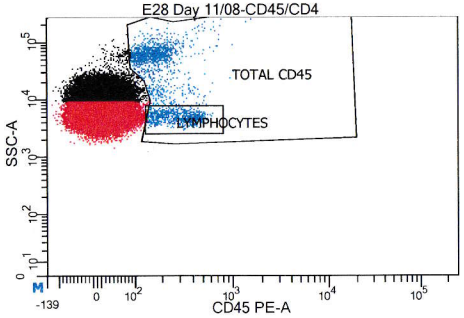


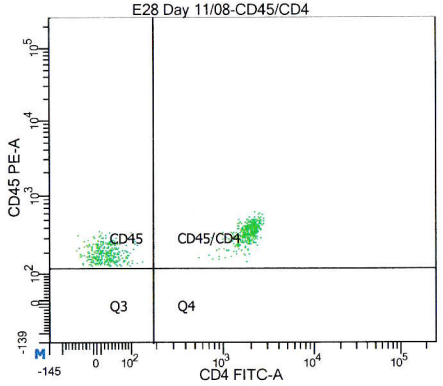
Experiment Name:	Chicken new batch
Specimen Name:	E26 Day 11/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:52:4...
$OP:	Administrator

Population	#Events
CD8	190
CD4	446

BD FACSDiva 8.0.1





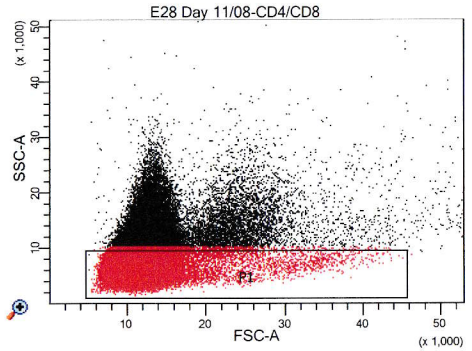


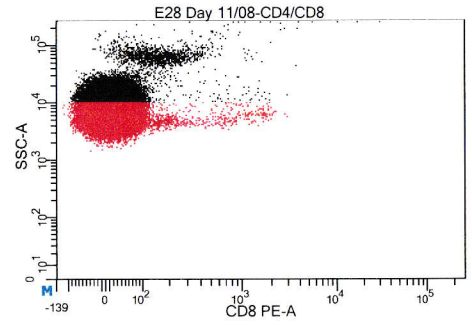
Experiment Name:	Chicken new batch
Specimen Name:	E28 Day 11/08
Tube Name:	CD45/CD4
Record Date:	Aug 11, 2017 3:54:1...
$OP:	Administrator

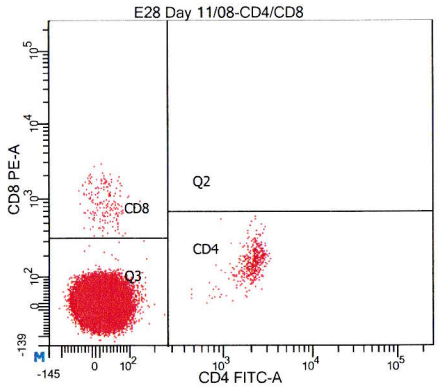
Population	#Events	%Parent
LYMPHOCYTES	782	0.4
CD45	359	45.9
CD45/CD4	423	54.1
TOTAL CD45	2,037	0.9

Printed by: Administrator

BD FACSDiva 8.0.1



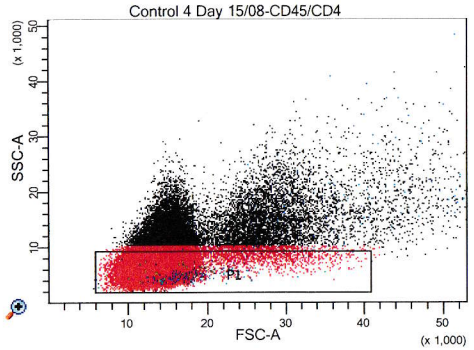


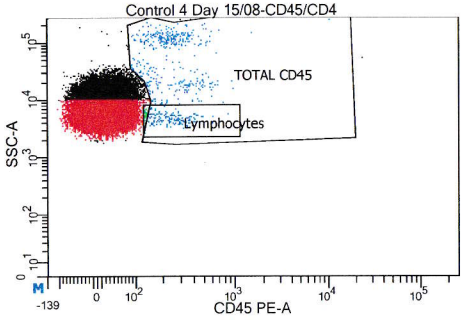


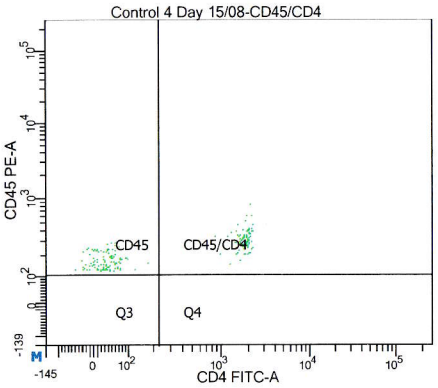
Experiment Name:	Chicken new batch
Specimen Name:	E28 Day 11/08
Tube Name:	CD4/CD8
Record Date:	Aug 11, 2017 3:55:5...
$OP:	Administrator

Population	#Events
CD8	196
CD4	466

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	Control 4 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 2:42:3...
$OP:	Administrator

Population	#Events	%Parent
Lymphocytes	191	0.2
CD45	105	55.0
CD45/CD4	86	45.0
TOTAL CD45	553	0.7

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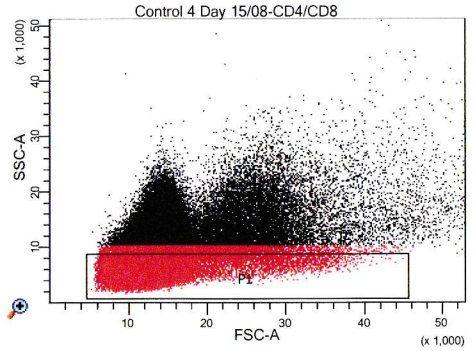
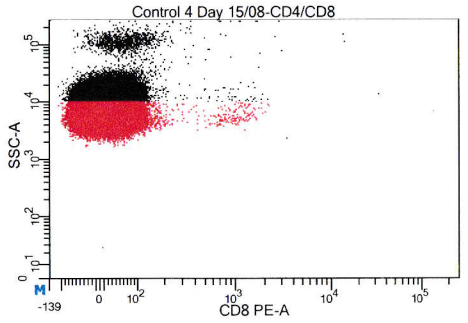
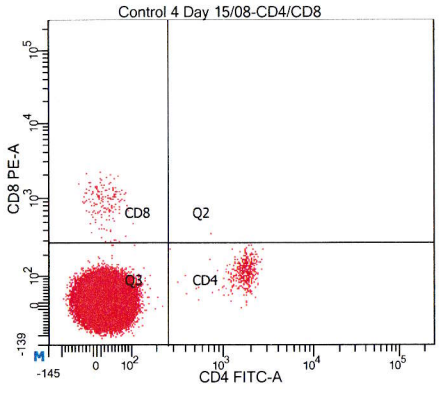
BD FACSDiva 8.0.1



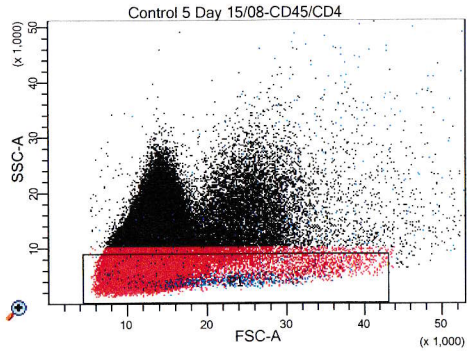


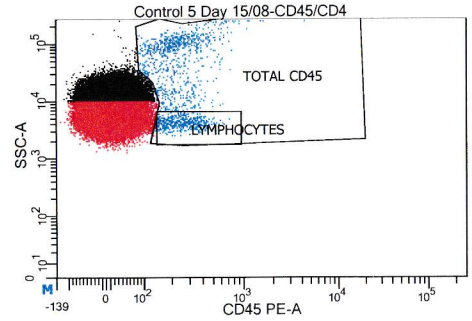


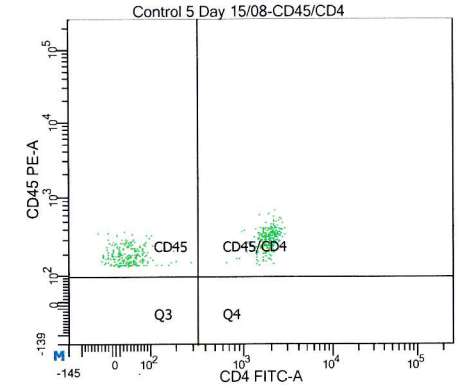
Experiment Name:	Chicken new batch
Specimen Name:	Control 4 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 2:44:2...
$OP:	Administrator

Population	#Events
CD8	169
CD4	442

BD FACSDiva 8.0.1





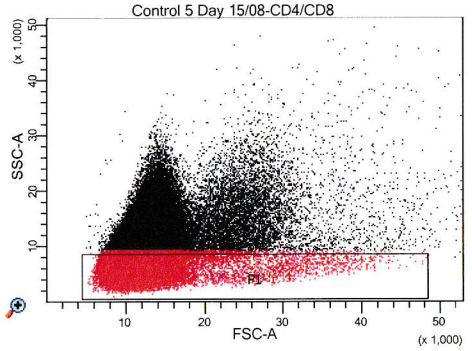


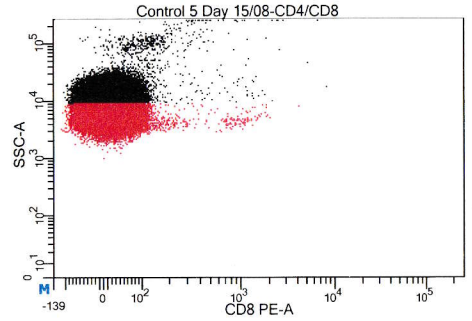
Experiment Name:	Chicken new batch
Specimen Name:	Control 5 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 2:46:3...
$OP:	Administrator

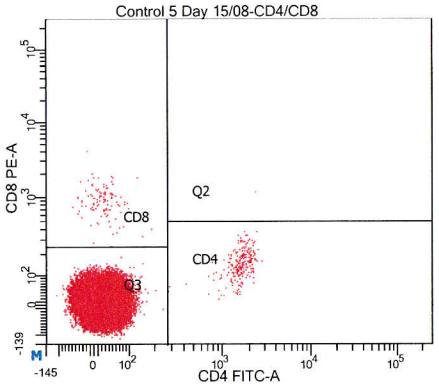
Population	#Events	%Parent
LYMPHOCYTES	510	0.1
CD45	240	47.1
CD45/CD4	270	52.9
TOTAL CD45	1,331	0.3

Printed by: Administrator

BD FACSDiva 8.0.1



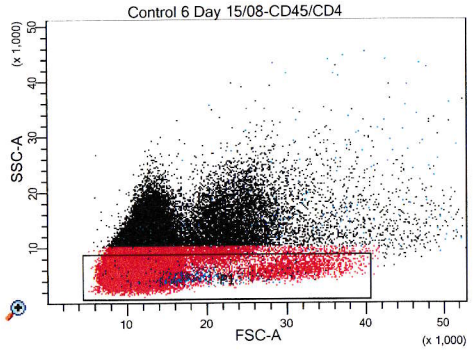


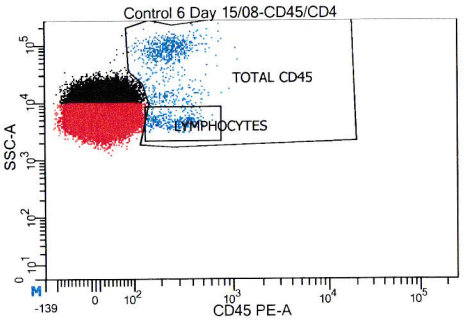


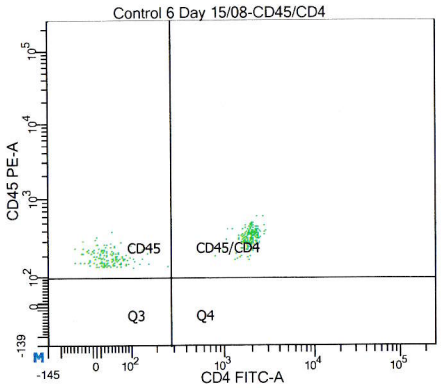
Experiment Name:	Chicken new batch
Specimen Name:	Control 5 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 2:48:3...
$OP:	Administrator

Population	#Events
CD8	113
CD4	233

BD FACSDiva 8.0.1





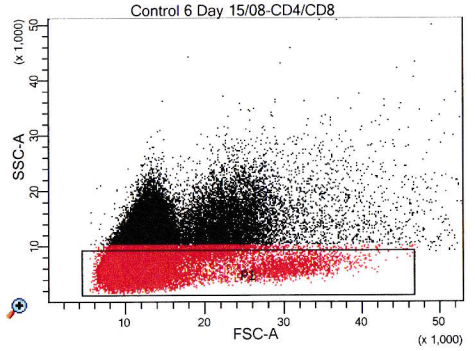


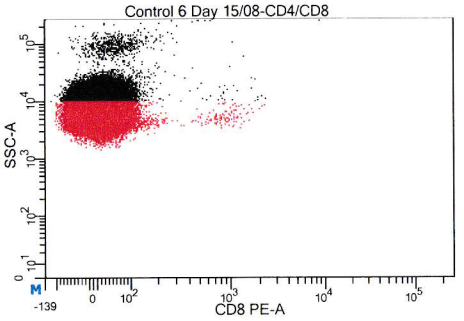
Experiment Name:	Chicken new batch
Specimen Name:	Control 6 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 2:49:4...
$OP:	Administrator

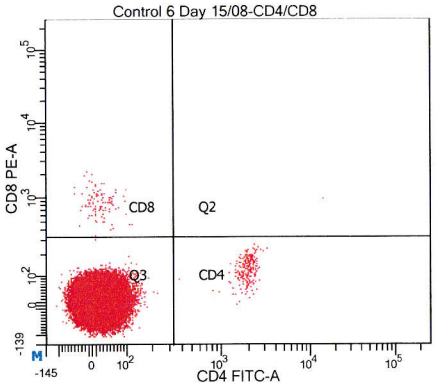
Population	#Events	%Parent
LYMPHOCYTES	346	0.1
CD45	139	40.2
CD45/CD4	207	59.8
TOTAL CD45	1,179	0.5

Printed by: Administrator

BD FACSDiva 8.0.1



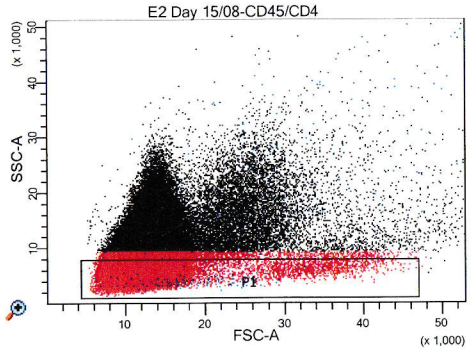


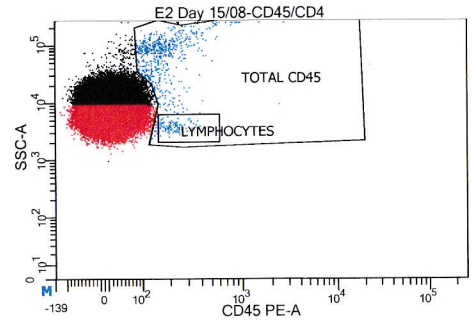


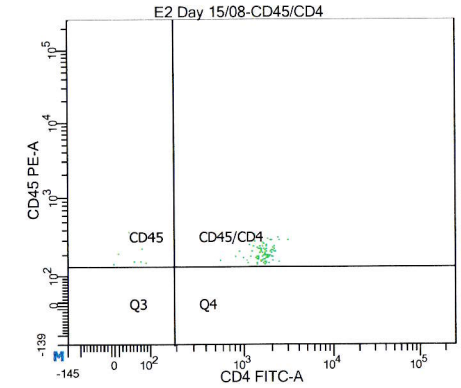
Experiment Name:	Chicken new batch
Specimen Name:	Control 6 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 2:50:4...
$OP:	Administrator

Population	#Events
CD8	105
CD4	231

BD FACSDiva 8.0.1





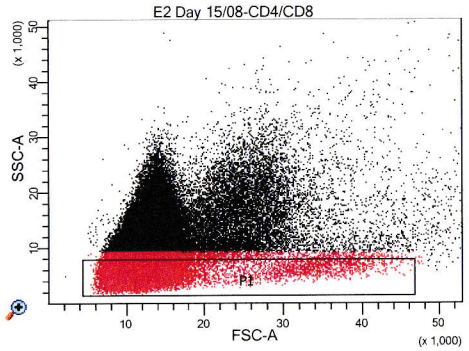


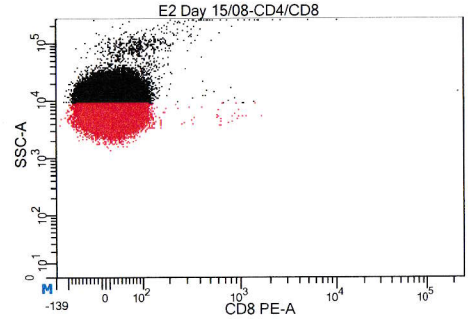
Experiment Name:	Chicken new batch
Specimen Name:	E2 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 2:52:2...
$OP:	Administrator

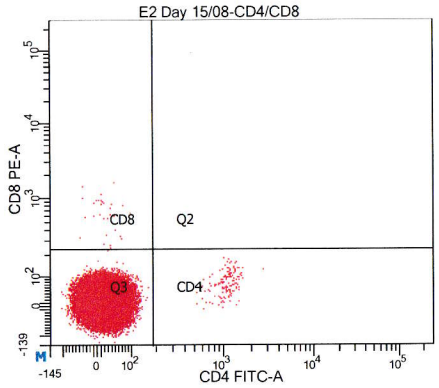
Population	#Events	%Parent
LYMPHOCYTES	104	0.0
CD45	7	6.7
CD45/CD4	97	93.3
TOTAL CD45	713	0.2

Printed by: Administrator

BD FACSDiva 8.0.1



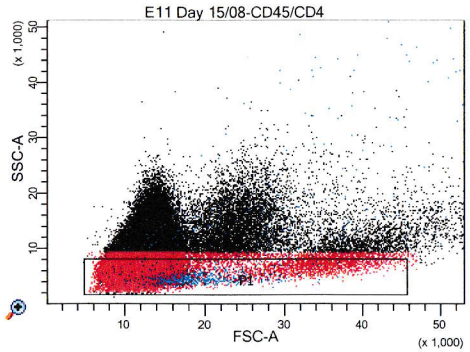


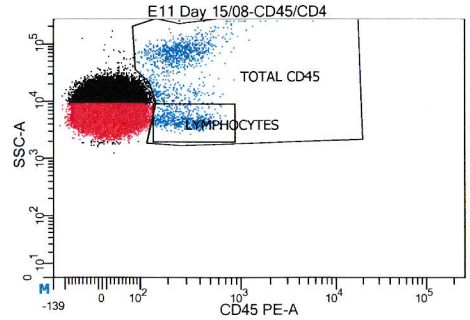


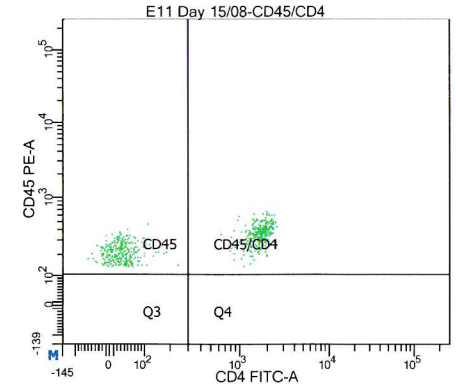
Experiment Name:	Chicken new batch
Specimen Name:	E2 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 2:54:0...
$OP:	Administrator

Population	#Events
CD8	31
CD4	149

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 2:55:3...
$OP:	Administrator

Population	#Events	%Parent
LYMPHOCYTES	647	0.2
CD45	310	47.9
CD45/CD4	337	52.1
TOTAL CD45	1,701	0.5

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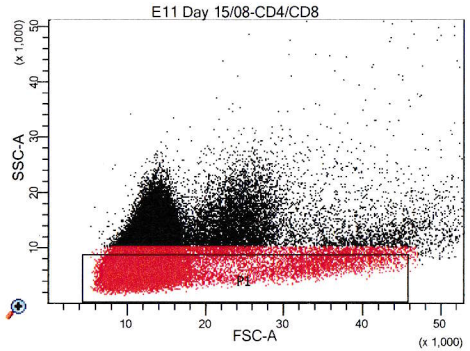
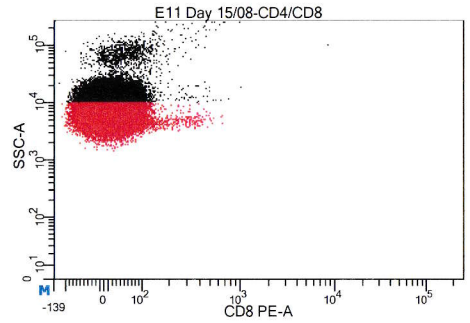
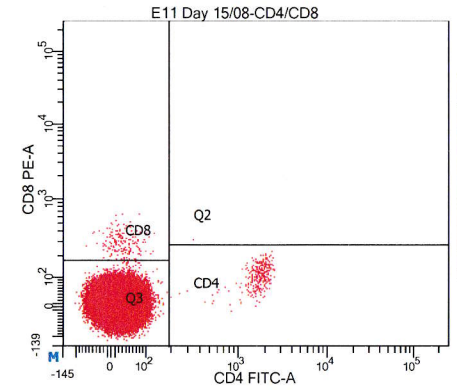
BD FACSDiva 8.0.1



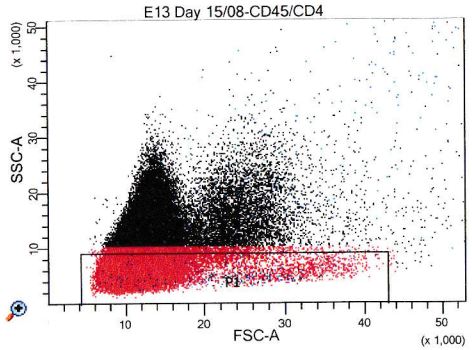


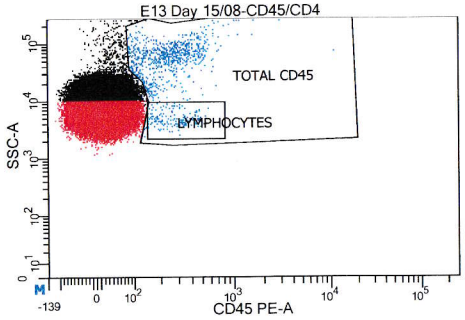


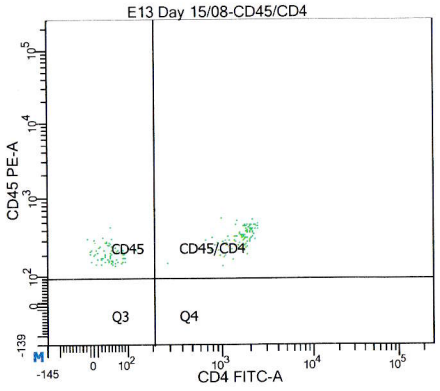
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 2:57:1...
$OP:	Administrator

Population	#Events
CD8	179
CD4	314

BD FACSDiva 8.0.1





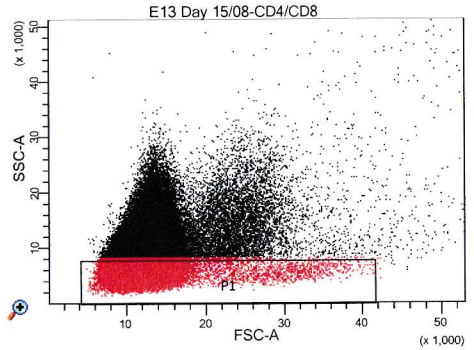


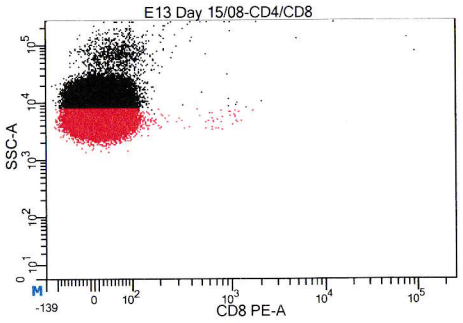
Experiment Name:	Chicken new batch
Specimen Name:	E13 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 2:59:0...
$OP:	Administrator

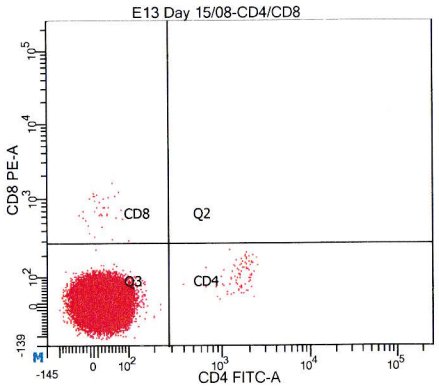
Population	#Events	%Parent
LYMPHOCYTES	167	0.0
CD45	72	43.1
CD45/CD4	95	56.9
TOTAL CD45	978	0.3

Printed by: Administrator

BD FACSDiva 8.0.1



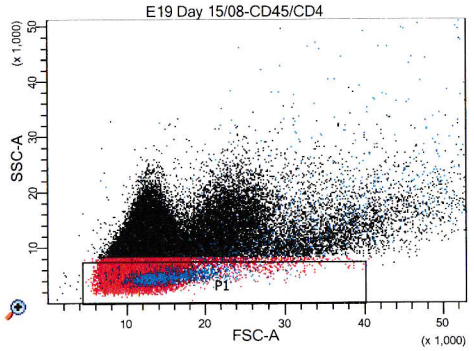


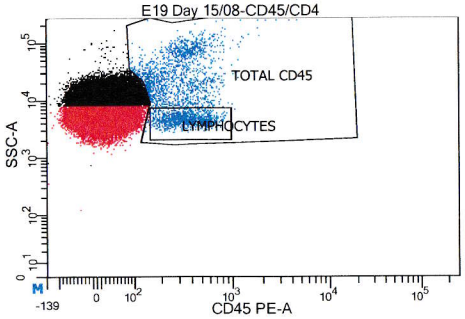


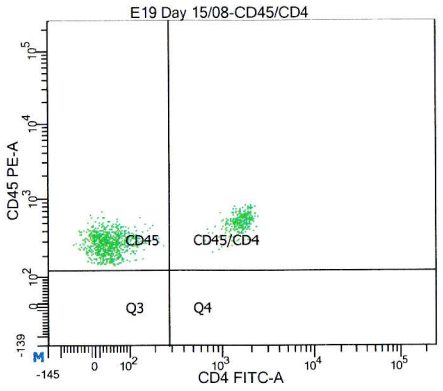
Experiment Name:	Chicken new batch
Specimen Name:	E13 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 3:00:5...
$OP:	Administrator

Population	#Events
CD8	37
CD4	84

BD FACSDiva 8.0.1





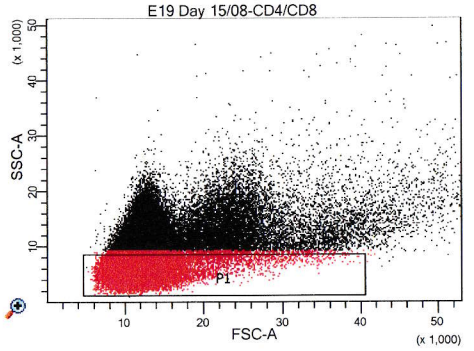


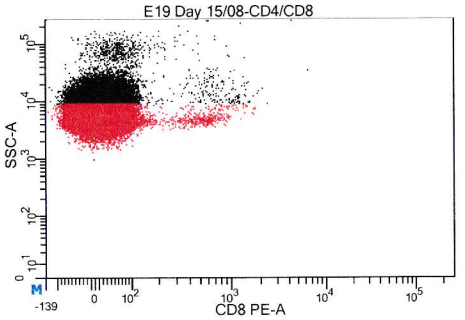
Experiment Name:	Chicken new batch
Specimen Name:	E19 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 3:02:4...
$OP:	Administrator

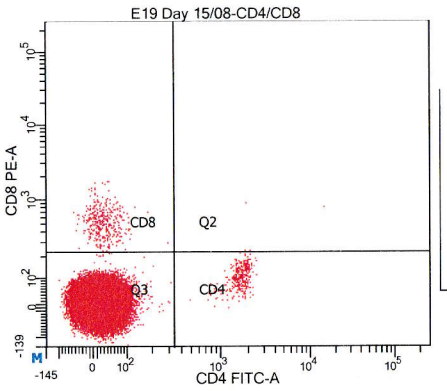
Population	#Events	%Parent
LYMPHOCYTES	1,228	0.4
CD45	903	73.5
CD45/CD4	325	26.5
TOTAL CD45	2,843	0.8

Printed by: Administrator

BD FACSDiva 8.0.1



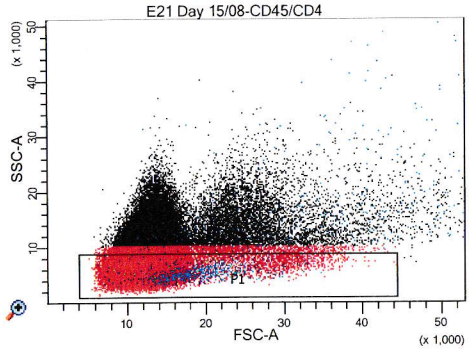


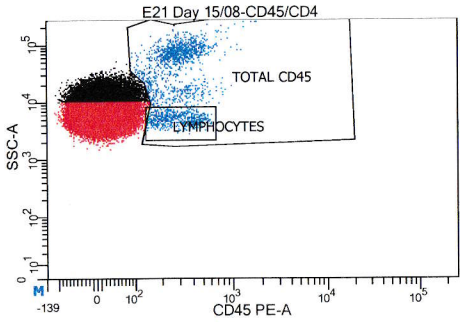


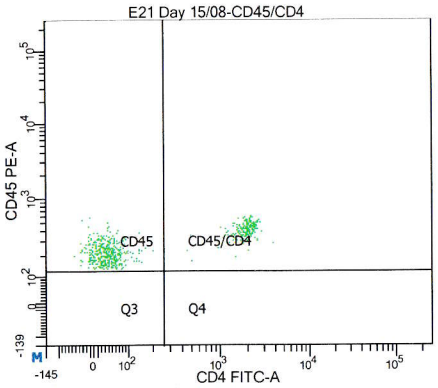
Experiment Name:	Chicken new batch
Specimen Name:	E19 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 3:04:2...
$OP:	Administrator

Population	#Events
CD8	389
CD4	339

BD FACSDiva 8.0.1





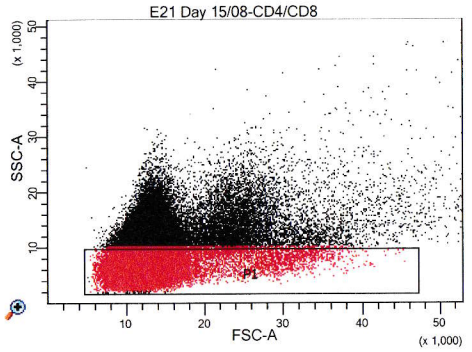


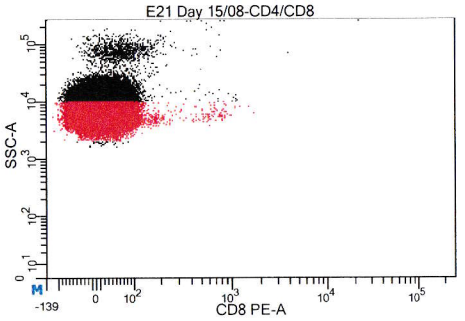
Experiment Name:	Chicken new batch
Specimen Name:	E21 Day 15/08
Tube Name:	CD45/CD4
Record Date:	Aug 16, 2017 3:05:5...
$OP:	Administrator

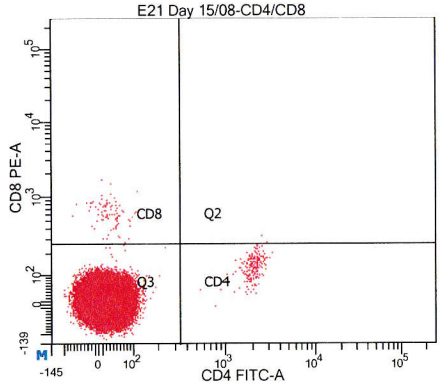
Population	#Events	%Parent
LYMPHOCYTES	578	0.2
CD45	383	66.3
CD45/CD4	195	33.7
TOTAL CD45	1,947	0.8

Printed by: Administrator

BD FACSDiva 8.0.1



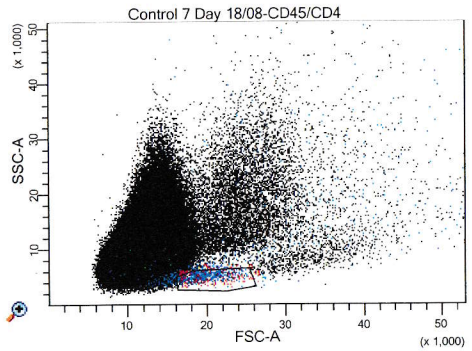


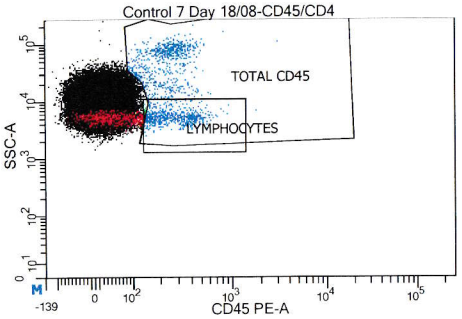


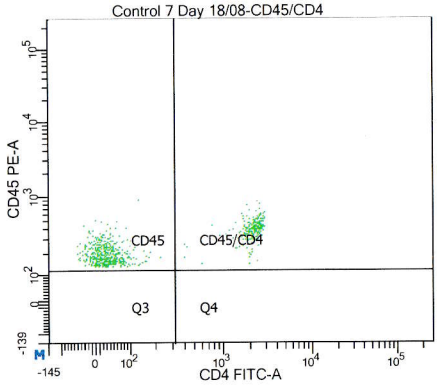
Experiment Name:	Chicken new batch
Specimen Name:	E21 Day 15/08
Tube Name:	CD4/CD8
Record Date:	Aug 16, 2017 3:07:1...
$OP:	Administrator

Population	#Events
CD8	90
CD4	207

BD FACSDiva 8.0.1





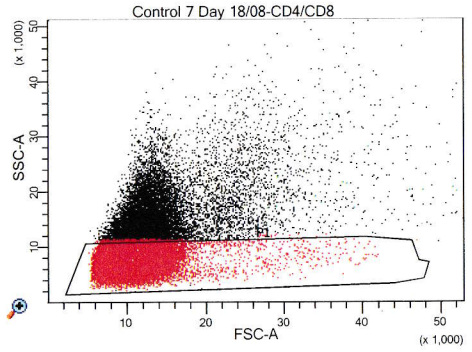


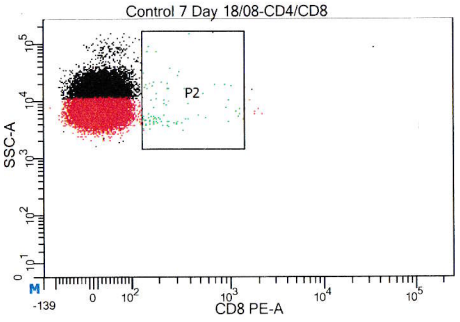
Experiment Name:	Chicken new batch
Specimen Name:	Control 7 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 2:48:...
$OP:	Administrator

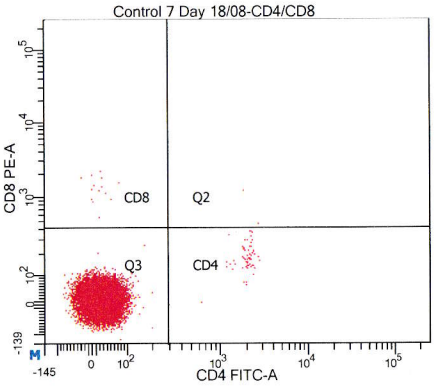
Population	#Events
LYMPHOCYTES	688
CD45	440
CD45/CD4	248
TOTAL CD45	1,325

Printed by: Administrator

BD FACSDiva 8.0.1



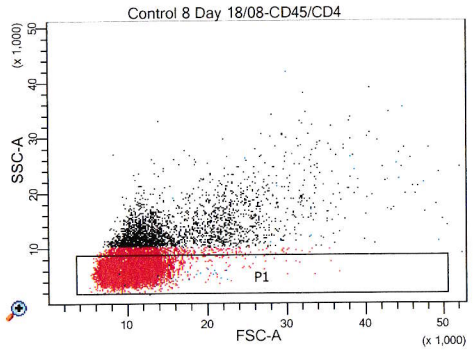


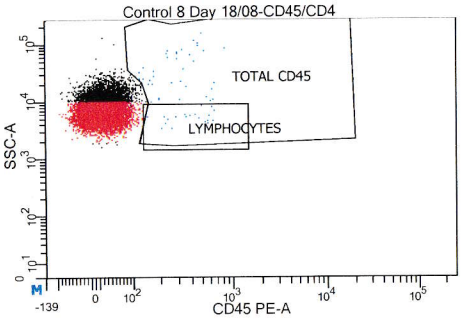


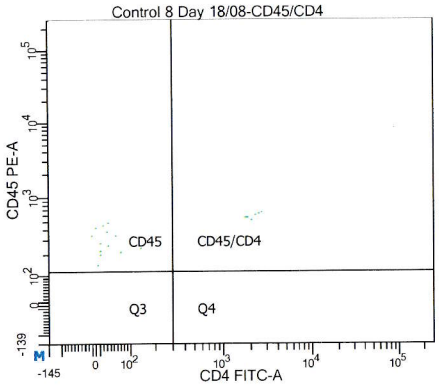
Experiment Name:	Chicken new batch
Specimen Name:	Control 7 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 2:49:...
$OP:	Administrator

Population	#Events
CD8	14
CD4	59

BD FACSDiva 8.0.1





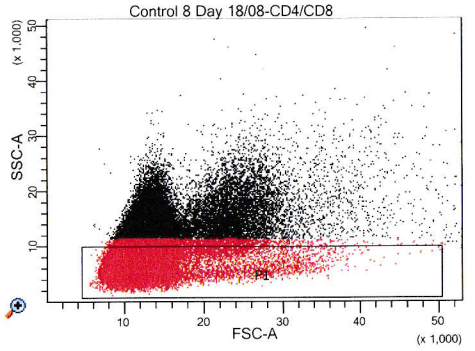


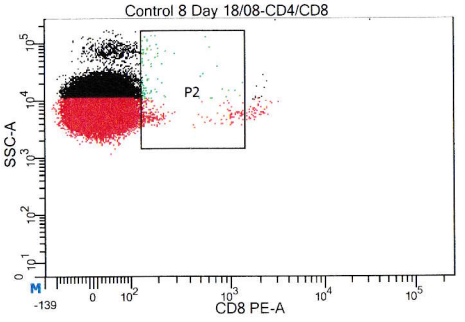
Experiment Name:	Chicken new batch
Specimen Name:	Control 8 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 2:49:...
$OP:	Administrator

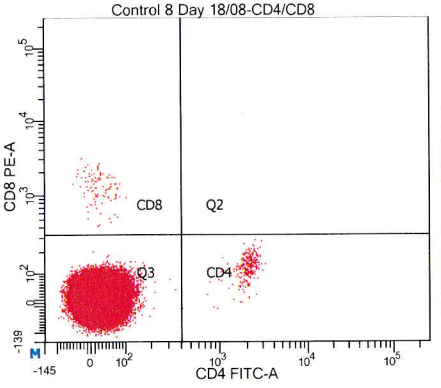
Population	#Events
LYMPHOCYTES	20
CD45	13
CD45/CD4	7
TOTAL CD45	60

Printed by: Administrator

BD FACSDiva 8.0.1



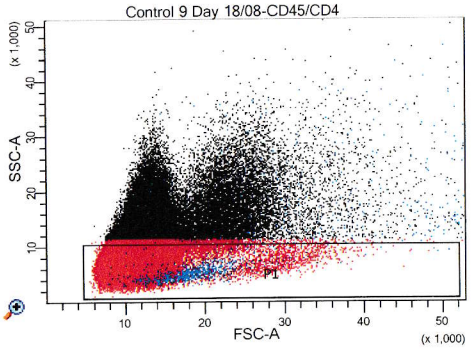


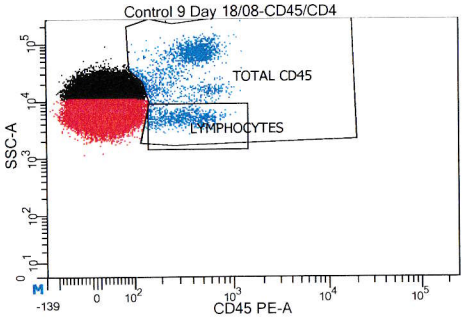


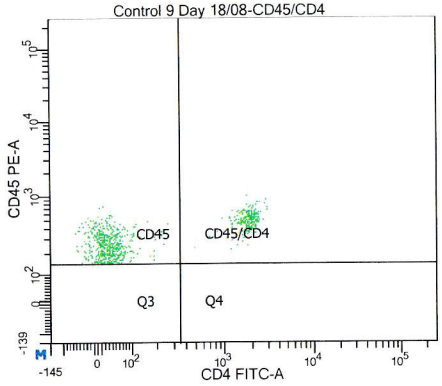
Experiment Name:	Chicken new batch
Specimen Name:	Control 8 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 2:51:...
$OP:	Administrator

Population	#Events
CD8	109
CD4	309

BD FACSDiva 8.0.1





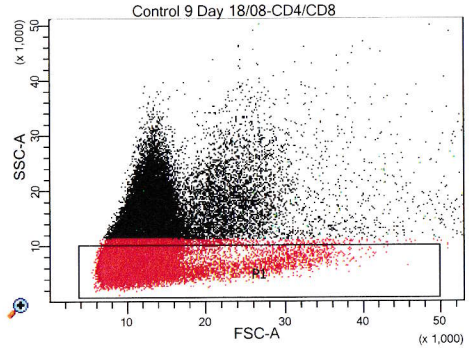


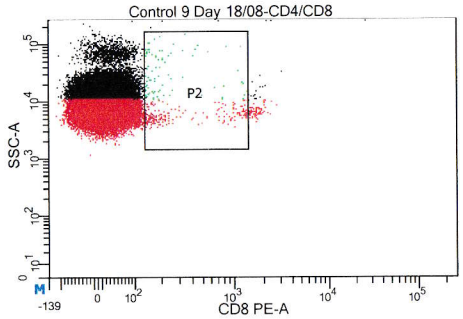
Experiment Name:	Chicken new batch
Specimen Name:	Control 9 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 2:52:...
$OP:	Administrator

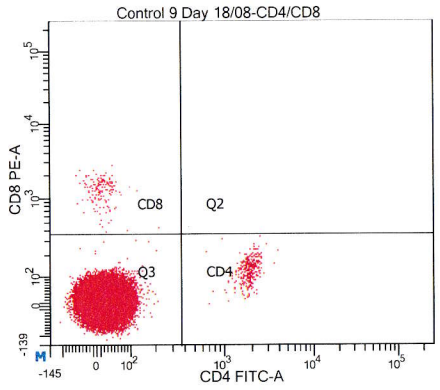
Population	#Events
LYMPHOCYTES	758
CD45	516
CD45/CD4	242
TOTAL CD45	2,623

Printed by: Administrator

BD FACSDiva 8.0.1



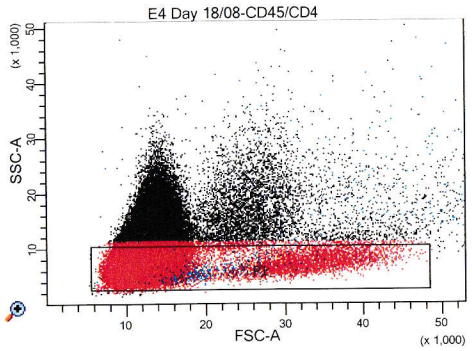


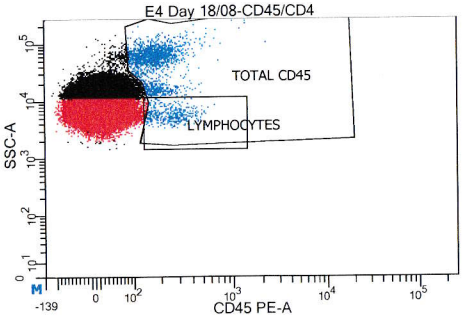


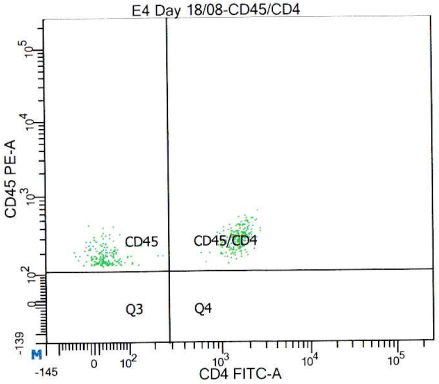
Experiment Name:	Chicken new batch
Specimen Name:	Control 9 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 2:53:...
$OP:	Administrator

Population	#Events
CD8	152
CD4	318

BD FACSDiva 8.0.1





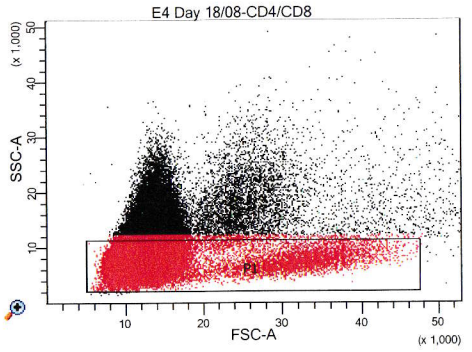


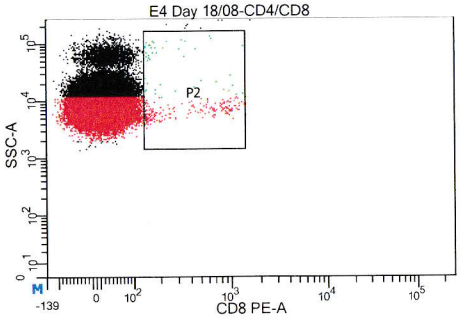
Experiment Name:	Chicken new batch
Specimen Name:	E4 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 2:54:4...
$OP:	Administrator

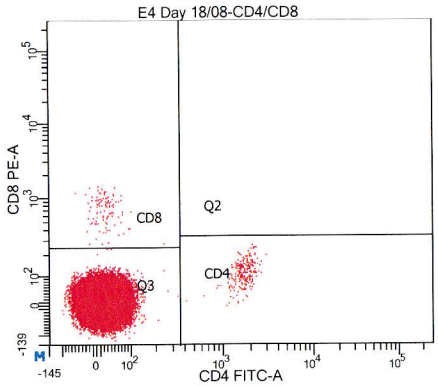
Population	#Events
LYMPHOCYTES	405
CD45	176
CD45/CD4	229
TOTAL CD45	2,375

Printed by: Administrator

BD FACSDiva 8.0.1



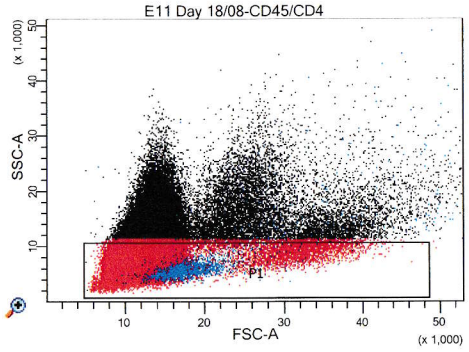


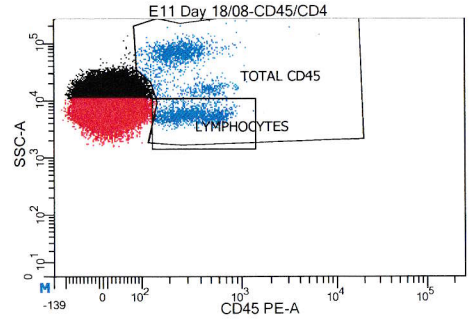


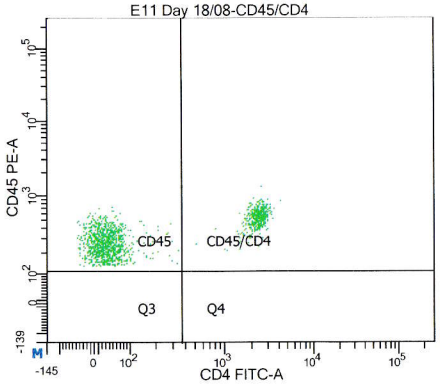
Experiment Name:	Chicken new batch
Specimen Name:	E4 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 2:55:4...
$OP:	Administrator

Population	#Events
CD8	110
CD4	235

BD FACSDiva 8.0.1





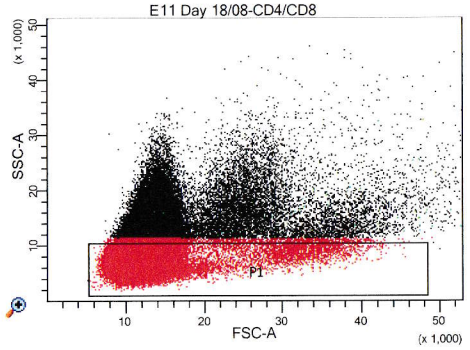


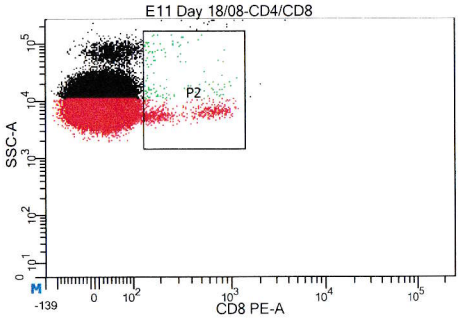
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 2:57:1...
$OP:	Administrator

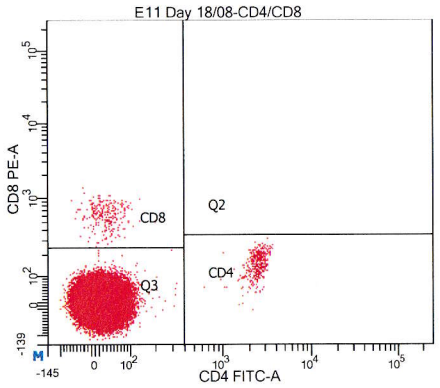
Population	#Events
LYMPHOCYTES	1,561
CD45	1,028
CD45/CD4	533
TOTAL CD45	3,139

Printed by: Administrator

BD FACSDiva 8.0.1



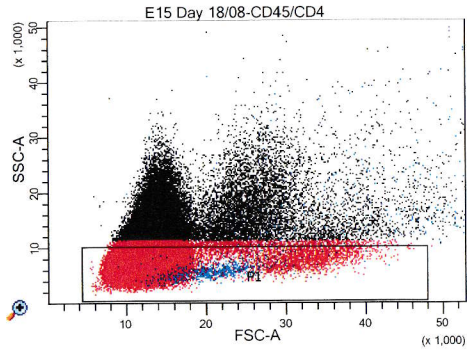


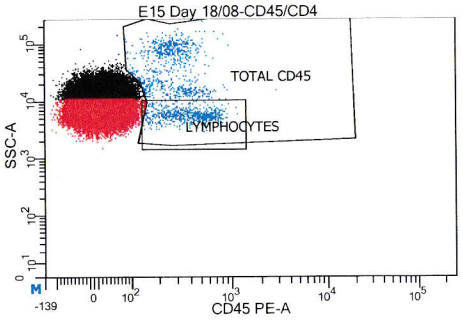


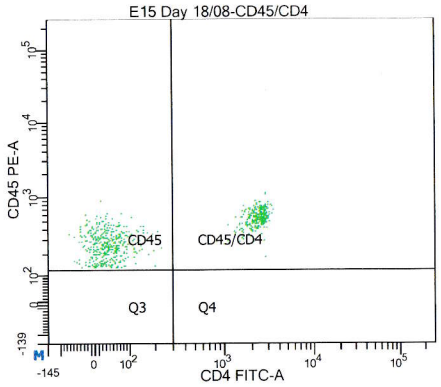
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 2:58:2...
$OP:	Administrator

Population	#Events
CD8	254
CD4	446

BD FACSDiva 8.0.1





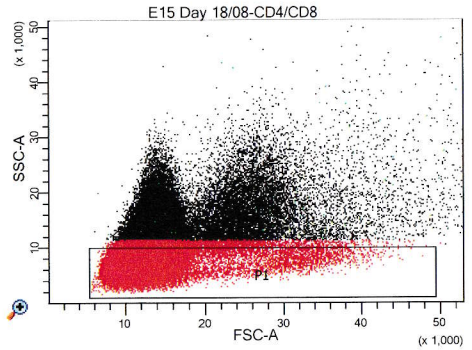


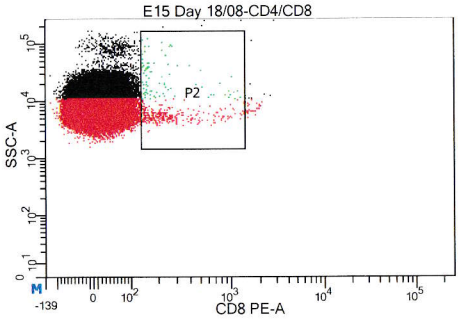
Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 2:59:...
$OP:	Administrator

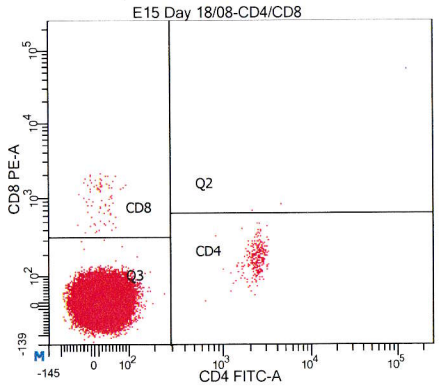
Population	#Events
LYMPHOCYTES	744
CD45	436
CD45/CD4	308
TOTAL CD45	1,489

Printed by: Administrator

BD FACSDiva 8.0.1



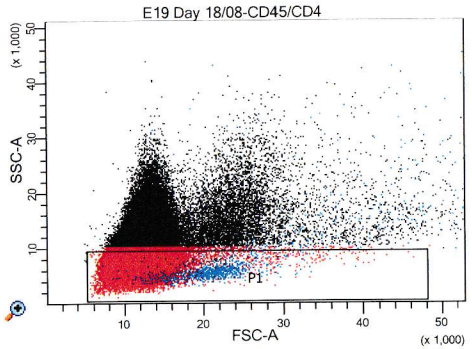


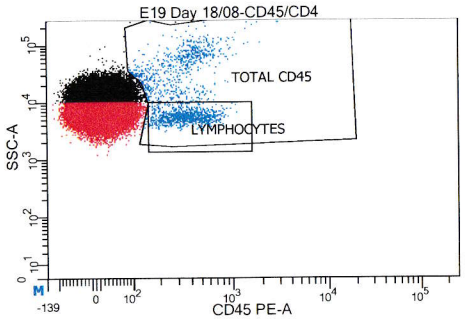


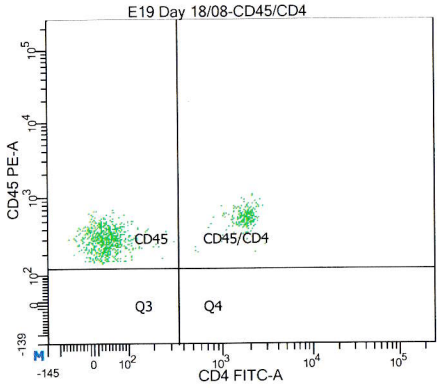
Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 3:00:...
$OP:	Administrator

Population	#Events
CD8	81
CD4	281

BD FACSDiva 8.0.1





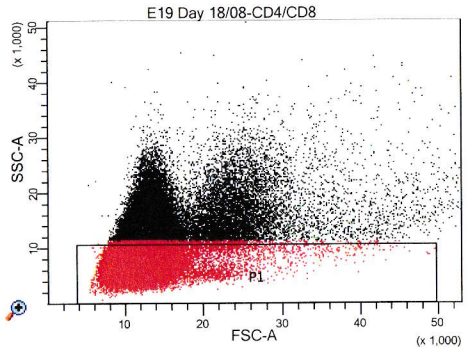


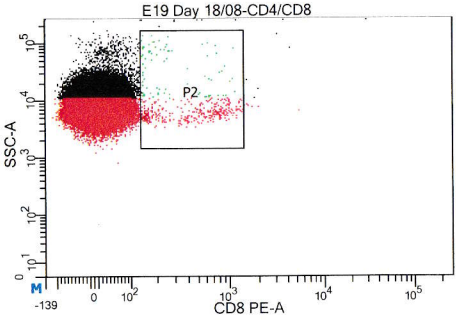
Experiment Name:	Chicken new batch
Specimen Name:	E19 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 3:02:...
$OP:	Administrator

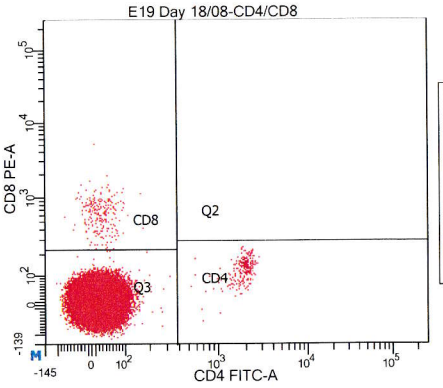
Population	#Events
LYMPHOCYTES	984
CD45	737
CD45/CD4	247
TOTAL CD45	1,767

Printed by: Administrator

BD FACSDiva 8.0.1



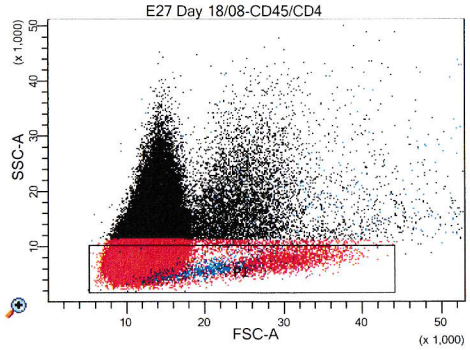


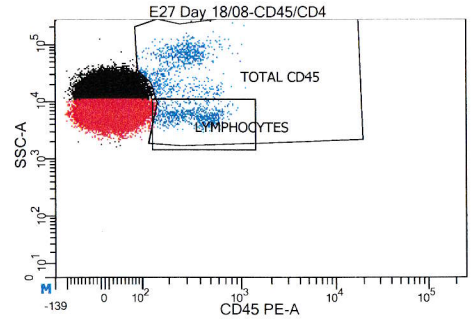


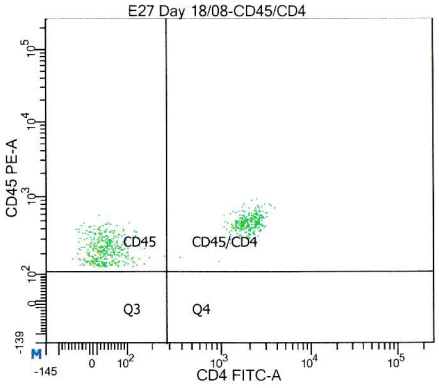
Experiment Name:	Chicken new batch
Specimen Name:	E19 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 3:03:3...
$OP:	Administrator

Population	#Events
CD8	234
CD4	251

BD FACSDiva 8.0.1





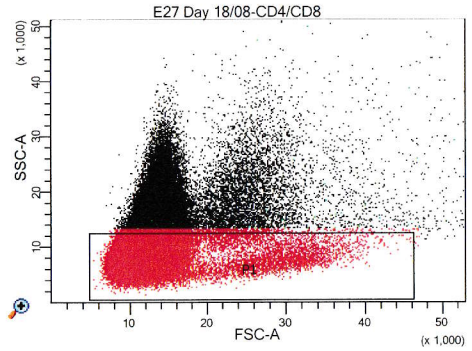


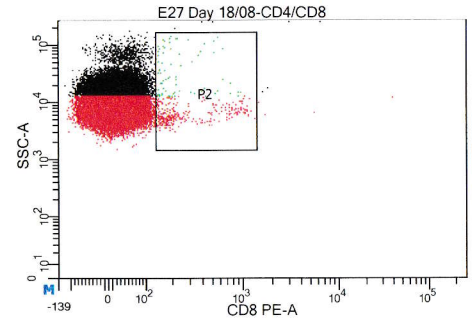
Experiment Name:	Chicken new batch
Specimen Name:	E27 Day 18/08
Tube Name:	CD45/CD4
Record Date:	Aug 18, 2017 3:04:...
$OP:	Administrator

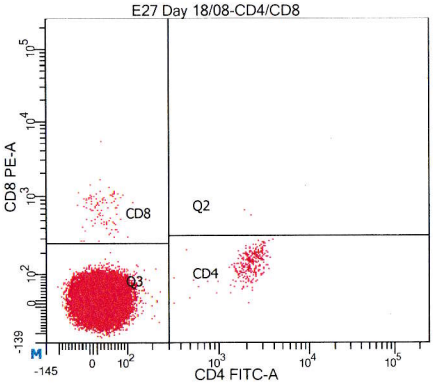
Population	#Events
LYMPHOCYTES	714
CD45	403
CD45/CD4	311
TOTAL CD45	1,549

Printed by: Administrator

BD FACSDiva 8.0.1



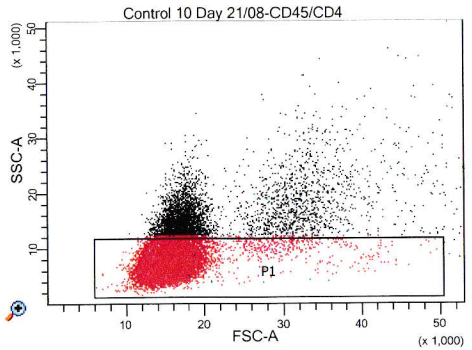


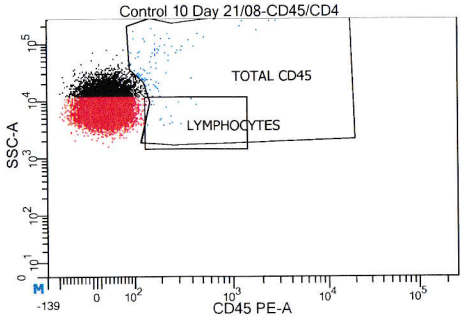


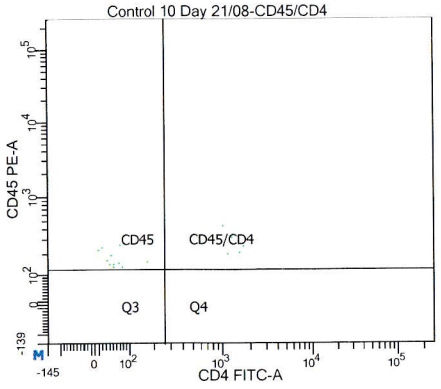
Experiment Name:	Chicken new batch
Specimen Name:	E27 Day 18/08
Tube Name:	CD4/CD8
Record Date:	Aug 18, 2017 3:06:...
$OP:	Administrator

Population	#Events
CD8	103
CD4	315

BD FACSDiva 8.0.1





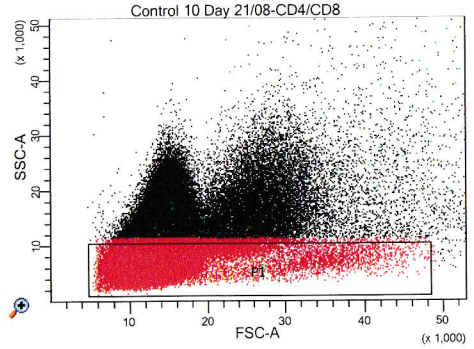


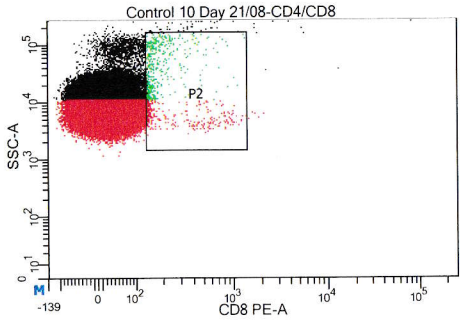
Experiment Name:	Chicken new batch
Specimen Name:	Control 10 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:35:5...
$OP:	Administrator

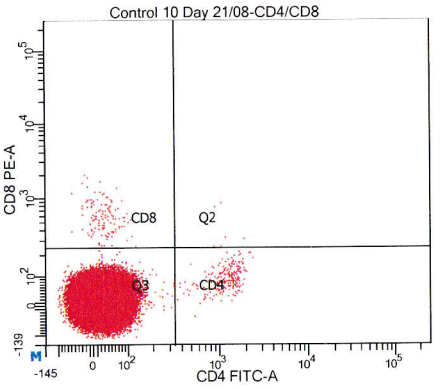
Population	#Events
LYMPHOCYTES	17
CD45	11
CD45/CD4	6
TOTAL CD45	103

Printed by: Administrator

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	Control 10 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:38:0...
$OP:	Administrator

Population	#Events
CD8	137
CD4	227

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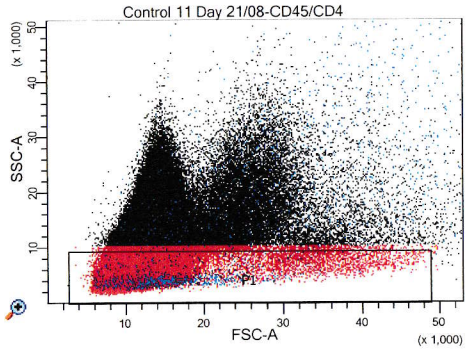

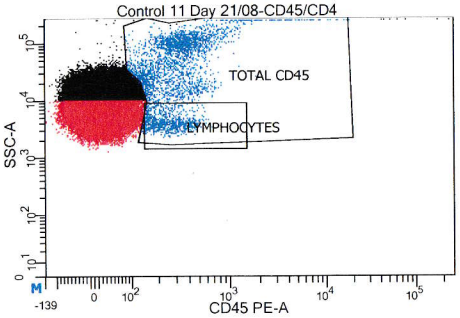

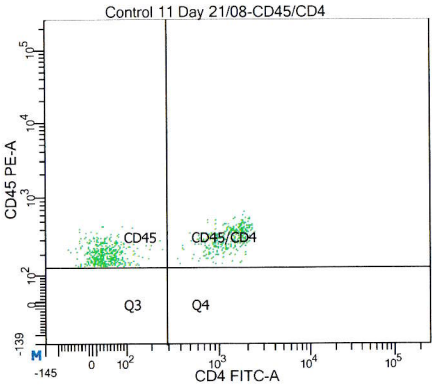

Experiment Name:	Chicken new batch
Specimen Name:	Control 11 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:40:2...
$OP:	Administrator

Population	#Events
LYMPHOCYTES	845
CD45	499
CD45/CD4	346
TOTAL CD45	3,472

Printed by: Administrator

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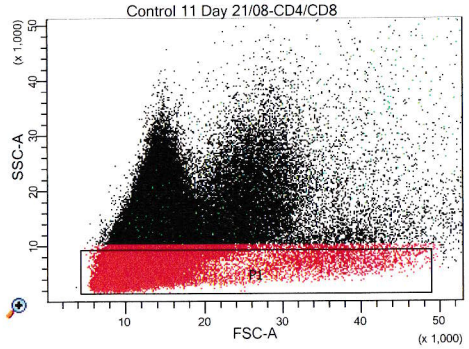

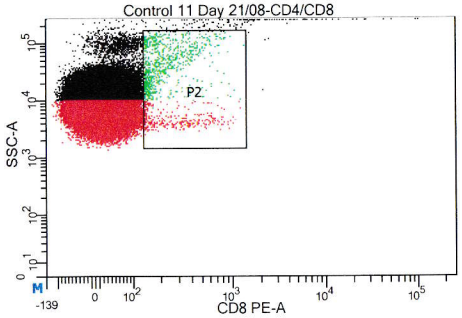

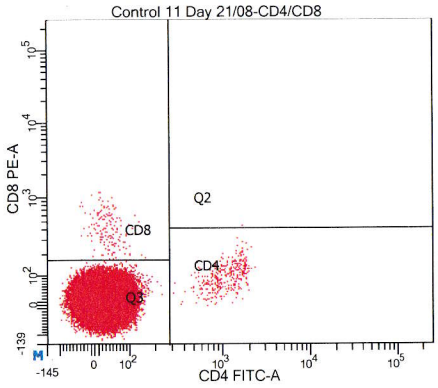

Experiment Name:	Chicken new batch
Specimen Name:	Control 11 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:42:...
$OP:	Administrator

Population	#Events
CD8	159
CD4	357

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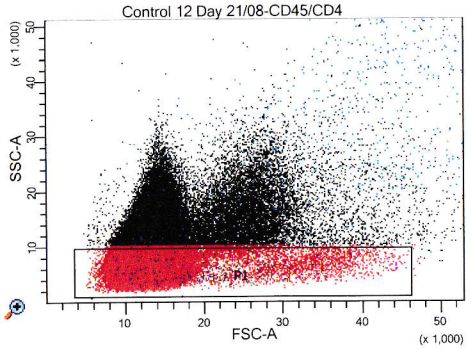
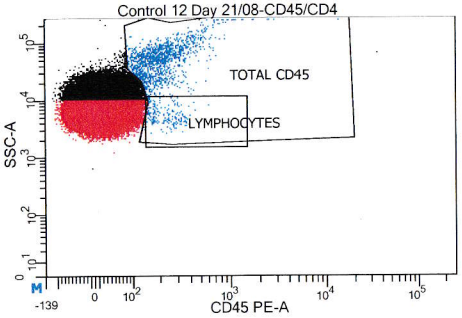
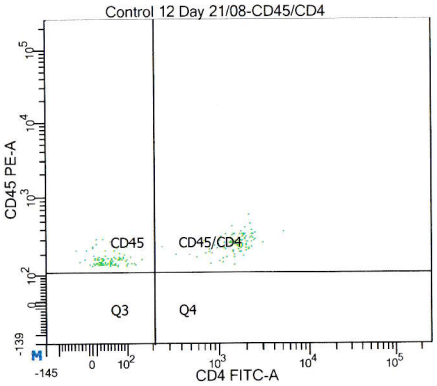
Control 12 Day 21/08-CD45/CD4



Control 12 Day 21/08-CD45/CD4



Control 12 Day 21/08-CD45/CD4



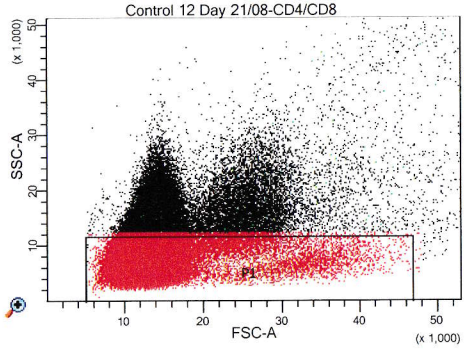
Experiment Name:	Chicken new batch
Specimen Name:	Control 12 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:44:1...
$OP:	Administrator

Population	#Events
LYMPHOCYTES	216
CD45	103
CD45/CD4	113
TOTAL CD45	1,577

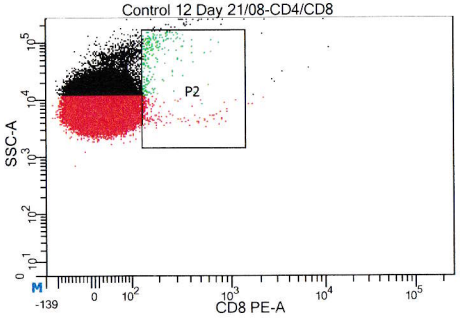
Printed by: Administrator

BD FACSDiva 8.0.1

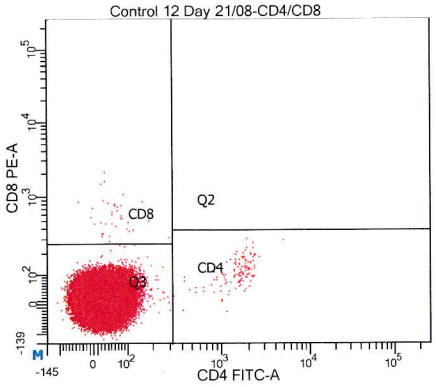
Control 12 Day 21/08-CD4/CD8



Control 12 Day 21/08-CD4/CD8



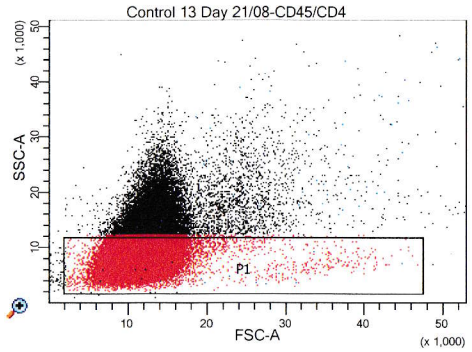
Control 12 Day 21/08-CD4/CD8

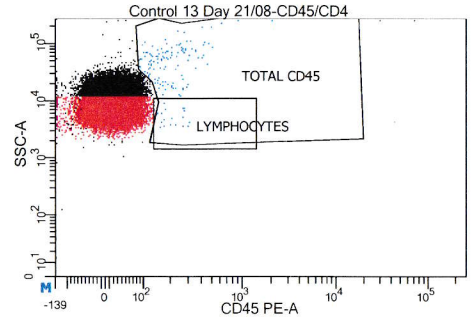


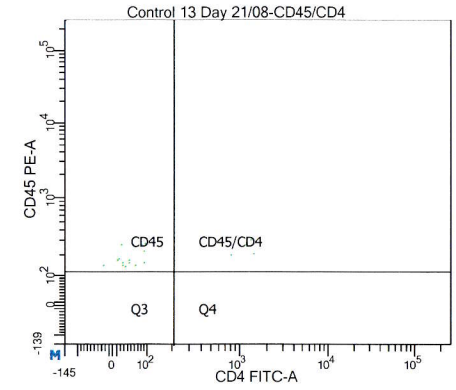
Experiment Name:	Chicken new batch
Specimen Name:	Control 12 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:46:...
$OP:	Administrator

Population	#Events
CD8	37
CD4	122

BD FACSDiva 8.0.1





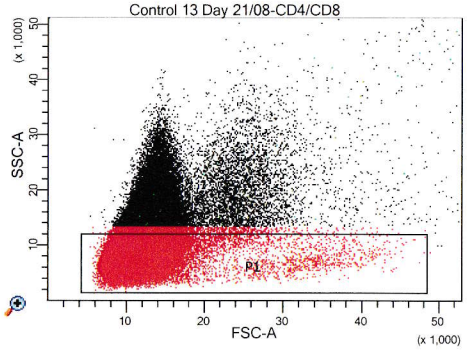


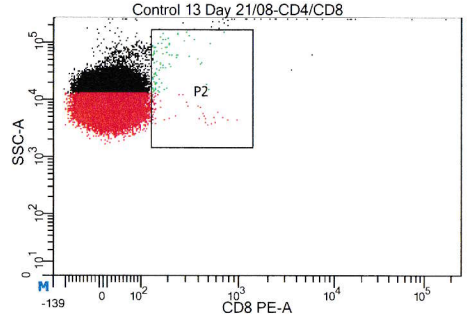
Experiment Name:	Chicken new batch
Specimen Name:	Control 13 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:48:5...
$OP:	Administrator

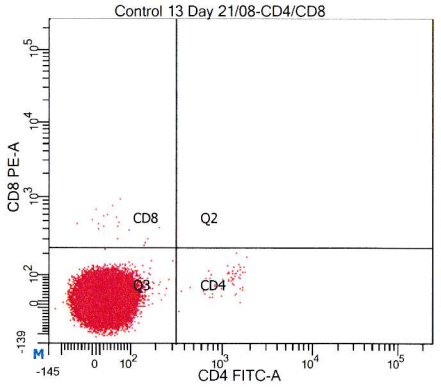
Population	#Events
LYMPHOCYTES	21
CD45	16
CD45/CD4	5
TOTAL CD45	143

Printed by: Administrator

BD FACSDiva 8.0.1



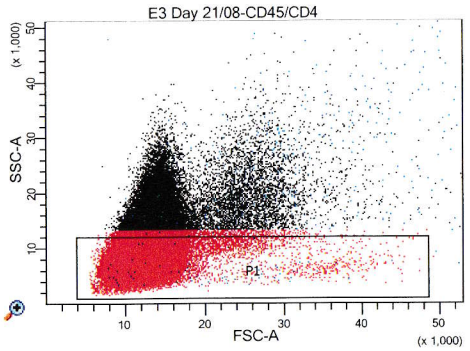


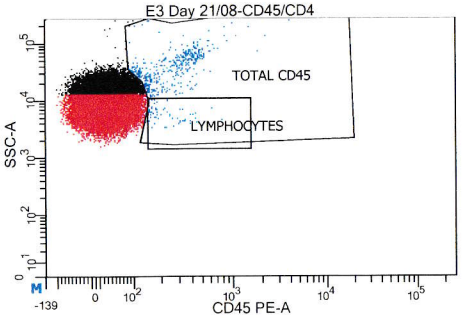


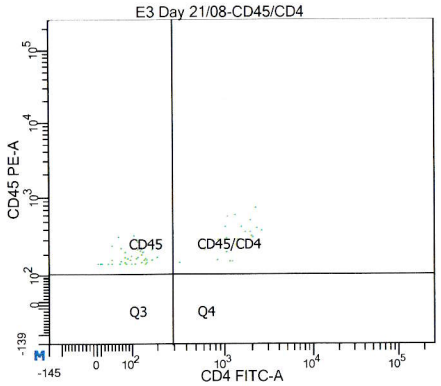
Experiment Name:	Chicken new batch
Specimen Name:	Control 13 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:50:0...
$OP:	Administrator

Population	#Events
CD8	20
CD4	58

BD FACSDiva 8.0.1





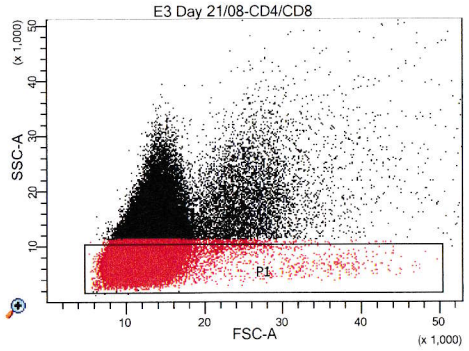


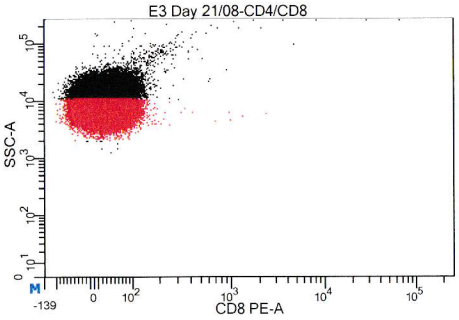
Experiment Name:	Chicken new batch
Specimen Name:	E3 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:51:0...
$OP:	Administrator

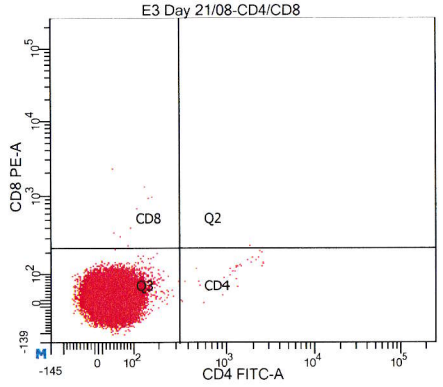
Population	#Events
LYMPHOCYTES	63
CD45	39
CD45/CD4	24
TOTAL CD45	536

Printed by: Administrator

BD FACSDiva 8.0.1



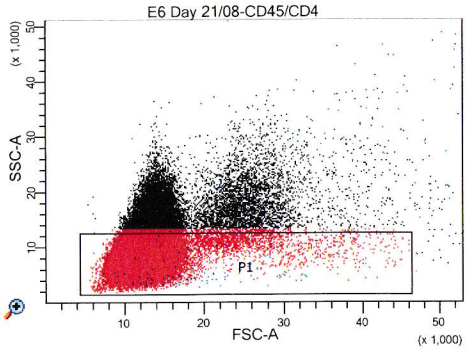


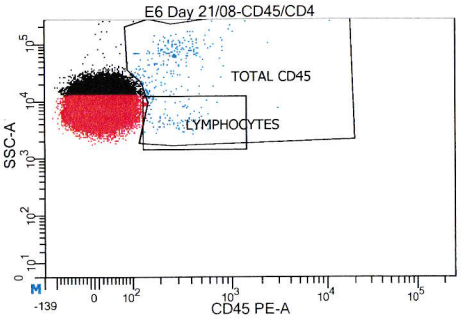


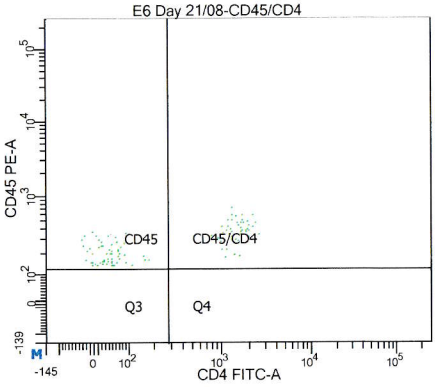
Experiment Name:	Chicken new batch
Specimen Name:	E3 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:52:...
$OP:	Administrator

Population	#Events
CD8	9
CD4	33

BD FACSDiva 8.0.1





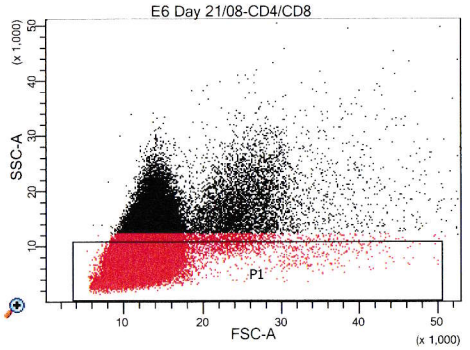


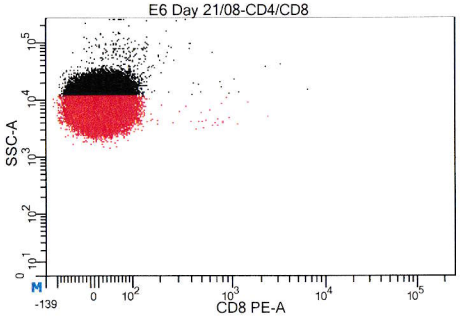
Experiment Name:	Chicken new batch
Specimen Name:	E6 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:53:1...
$OP:	Administrator

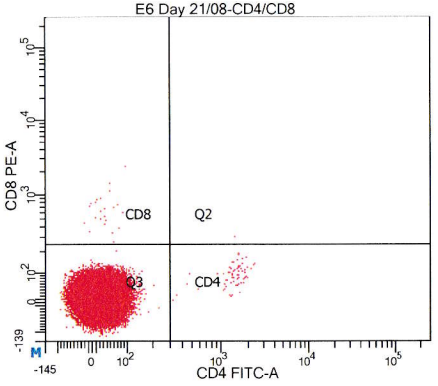
Population	#Events
LYMPHOCYTES	104
CD45	58
CD45/CD4	46
TOTAL CD45	340

Printed by: Administrator

BD FACSDiva 8.0.1



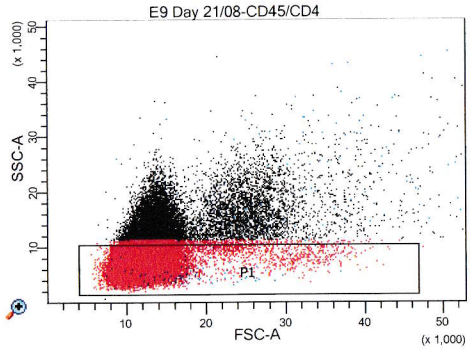


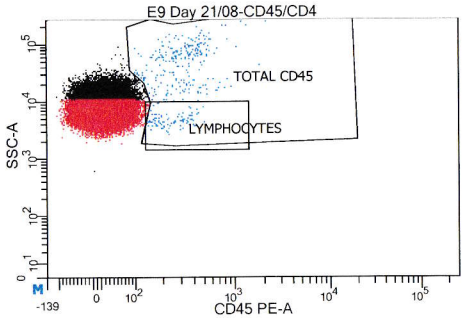


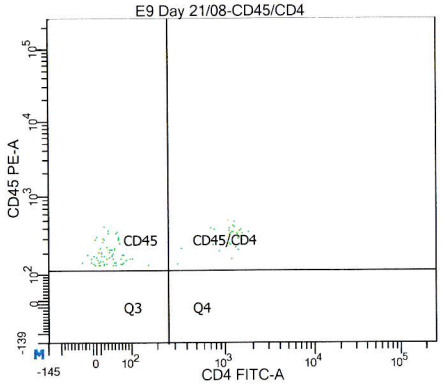
Experiment Name:	Chicken new batch
Specimen Name:	E6 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:54:1...
$OP:	Administrator

Population	#Events
CD8	23
CD4	66

BD FACSDiva 8.0.1





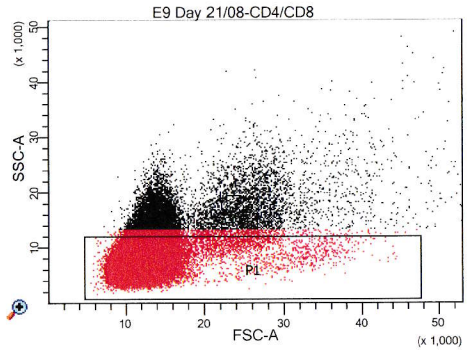


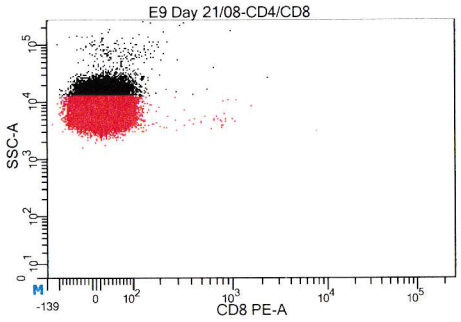
Experiment Name:	Chicken new batch
Specimen Name:	E9 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:55:...
$OP:	Administrator

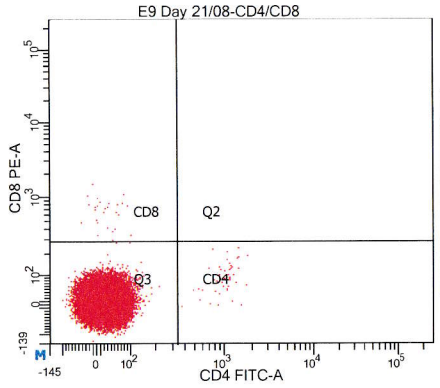
Population	#Events
LYMPHOCYTES	99
CD45	67
CD45/CD4	32
TOTAL CD45	344

Printed by: Administrator

BD FACSDiva 8.0.1



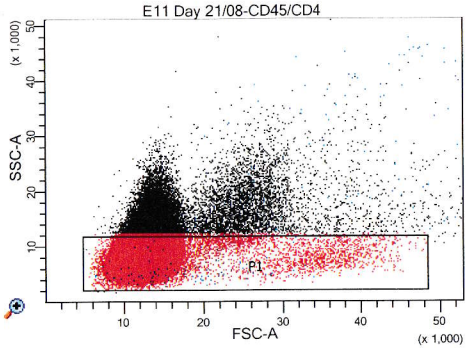


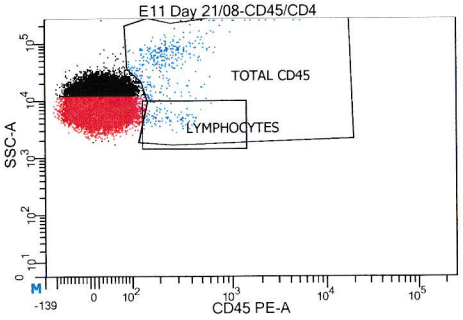


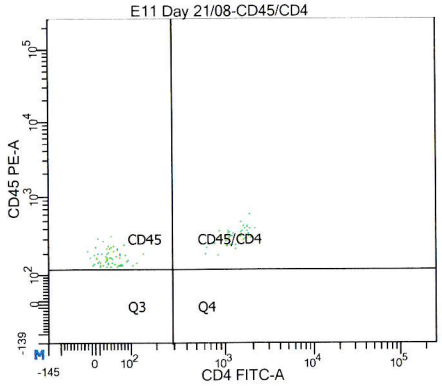
Experiment Name:	Chicken new batch
Specimen Name:	E9 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:55:4...
$OP:	Administrator

Population	#Events
CD8	28
CD4	46

BD FACSDiva 8.0.1





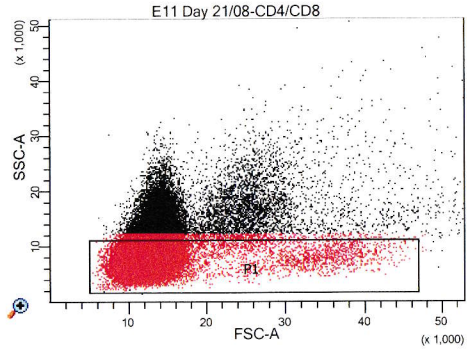


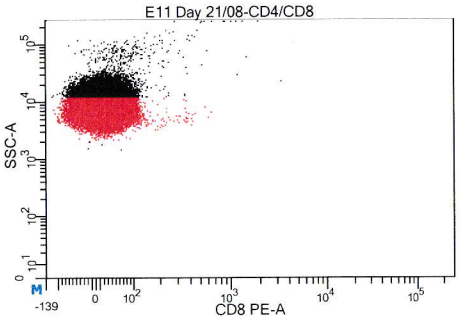
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:56:4...
$OP:	Administrator

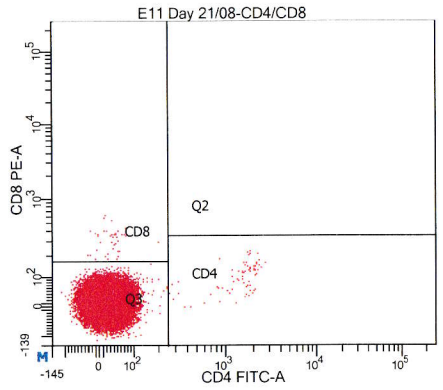
Population	#Events
LYMPHOCYTES	102
CD45	61
CD45/CD4	41
TOTAL CD45	458

Printed by: Administrator

BD FACSDiva 8.0.1



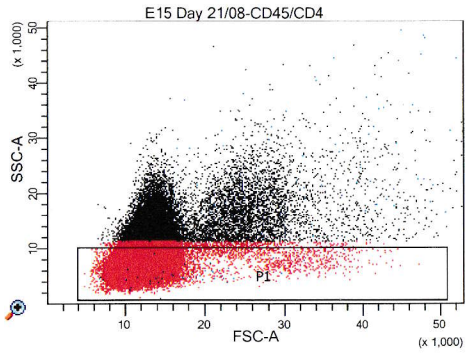


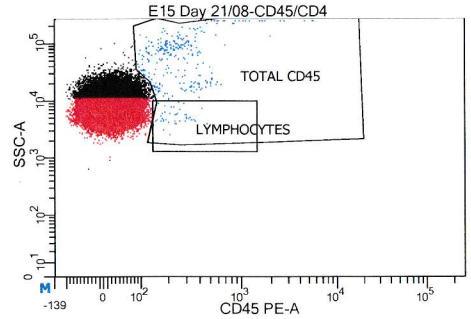


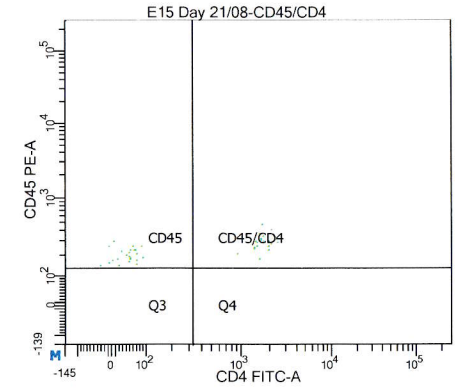
Experiment Name:	Chicken new batch
Specimen Name:	E11 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:57:4...
$OP:	Administrator

Population	#Events
CD8	40
CD4	70

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 3:58:...
$OP:	Administrator

Population	#Events
LYMPHOCYTES	44
CD45	24
CD45/CD4	20
TOTAL CD45	318

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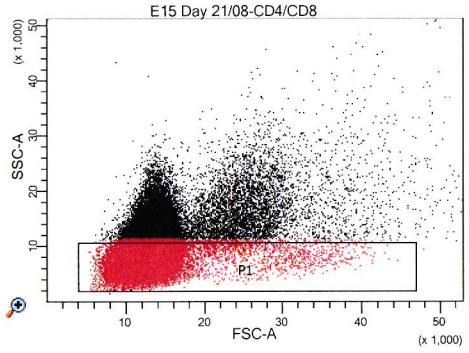
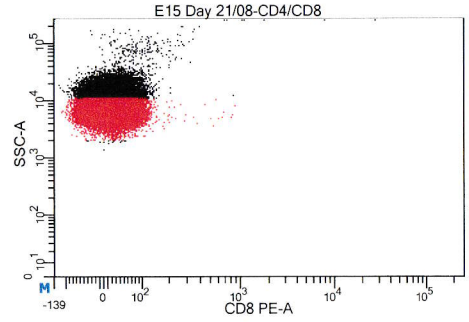
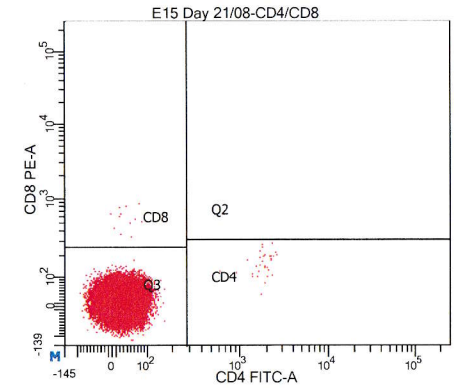
BD FACSDiva 8.0.1



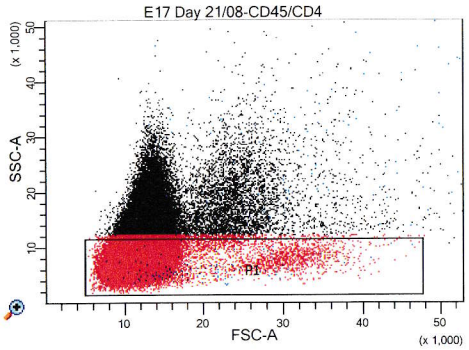


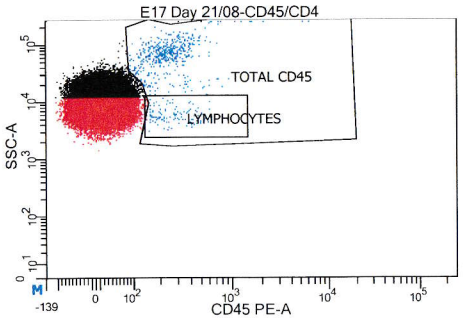


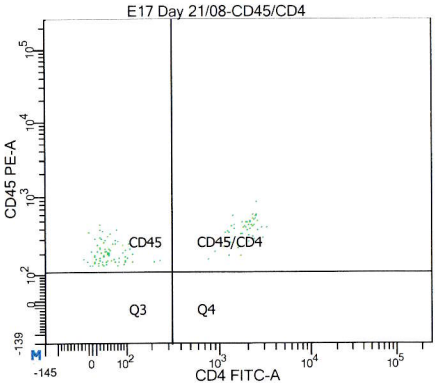
Experiment Name:	Chicken new batch
Specimen Name:	E15 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 3:59:2...
$OP:	Administrator

Population	#Events
CD8	12
CD4	32

BD FACSDiva 8.0.1



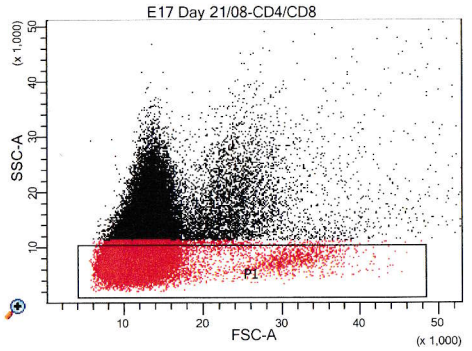


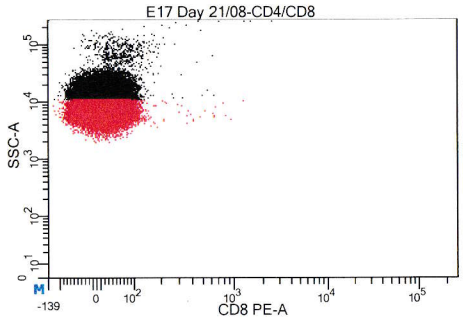


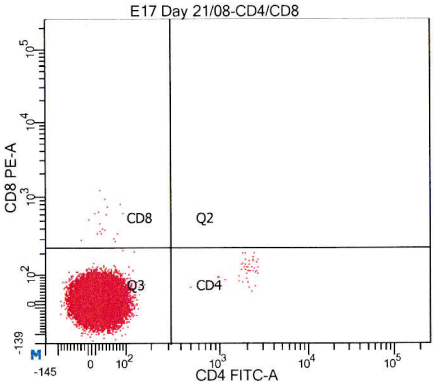
Experiment Name:	Chicken new batch
Specimen Name:	E17 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 4:00:2...
$OP:	Administrator

Population	#Events
LYMPHOCYTES	127
CD45	78
CD45/CD4	49
TOTAL CD45	628

BD FACSDiva 8.0.1



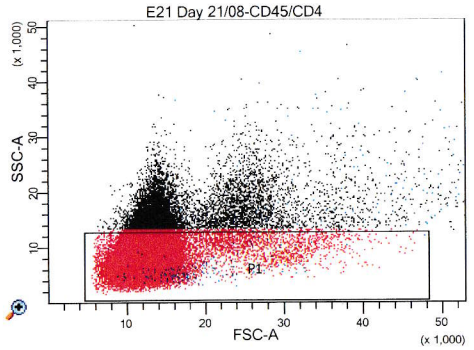


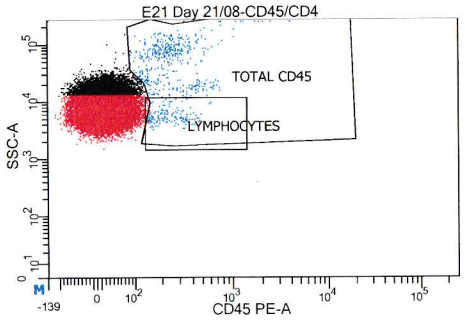


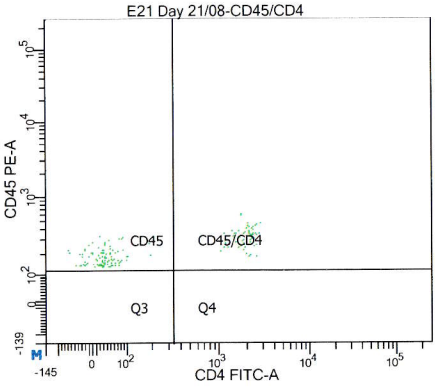
Experiment Name:	Chicken new batch
Specimen Name:	E17 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 4:01:...
$OP:	Administrator

Population	#Events
CD8	19
CD4	43

BD FACSDiva 8.0.1





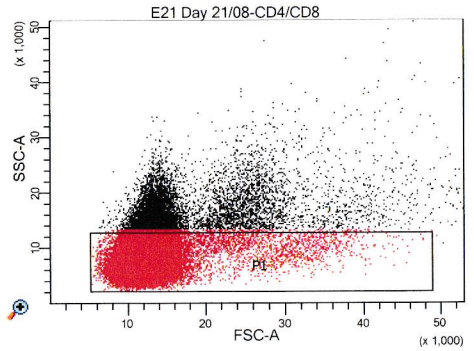


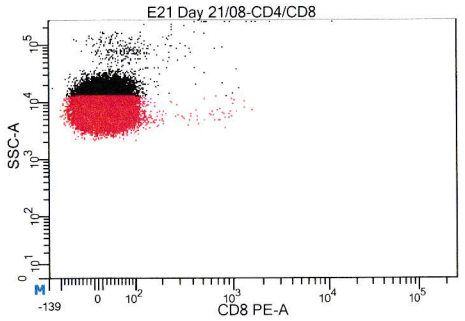
Experiment Name:	Chicken new batch
Specimen Name:	E21 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 4:02:1...
$OP:	Administrator

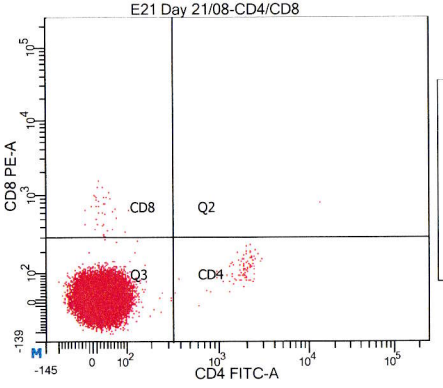
Population	#Events
LYMPHOCYTES	164
CD45	100
CD45/CD4	64
TOTAL CD45	585

Printed by: Administrator

BD FACSDiva 8.0.1



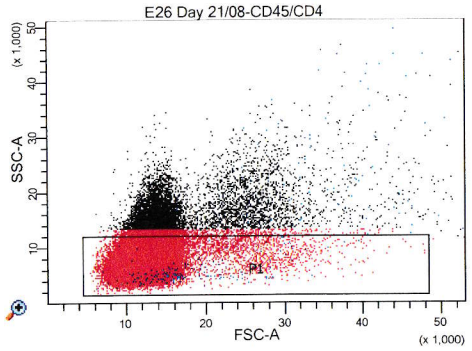


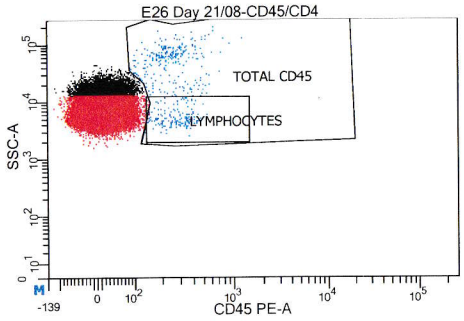


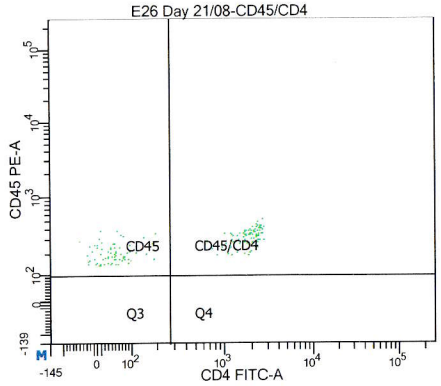
Experiment Name:	Chicken new batch
Specimen Name:	E21 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 4:03:0...
$OP:	Administrator

Population	#Events
CD8	37
CD4	87

BD FACSDiva 8.0.1





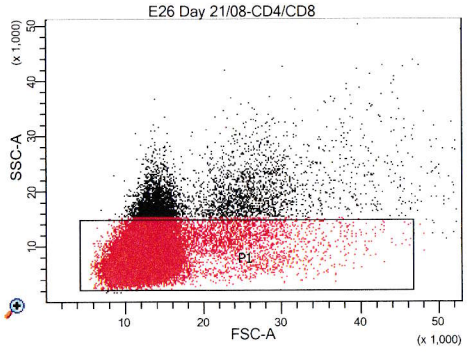


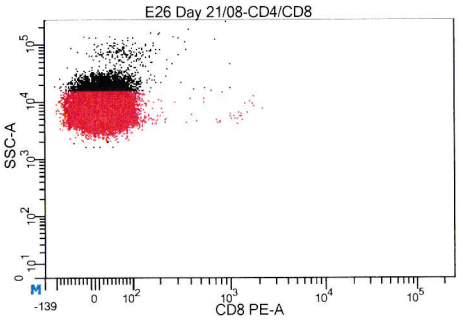
Experiment Name:	Chicken new batch
Specimen Name:	E26 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 4:03:5...
$OP:	Administrator

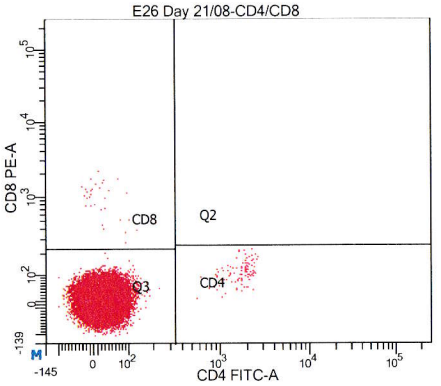
Population	#Events
LYMPHOCYTES	168
CD45	72
CD45/CD4	96
TOTAL CD45	442

Printed by: Administrator

BD FACSDiva 8.0.1



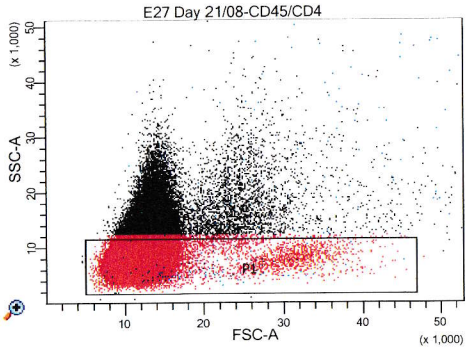


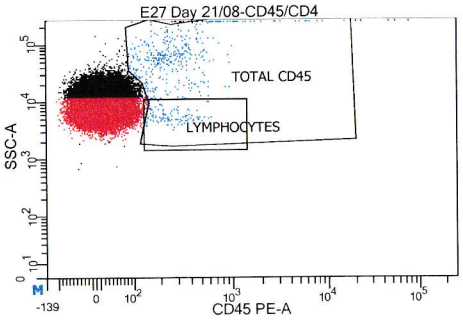


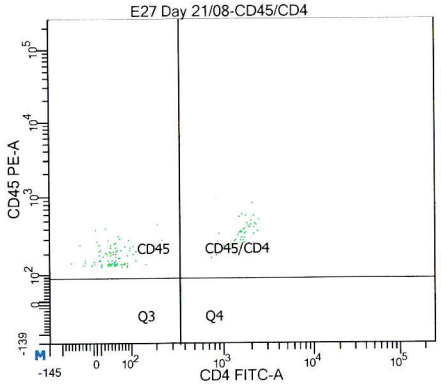
Experiment Name:	Chicken new batch
Specimen Name:	E26 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 4:04:3...
$OP:	Administrator

Population	#Events
CD8	32
CD4	102

BD FACSDiva 8.0.1





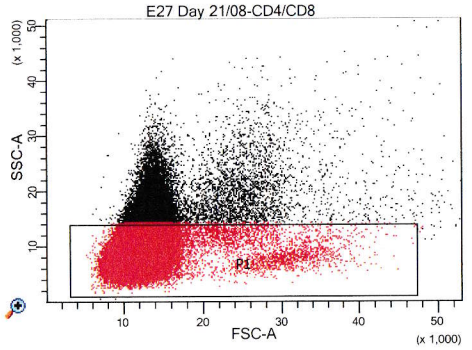


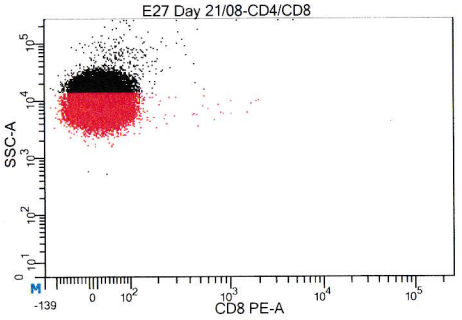
Experiment Name:	Chicken new batch
Specimen Name:	E27 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 4:05:3...
$OP:	Administrator

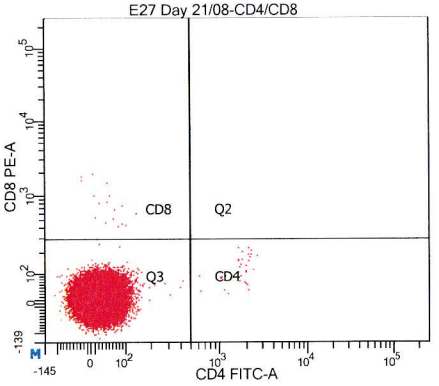
Population	#Events
LYMPHOCYTES	128
CD45	85
CD45/CD4	43
TOTAL CD45	498

Printed by: Administrator

BD FACSDiva 8.0.1



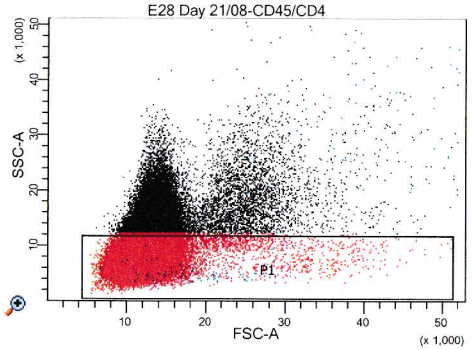


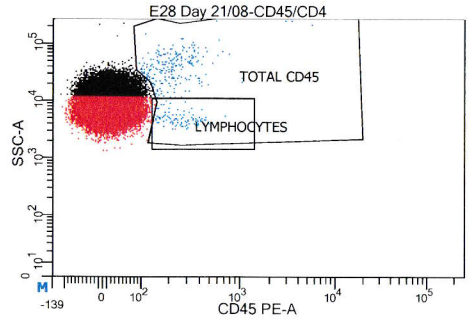


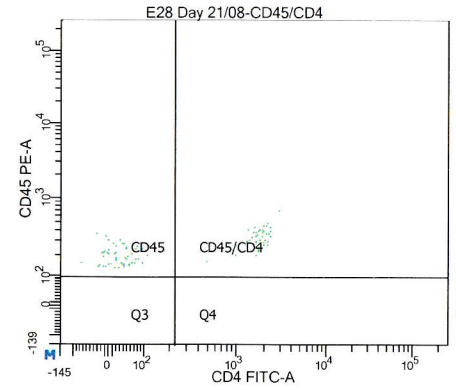
Experiment Name:	Chicken new batch
Specimen Name:	E27 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 4:06:...
$OP:	Administrator

Population	#Events
CD8	19
CD4	36

BD FACSDiva 8.0.1





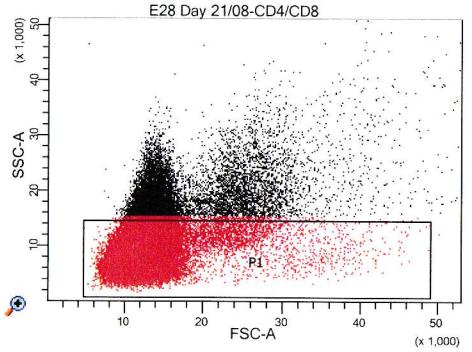


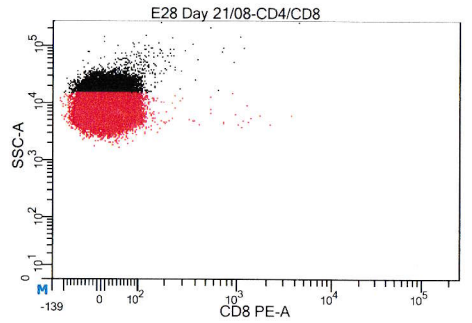
Experiment Name:	Chicken new batch
Specimen Name:	E28 Day 21/08
Tube Name:	CD45/CD4
Record Date:	Aug 22, 2017 4:07:2...
$OP:	Administrator

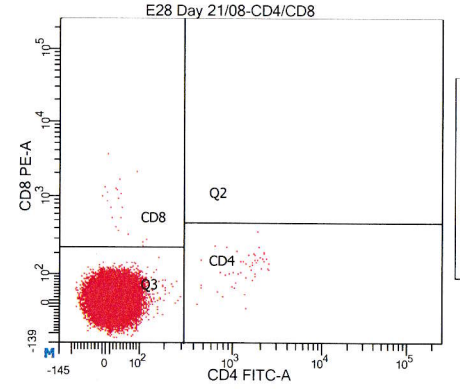
Population	#Events
LYMPHOCYTES	101
CD45	54
CD45/CD4	47
TOTAL CD45	323

Printed by: Administrator

BD FACSDiva 8.0.1







Experiment Name:	Chicken new batch
Specimen Name:	E28 Day 21/08
Tube Name:	CD4/CD8
Record Date:	Aug 22, 2017 4:08:1...
$OP:	Administrator

Population	#Events
CD8	22
CD4	49

APPENDIX C

1STC3PJR_2018-03-16.ab1 (1st Injection with *Av. paragallinarum* SA-3 Strain- 1IC)

GCCCGAGAACGTATTCACCGCAACATTCTGATTTGCGATTACTAGCGATTCCGACTTCA
TGGAGTCGAGTTGCAGACTCCAATCCGGACTTAGATGCACTTTCTGAGATTCGCTCCC
CCTCGCAGGCTCGCTTCCCTCTGTATGCACCATTGTAGCACGTGTGTAGCCCTACTCG
TAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCT
CCTTTGAGTTCCCACCCGAAGTGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCTAA
GCTCCCGAAGGCACAAACTCATCTCTGAGTTCTTCTTAGGATGTCAAGAGTAGGTAAGG
TTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA
ATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTATCACGTTAG
CTACGGGCACCAAGCCTAAAGCCCAATCCCCAAATCGACAGCGTTTACAGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACATGAGCGTCAGTAG

2NDC3PJR_2018-03-16.ab1 (2nd Injection with *Av. paragallinarum* SA-3 Strain- 2IC)

TGTACAAGGCCCGAGAACGTATTCACCGCAACATTCTGATTTGCGATTACTAGCGATTC
CGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTTAGATGCACTTTCTGAGATT
CGCTCCCCCTCGCAGGCTCGCTTCCCTCTGTATGCACCATTGTAGCACGTGTGTAGCC
CTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTG
GCAGTCTCCTTTGAGTTCCCACCCGAAGTGCTGGCAACAAAGGATAAGGGTTGCGCTC
GTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCT
GTCTCTAAGCTCCCGAAGGCACAAACTCATCTCTGAGTTCTTCTTAGGATGTCAAGAGT
AGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC
CCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAT
CACGTTAGCTACGGGCACCAAGCCTAAAGCCCAATCCCCAAATCGACAGCGTTTACAG

CGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACATGAGCGTC
AGTAGC

PCC3PJR 2018-03-16.ab1 (*Av. paragallinarum* SA-3 strain positive control- $C3^{+}$)

CAAGGCCCGAGAACGTATTCACCGCAACATTCTGATTTGCGATTACTAGCGATTCCGAC
TTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTTAGATGCACTTTCTGAGATTCGCT
CCCCCTCGCAGGCTCGCTTCCCTCTGTATGCACCATTGTAGCACGTGTGTAGCCCTAC
TCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAG
TCTCCTTTGAGTTCCCACCCGAAGTGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCT
CTAAGCTCCCGAAGGCACAAACTCATCTCTGAGTTCTTCTTAGGATGTCAAGAGTAGGT
AAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC
GTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTATCACG
TTAGCTACGGGCACCAAGCCTAAAGCCCAATCCCCAAATCGACAGCGTTTACAGCGTG
GACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACATGAGCGTCAGTAG

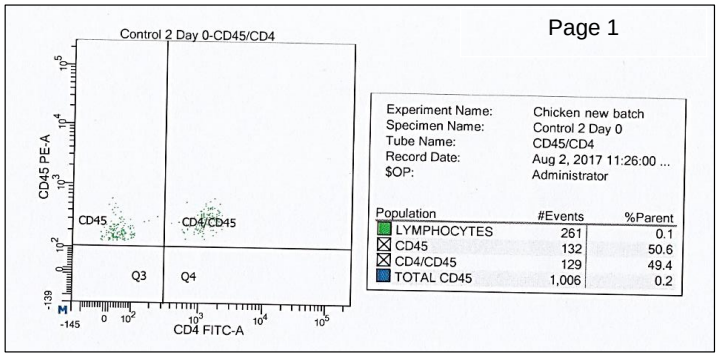
APPENDIX D

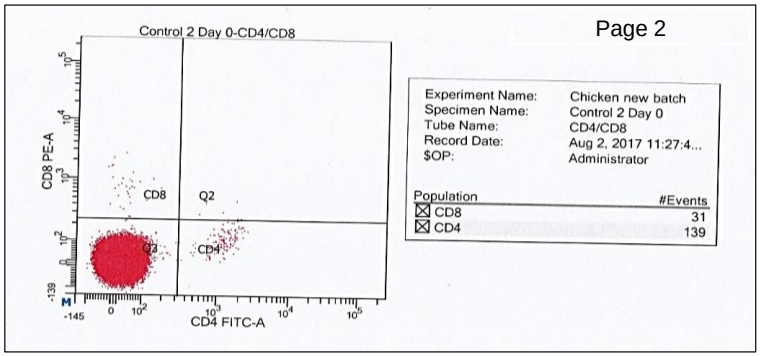
Flow cytometry ratio calculations for one of the chickens, Control 2 with score 0 on Day 0

1st Page			
Cell Populations	**Cell Counts**	**% of Total Leukocytes**	**% of Total Lymphocytes**
Total Lymphocyte count	261	25.94	-
CD8 (Cytotoxic T cells)/ B lymphocytes/NK cells	132	13.12	50.57
Estimated CD8/CD45 (Cytotoxic T cells)	29	2.86	11.02
CD4/CD45 (T helper cells)	129	12.82	49.43
Total Leukocyte Count (CD45)	1006	-	-

2nd Page		
Cell Populations	**Cell Counts**	**% of Total Lymphocytes**
CD8 (Cytotoxic T lymphocytes)	31	11.02
CD4 (T helper lymphocytes)	139	49.43
Calculated Total Lymphoctye Count	281	-
Ratio of CD4:CD8	4.5	

Summary:	**% of Total Leukocytes**
CD4 (T helper lymphocytes)	12.82
CD8(Cytotoxic T cells)	2.86
T cells (CD4 and CD8)	15.68
B cells and Natural killer cells	10.26
Ratio of CD4:CD8	4.5:1





Values might be subjected to slight changes due to using Excel spreadsheet and formulas for calculations:

Fomula: T= C/%TL x 100

T= Calculated Total Lymphocyte count or Leucocyte count

C= Cell Count from table of populations/results from first or second page provided

%TL= % of Total Lymphocytes or Leucocytes

Calculations:

CD4 (T helper lymphocytes) cell count= 139 will have the same % of Total Lymphocytes of 49.34% from page 1

Estimated Total Lymphocyte count using data from page 2= 139/49.34 x 100 = 281.4 ~ 281 cells

We then use the Calculated Total Lymphocyte count to Calculate the % of Total Lymphocytes of CD8 (Cytotoxic T lymphocytes)= CD8 cell count/ estimated total lymphocyte count X 100= 31/281 x 100= 11.02%

We then calculate the Estimated CD8/ CD45 (Cytotoxic T cells) cell count for page 1= % of Total Lymphocytes of CD8 (Cytotoxic T lymphocytes)/100 x Total Lymphocyte count from page 1= 11.02/100 x 261= 28.71 ~ 29 cells

Finally, we calculate the CD8/ CD45 (Cytotoxic T cells) % of Total Leukocytes= Estimated CD8/ CD45 (Cytotoxic T cells) cell count for page 1/ Total Leukocyte count from page 1= 28.71/1006 x 100 = 2.85%

Summary (As explained)

CD4 (T helper lymphocytes) % of Total Leukocytes= CD4 cell count/ total leukocyte count X 100= 129/1006 x 100 = 12.82%

CD8 (Cytotoxic T lymphocytes) % of Total Leukocytes= CD8 cell count/ total leukocyte count X 100= 28.71/1006 x 100 = 2.85%

T cell % of Total Leukocytes= (CD4 + CD8) % of Total Leukocytes= (12.82 + 2.85)% = 15. 67%

B cells and Natural killer cells % of Total Leukocytes= Lymphocyte % of Total Leukocytes - T cell % of Total Leukocytes= (25.94 - 15.65)%= 10.29%

Ratio of CD4: CD8 count= 139 (139/31):31 (31/31) = 4.48:1 ~ 4.5:1

Day	% of Total Leukocytes- Student t-Test					
	Chicken ID	Cell Count	% of Total Leukocytes	Chicken ID	Cell count	% of Total Leukocytes
0	Chicken(Control 2)	261	25.94	Chicken(Control 2)	261	25.94
0	Chicken(Control 1)	102	100.00	Chicken(Control 1)	102	100.00
0	E3	90	30.30	E3	90	30.30
0	E4	27	11.64	E4	27	11.64
0	E5	14	8.86	E5	14	8.86
1	Chicken(Control 2)	261	25.94	E6	246	18.71
1	Chicken(Control 1)	102	100.00	E7	359	12.64
1	E3	90	30.30	E8*	37	2.34
1	E4	27	11.64	E9	151	6.46
1	E5	14	8.86	E11	281	23.42
3	Chicken(Control 2)	261	25.94	E12	42	32.06
3	Chicken(Control 1)	102	100.00	E13	212	42.23
3	E3	90	30.30	E16	90	25.00
3	E4	27	11.64	E20	272	43.52
3	E5	14	8.86	E21	387	16.77
4	Chicken(Control 2)	261	25.94	E4	200	7.92
4	Chicken(Control 1)	102	100.00	E11	241	12.13
4	E3	90	30.30	E17	221	7.66
4	E4	27	11.64	E24	147	8.98
4	E5	14	8.86	E17	221	7.66
4				E24	147	8.98
4				E25	346	15.56
7	Chicken(Control 2)	261	25.94	E15	199	42.34
7	Chicken(Control 1)	102	100.00	E16	572	34.77
7	E3	90	30.30	E22	445	28.88
7	E4	27	11.64	E24	110	30.56
7	E5	14	8.86	E26	633	16.74
7				E28	524	11.64
7				E30	447	42.09
10	Chicken(Control 2)	261	25.94	E6	282	32.27
10	Chicken(Control 1)	102	100.00	E9	208	25.43
10	E3	90	30.30	E11	321	27.7
10	E4	27	11.64	E17	202	23.63
10	E5	14	8.86	E22	155	26.14
11	Chicken (Control 1)	507	34.05	E15	453	32.97
11	Chicken (Control 2)	353	29.94	E22	465	49.21
11	Chicken (Control 3)	973	54.3	E26	636	40.54
11				E28	782	38.39
15	Chicken (Control 4)	191	34.54	E2	104	14.59
15	Chicken (Control 5)	510	38.32	E11	647	38.04
15	Chicken (Control 6)	346	29.35	E13	167	17.08
15				E19	1228	43.19
15				E21	578	29.69
18	Chicken (Control 7)	688	51.92	E4	405	17.05
18	Chicken (Control 8)	20	33.33	E11	1561	49.73
18	Chicken (Control 9)	758	28.9	E15	744	49.97
18				E19	984	55.69
18				E27	714	46.09
21	Chicken (Control 10)	17	16.5	E3	63	11.75
21	Chicken (Control 11)	845	24.34	E6	104	30.59
21	Chicken (Control 12)	216	13.7	E9	99	28.78
21	Chicken (Control 12)	21	14.69	E11	102	22.27
21				E15	44	13.84
21				E17	127	20.22
21				E21	164	28.03
21				E26	168	38.01
21				E27	128	25.7
21				E28	101	31.27

Null hypothesis:

H_0: There is no significant difference between the means of the experimental and control chicken groups ($\mu_d=0$)

H_A: There is a significant difference between the means of the experimental and control chicken groups ($\mu_d \neq 0$)

% of Total Leukocytes

Day 0

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	35.348	35.348
Variance	1389.38932	1389.38932
Observations	5	5
Hypothesized Mean Difference	0	
df	8	
t Stat	0	
P(T<=t) one-tail	0.5	
t Critical one-tail	1.85954803	
P(T<=t) two-tail	1	
t Critical two-tail	2.306004135	
However:	p=1.000	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 1

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	35.348	12.714
Variance	1389.38932	74.32708
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	1.322872061	
P(T<=t) one-tail	0.128218026	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.256436053	
t Critical two-tail	2.776445105	
However:	p=0.256	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 3

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	35.348	31.916
Variance	1389.38932	129.57113
Observations	5	5
Hypothesized Mean Difference	0	
df	5	
t Stat	0.196906045	
P(T<=t) one-tail	0.425827421	
t Critical one-tail	2.01504837 3	
P(T<=t) two-tail	0.851654842	
t Critical two-tail	2.570581836	
However:	p=0.852	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 4

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	35.348	9.841428571
Variance	1389.38932	8.772147619
Observations	5	7
Hypothesized Mean Difference	0	
df	4	
t Stat	1.52668004	
P(T<=t) one-tail	0.100773566	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.201547132	
t Critical two-tail	2.776445105	
However:	p=0.202	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 7

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	35.348	29.57428571
Variance	1389.38932	139.0688619
Observations	5	7
Hypothesized Mean Difference	0	
df	5	
t Stat	0.334605568	
P(T<=t) one-tail	0.375751268	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.751502537	
t Critical two-tail	2.570581836	
However:	p=0.752	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 10

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	35.348	27.034
Variance	1389.38932	10.70463
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	0.496839862	
P(T<=t) one-tail	0.322684319	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.645368638	
t Critical two-tail	2.776445105	
However:	p=0.645	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 11

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	39.43	40.2775
Variance	170.0607	45.60689167
Observations	3	4
Hypothesized Mean Difference	0	
df	3	
t Stat	-0.102707565	
P(T<=t) one-tail	0.462337783	
t Critical one-tail	2.353363435	
P(T<=t) two-tail	0.924675566	
t Critical two-tail	3.182446305	
However:	p=0.925	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 15

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	34.07	28.518
Variance	20.2809	158.03167
Observations	3	5
Hypothesized Mean Difference	0	
df	5	
t Stat	0.89633965	
P(T<=t) one-tail	0.205572708	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.411145416	
t Critical two-tail	2.570581836	
However:	p=0.411	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 18

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	38.05	43.706
Variance	149.1889	233.84208
Observations	3	5
Hypothesized Mean Difference	0	
df	5	
t Stat	-0.57577146	
P(T<=t) one-tail	0.294856307	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.589712614	
t Critical two-tail	2.570581836	
However:	p=0.590	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day 21

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	Variable 2
Mean	17.3075	25.046
Variance	23.32449167	66.01873778
Observations	4	10
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.194668235	
P(T<=t) one-tail	0.026458188	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.052916375	
t Critical two-tail	2.228138852	
However:	p=0.053	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day	CD8 Cytotoxic T cell Results- Student t-Test					
	Chicken ID	Cell Count	% of Total Leukocytes	Chicken ID	Cell count	% of Total Leukocytes
0	Chicken(Control 2)	29	2.86	Chicken(Control 2)	29	2.86
0	Chicken(Control 1)	23	22.13	Chicken(Control 1)	23	22.13
0	E3	0	0.00	E3	0	0.00
0	E4	11	4.90	E4	11	4.90
0	E5	2	1.27	E5	2	1.27
1	Chicken(Control 2)	29	2.86	E6	48	3.62
1	Chicken(Control 1)	23	22.13	E7	49	1.74
1	E3	0	0.00	E8*	19	1.20
1	E4	11	4.90	E9	23	1.00
1	E5	2	1.27	E11	83	6.95
3	Chicken(Control 2)	29	2.86	E12	9	7.05
3	Chicken(Control 1)	23	22.13	E13	84	16.79
3	E3	0	0.00	E16	26	7.12
3	E4	11	4.90	E20	48	7.66
3	E5	2	1.27	E21	37	1.59
4	Chicken(Control 2)	29	2.86	E4	36	1.43
4	Chicken(Control 1)	23	22.13	E11	29	1.48
4	E3	0	0.00	E17	67	2.31
4	E4	11	4.90	E24	31	1.91
4	E5	2	1.27	E17	67	2.31
4				E24	31	1.91
4				E25	17	0.78
7	Chicken(Control 2)	29	2.86	E15	26	5.52
7	Chicken(Control 1)	23	22.13	E16	82	4.98
7	E3	0	0.00	E22	72	4.69
7	E4	11	4.90	E24	1	0.40
7	E5	2	1.27	E26	213	5.63
7				E28	129	2.89
7				E30	186	17.47
10	Chicken(Control 2)	29	2.86	E6	70	8.06
10	Chicken(Control 1)	23	22.13	E9	62	7.53
10	E3	0	0.00	E11	91	7.83
10	E4	11	4.90	E17	55	6.47
10	E5	2	1.27	E22	16	2.62
11	Chicken (Control 1)	104	6.97	E15	87	6.31
11	Chicken (Control 2)	68	5.76	E22	79	8.36
11	Chicken (Control 3)	151	8.41	E26	181	11.57
11				E26	178	8.73
15	Chicken (Control 4)	33	5.95	E2	20	2.83
15	Chicken (Control 5)	131	9.84	E11	192	11.29
15	Chicken (Control 6)	94	7.98	E13	42	4.28
15				E19	373	13.12
15				E21	85	4.35
18	Chicken (Control 7)	59	4.44	E4	107	4.51
18	Chicken (Control 8)	2	4.12	E11	304	9.67
18	Chicken (Control 9)	116	4.41	E15	89	5.96
18				E19	230	13.03
18				E27	102	6.57
21	Chicken (Control 10)	4	3.52	E3	7	1.22
21	Chicken (Control 11)	154	4.44	E6	16	4.71
21	Chicken (Control 12)	34	2.17	E9	19	5.66
21	Chicken (Control 12)	2	1.21	E11	23	5.12
21				E15	8	2.36
21				E17	22	3.45
21				E21	27	4.65
21				E26	30	6.81
21				E27	23	4.56
21				E28	21	6.53

Null hypothesis:

H_0: There is no significant difference between the means of the experimental and control chicken groups (μ_e=0)

H_A: There is a significant difference between the means of the experimental and control chicken groups (μ_e≠0)

% CD8 Cells of Total Leukocyte

t-Test: Two-Sample Assuming Unequal Variances	**Day 0**	
	Variable 1	*Variable 2*
Mean	6.230212193	6.230212193
Variance	82.31026361	82.31026361
Observations	5	5
Hvoothesized Mean Difference	0	
df	8	
t Stat	0	
P(T<=t) one-tail	0.5	
t Critical one-tail	1.859548038	
P(T<=t) two-tail	1	
t Critical two-tail	2.306004135	
However:	p=1.000	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 1**	
	Variable 1	*Variable 2*
Mean	6.230212193	2.903843087
Variance	82.31026361	6.188317129
Observations	5	5
Hvoothesized Mean Difference	0	
df	5	
t Stat	0.790655484	
P(T<=t) one-tail	0.232488373	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.464976746	
t Critical two-tail	2.570581836	
However:	p=0.465	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 3**	
	Variable 1	*Variable 2*
Mean	6.230212193	8.042268577
Variance	82.31026361	30.04620348
Observations	5	5
Hypothesized Mean Difference	0	
df	7	
t Stat	-0.382258958	
P(T<=t) one-tail	0.356805079	
t Critical one-tail	1.894578605	
P(T<=t) two-tail	0.713610158	
t Critical two-tail	2.364624252	
However:	p=0.714	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 4**	
	Variable 1	*Variable 2*
Mean	6.230212193	1.730639787
Variance	82.31026361	0.298119753
Observations	5	7
Hypothesized Mean Difference	0	
df	4	
t Stat	1.107562391	
P(T<=t) one-tail	0.165082972	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.330165943	
t Critical two-tail	2.776445105	
However:	p=0.330	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 7**	
	Variable 1	*Variable 2*
Mean	6.230212193	5.939015205
Variance	82.31026361	29.29543978
Observations	5	7
Hypothesized Mean Difference	0	
df	6	
t Stat	0.064085117	
P(T<=t) one-tail	0.475492094	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.950984188	
t Critical two-tail	2.446911851	
However:	p=0.951	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 10**	
	Variable 1	*Variable 2*
Mean	6.230212193	6.502341318
Variance	82.31026361	5.084669262
Observations	5	5
Hvoothesized Mean Difference	0	
df	4	
t Stat	-0.065090394	
P(T<=t) one-tail	0.475612623	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.951225246	
t Critical two-tail	2.776445105	
However:	p=0.951	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 11**	
	Variable 1	*Variable 2*
Mean	7.05007715	8.741107781
Variance	1.760500027	4.683449724
Observations	3	4
Hvoothesized Mean Difference	0	
df	5	
t Stat	-1.275497525	
P(T<=t) one-tail	0.129089209	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.258178418	
t Critical two-tail	2.570581836	
However:	p=0.258	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 15**	
	Variable 1	*Variable 2*
Mean	7.921592332	7.17567084
Variance	3.789244603	21.87513125
Observations	3	5
Hvoothesized Mean Difference	0	
df	6	
t Stat	0.31439658	
P(T<=t) one-tail	0.381932505	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.76386501	
t Critical two-tail	2.446911851	
However:	p=0.764	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 18**	
	Variable 1	*Variable 2*
Mean	4.322230807	7.948570958
Variance	0.032322139	11.61546819
Observations	3	5
Hvoothesized Mean Difference	0	
df	4	
t Stat	-2.373725106	
P(T<=t) one-tail	0.038254966	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.076509933	
t Critical two-tail	2.776445105	
However:	p=0.077	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances	**Day 21**	
	Variable 1	*Variable 2*
Mean	2.833224733	4.507670421
Variance	2.04228688	3.081561401
Observations	4	10
Hvoothesized Mean Difference	0	
df	7	
t Stat	-1.850551947	
P(T<=t) one-tail	0.053340327	
t Critical one-tail	1.894578605	
P(T<=t) two-tail	0.106680654	
t Critical two-tail	2.364624252	
However:	p=0.107	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day	CD4 T-Helper cell Results- Student t-Test					
	Chicken ID	Cell Count	% of Total Leukocytes	Chicken ID	Cell count	% of Total Leukocytes
0	Chicken(Control 2)	129	12.82	Chicken(Control 2)	129	12.82
0	Chicken(Control 1)	53	51.96	Chicken(Control 1)	53	51.96
0	E3	26	8.75	E3	26	8.75
0	E4	12	5.17	E4	12	5.17
0	E5	7	4.43	E5	7	4.43
1	Chicken(Control 2)	129	12.82	E6	134	10.19
1	Chicken(Control 1)	53	51.96	E7	254	8.94
1	E3	26	8.75	E8*	19	1.20
1	E4	12	5.17	E9	67	2.86
1	E5	7	4.43	E11	134	11.17
3	Chicken(Control 2)	129	12.82	E12	19	14.50
3	Chicken(Control 1)	53	51.96	E13	59	11.75
3	E3	26	8.75	E16	31	8.61
3	E4	12	5.17	E20	105	16.80
3	E5	7	4.43	E21	124	5.37
4	Chicken(Control 2)	129	12.82	E4	79	3.13
4	Chicken(Control 1)	53	51.96	E11	92	4.63
4	E3	26	8.75	E17	96	3.33
4	E4	12	5.17	E24	85	5.19
4	E5	7	4.43	E17	96	3.33
4				E24	85	5.19
4				E25	229	10.30
7	Chicken(Control 2)	129	12.82	E15	94	20.00
7	Chicken(Control 1)	53	51.96	E16	211	12.83
7	E3	26	8.75	E22	194	12.59
7	E4	12	5.17	E24	37	10.28
7	E5	7	4.43	E26	356	9.42
7				E28	312	6.96
7				E30	404	38.04
10	Chicken(Control 2)	129	12.82	E6	132	15.10
10	Chicken(Control 1)	53	51.96	E9	85	10.39
10	E3	26	8.75	E11	135	11.65
10	E4	12	5.17	E17	90	10.53
10	E5	7	4.43	E22	80	13.49
11	Chicken (Control 1)	238	15.98	E15	259	18.85
11	Chicken (Control 2)	192	16.28	E22	293	31.01
11	Chicken (Control 3)	407	22.71	E26	426	27.15
11				E28	423	20.77
15	Chicken (Control 4)	86	15.55	E2	97	13.60
15	Chicken (Control 5)	270	20.29	E11	337	19.81
15	Chicken (Control 6)	207	17.56	E13	95	9.71
15				E19	325	11.43
15				E21	195	10.02
18	Chicken (Control 7)	248	18.72	E4	229	9.64
18	Chicken (Control 8)	7	11.67	E11	533	16.98
18	Chicken (Control 9)	242	9.23	E15	308	20.69
18				E19	247	13.98
18				E27	311	20.08
21	Chicken (Control 10)	6	5.83	E3	24	4.48
21	Chicken (Control 11)	346	9.97	E6	46	13.53
21	Chicken (Control 12)	113	7.17	E9	32	9.30
21	Chicken (Control 12)	5	3.50	E11	41	8.95
21				E15	20	6.29
21				E17	49	7.80
21				E21	64	10.94
21				E26	96	21.72
21				E27	43	8.63
21				E28	47	14.55

Null hypothesis:
H_0: There is no significant difference between the means of the experimental and control chicken groups (μ_e=0)
H_A: There is a significant difference between the means of the experimental and control chicken groups ($\mu_e \neq 0$)

% CD4 Cells of Total Leukocytes

t-Test: Two-Sample Assuming Unequal Variances — **Day 0**

	Variable 1	*Variable 2*
Mean	16.62816965	16.62816965
Variance	401.2230467	401.2230467
Observations	5	5
Hypothesized Mean Difference	0	
df	8	
t Stat	0	
P(T<=t) one-tail	0.5	
t Critical one-tail	1.859548038	
P(T<=t) two-tail	1	
t Critical two-tail	2.306004135	
However:	p=1.000	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 1**

	Variable 1	*Variable 2*
Mean	16.62816965	6.873794094
Variance	401.2230467	20.48292063
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	1.062134709	
P(T<=t) one-tail	0.174023218	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.348046435	
t Critical two-tail	2.776445105	
However:	p=0.348	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 3**

	Variable 1	*Variable 2*
Mean	16.62816965	11.40810659
Variance	401.2230467	20.75629948
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	0.568218218	
P(T<=t) one-tail	0.300133048	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.600266097	
t Critical two-tail	2.776445105	
However:	p=0.600	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 4**

	Variable 1	*Variable 2*
Mean	16.62816965	5.013806908
Variance	401.2230467	6.238089061
Observations	5	7
Hypothesized Mean Difference	0	
df	4	
t Stat	1.289404609	
P(T<=t) one-tail	0.133382123	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.266764247	
t Critical two-tail	2.776445105	
However:	p=0.267	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 7**

	Variable 1	*Variable 2*
Mean	16.62816965	15.7304878
Variance	401.2230467	113.4651399
Observations	5	7
Hypothesized Mean Difference	0	
df	6	
t Stat	0.091403434	
P(T<=t) one-tail	0.465073634	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.930147268	
t Critical two-tail	2.446911851	
However:	p=0.930	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 10**

	Variable 1	*Variable 2*
Mean	16.62816965	12.23183724
Variance	401.2230467	4.116470998
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	0.488276757	
P(T<=t) one-tail	0.325455902	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.650911803	
t Critical two-tail	2.776445105	
However:	p=0.651	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 11**

	Variable 1	*Variable 2*
Mean	18.32697422	24.44306188
Variance	14.44435684	31.73320181
Observations	3	4
Hypothesized Mean Difference	0	
df	5	
t Stat	-1.712975724	
P(T<=t) one-tail	0.073695298	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.147390596	
t Critical two-tail	2.570581836	
However:	p=0.147	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 15**

	Variable 1	*Variable 2*
Mean	17.7980962	12.91541191
Variance	5.646104845	17.22468582
Observations	3	5
Hypothesized Mean Difference	0	
df	6	
t Stat	2.115526318	
P(T<=t) one-tail	0.039384442	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.078768885	
t Critical two-tail	2.446911851	
However:	p=0.079	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 18**

	Variable 1	*Variable 2*
Mean	13.2032416	16.27260453
Variance	24.29011216	20.91830185
Observations	3	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.875875978	
P(T<=t) one-tail	0.215272402	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.430544804	
t Critical two-tail	2.776445105	
However:	p=0.431	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 21**

	Variable 1	*Variable 2*
Mean	6.613172031	10.619842
Variance	7.292403933	24.45567502
Observations	4	10
Hypothesized Mean Difference	0	
df	10	
t Stat	-1.939265882	
P(T<=t) one-tail	0.040589798	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.081179597	
t Critical two-tail	2.228138852	
However:	p=0.081	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day	Ratio of CD4:CD8 Results- Student t-Test			
	Chicken ID	Cell Count	Chicken ID	Cell count
0	Chicken(Control 2)	4.48	Chicken(Control 2)	4.48
0	Chicken(Control 1)	2.35	Chicken(Control 1)	2.35
0	E3	0.00	E3	0.00
0	E4	1.06	E4	1.06
0	E5	3.50	E5	3.50
1	Chicken(Control 2)	4.48	E6	2.81
1	Chicken(Control 1)	2.35	E7	5.15
1	E3	0.00	E8*	1.00
1	E4	1.06	E9	2.86
1	E5	3.50	E11	1.61
3	Chicken(Control 2)	4.48	E12	2.06
3	Chicken(Control 1)	2.35	E13	0.70
3	E3	0.00	E16	1.21
3	E4	1.06	E20	2.19
3	E5	3.50	E21	3.39
4	Chicken(Control 2)	4.48	E4	2.19
4	Chicken(Control 1)	2.35	E11	3.14
4	E3	0.00	E17	1.44
4	E4	1.06	E24	2.72
4	E5	3.50	E17	1.44
4			E24	2.72
4			E25	13.18
7	Chicken(Control 2)	4.48	E15	3.63
7	Chicken(Control 1)	2.35	E16	2.58
7	E3	0.00	E22	2.68
7	E4	1.06	E24	26.00
7	E5	3.50	E26	1.67
7			E28	2.41
7			E30	2.18
10	Chicken(Control 2)	4.48	E6	1.87
10	Chicken(Control 1)	2.35	E9	1.38
10	E3	0.00	E11	1.49
10	E4	1.06	E17	1.63
10	E5	3.50	E22	5.15
11	Chicken (Control 1)	2.29	E15	2.99
11	Chicken (Control 2)	2.83	E22	3.71
11	Chicken (Control 3)	2.70	E26	2.35
11			E28	2.38
15	Chicken (Control 4)	2.62	E2	4.81
15	Chicken (Control 5)	2.06	E11	1.75
15	Chicken (Control 6)	2.20	E13	2.27
15			E19	0.87
15			E21	2.30
18	Chicken (Control 7)	4.21	E4	2.14
18	Chicken (Control 8)	2.83	E11	1.76
18	Chicken (Control 9)	2.09	E15	3.47
18			E19	1.07
18			E27	3.06
21	Chicken (Control 10)	1.66	E3	3.67
21	Chicken (Control 11)	2.25	E6	2.87
21	Chicken (Control 12)	3.30	E9	1.64
21	Chicken (Control 12)	2.90	E11	1.75
21			E15	2.67
21			E17	2.26
21			E21	2.35
21			E26	3.19
21			E27	1.89
21			E28	2.23

Null hypothesis:
H_0: There is no significant difference between the means of the experimental and control chicken groups (μ_d=0)
H_A: There is a significant difference between the means of the experimental and control chicken groups ($\mu_d \neq 0$)

CD4/CD8 ratio

t-Test: Two-Sample Assuming Unequal Variances — **Day 0**

	Control	*Variable 2*
Mean	2.277582274	2.277582274
Variance	3.261943097	3.261943097
Observations	5	5
Hypothesized Mean Difference	0	
df	8	
t Stat	0	
P(T<=t) one-tail	0.5	
t Critical one-tail	1.859548038	
P(T<=t) two-tail	1	
t Critical two-tail	2.306004135	
However:	p=1.000	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 1**

	Control	*Variable 2*
Mean	2.277582274	2.684207704
Variance	3.261943097	2.525788472
Observations	5	5
Hypothesized Mean Difference	0	
df	8	
t Stat	-0.377942165	
P(T<=t) one-tail	0.357652189	
t Critical one-tail	1.859548038	
P(T<=t) two-tail	0.715304379	
t Critical two-tail	2.306004135	
However:	p=0.715	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 3**

	Control	*Variable 2*
Mean	2.277582274	1.909460056
Variance	3.261943097	1.060994583
Observations	5	5
Hypothesized Mean Difference	0	
df	6	
t Stat	0.395901862	
P(T<=t) one-tail	0.352935916	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.705871831	
t Critical two-tail	2.446911851	
However:	p=0.706	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 4**

	Control	*Variable 2*
Mean	2.277582274	3.834196915
Variance	3.261943097	17.41403556
Observations	5	7
Hypothesized Mean Difference	0	
df	9	
t Stat	-0.878433357	
P(T<=t) one-tail	0.20127548	
t Critical one-tail	1.833112933	
P(T<=t) two-tail	0.40255096	
t Critical two-tail	2.262157163	
However:	p=0.403	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 7**

	Control	*Variable 2*
Mean	2.277582274	5.877862358
Variance	3.261943097	79.0808419
Observations	5	7
Hypothesized Mean Difference	0	
df	7	
t Stat	-1.041498536	
P(T<=t) one-tail	0.16613675	
t Critical one-tail	1.894578605	
P(T<=t) two-tail	0.3322735	
t Critical two-tail	2.364624252	
However:	p=0.332	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 10**

	Control	*Variable 2*
Mean	2.277582274	2.303614547
Variance	3.261943097	2.56595284
Observations	5	5
Hypothesized Mean Difference	0	
df	8	
t Stat	-0.024112442	
P(T<=t) one-tail	0.490676759	
t Critical one-tail	1.859548038	
P(T<=t) two-tail	0.981353517	
t Critical two-tail	2.306004135	
However:	p=0.981	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 11**

	Control	*Variable 2*
Mean	2.605716785	2.855964513
Variance	0.077575787	0.41194195
Observations	3	4
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.697168737	
P(T<=t) one-tail	0.262046287	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.524092574	
t Critical two-tail	2.776445105	
However:	p=0.524	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 15**

	Control	*Variable 2*
Mean	2.292443839	2.400475425
Variance	0.082982723	2.142831752
Observations	3	5
Hypothesized Mean Difference	0	
df	4	
t Stat	-0.15994112	
P(T<=t) one-tail	0.440339595	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.88067919	
t Critical two-tail	2.776445105	
However:	p=0.881	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 18**

	Control	*Variable 2*
Mean	3.047084454	2.29846139
Variance	1.159691122	0.942754544
Observations	3	5
Hypothesized Mean Difference	0	
df	4	
t Stat	0.98715648	
P(T<=t) one-tail	0.18972524	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.379450481	
t Critical two-tail	2.776445105	
However:	p=0.379	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 21**

	Control	*Variable 2*
Mean	2.524878656	2.451977451
Variance	0.522949231	0.421241119
Observations	4	10
Hypothesized Mean Difference	0	
df	5	
t Stat	0.175341884	
P(T<=t) one-tail	0.43384533	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.86769066	
t Critical two-tail	2.570581836	
However:	p=0.868	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

Day	% B and NK cells of Total Leukocytes Results- Student t-Test			
	Chicken ID	% of Total Leukocytes	Chicken ID	% of Total Leukocytes
0	Chicken(Control 2)	10.26	Chicken(Control 2)	10.26
0	Chicken(Control 1)	25.91	Chicken(Control 1)	25.91
0	E3	21.55	E3	21.55
0	E4	1.57	E4	1.57
0	E5	3.16	E5	3.16
1	Chicken(Control 2)	10.26	E6	4.89
1	Chicken(Control 1)	25.91	E7	1.96
1	E3	21.55	E8*	-0.06
1	E4	1.57	E9	2.59
1	E5	3.16	E11	5.30
3	Chicken(Control 2)	10.26	E12	10.50
3	Chicken(Control 1)	25.91	E13	13.69
3	E3	21.55	E16	9.27
3	E4	1.57	E20	19.06
3	E5	3.16	E21	9.81
4	Chicken(Control 2)	10.26	E4	3.37
4	Chicken(Control 1)	25.91	E11	6.03
4	E3	21.55	E17	2.02
4	E4	1.57	E24	1.88
4	E5	3.16	E17	2.02
4			E24	1.88
4			E25	4.48
7	Chicken(Control 2)	10.26	E15	16.82
7	Chicken(Control 1)	25.91	E16	16.97
7	E3	21.55	E22	11.60
7	E4	1.57	E24	19.88
7	E5	3.16	E26	1.69
7			E28	1.84
7			E30	-13.42
10	Chicken(Control 2)	10.26	E6	9.10
10	Chicken(Control 1)	25.91	E9	7.51
10	E3	21.55	E11	8.22
10	E4	1.57	E17	6.63
10	E5	3.16	E22	10.03
11	Chicken (Control 1)	11.09	E15	7.81
11	Chicken (Control 2)	7.89	E22	9.85
11	Chicken (Control 3)	23.17	E26	1.82
11			E28	8.89
15	Chicken (Control 4)	13.04	E2	-1.85
15	Chicken (Control 5)	8.19	E13	6.93
15	Chicken (Control 6)	3.81	E13	3.08
15			E19	18.64
15			E21	15.32
18	Chicken (Control 7)	28.77	E4	2.90
18	Chicken (Control 8)	17.55	E11	23.08
18	Chicken (Control 9)	15.26	E15	23.32
18			E19	28.68
18			E27	19.45
21	Chicken (Control 10)	7.16	E3	6.05
21	Chicken (Control 11)	9.93	E6	12.34
21	Chicken (Control 12)	4.36	E9	13.81
21	Chicken (Control 12)	9.98	E11	8.20
21			E15	5.19
21			E17	8.97
21			E21	12.44
21			E26	9.48
21			E27	12.51
21			E28	10.19

Null hypothesis:
H_0: There is no significant difference between the means of the experimental and control chicken groups (μ_e=0)
H_A: There is a significant difference between the means of the experimental and control chicken groups ($\mu_e \neq 0$)

% B and NK cells of Total Leukocytes

t-Test: Two-Sample Assuming Unequal Variances — **Day 0**

	Variable 1	*Variable 2*
Mean	12.49082912	12.49082912
Variance	118.3860105	118.3860105
Observations	5	5
Hypothesized Mean Difference	0	
df	8	
t Stat	0	
P(T<=t) one-tail	0.5	
t Critical one-tail	1.859548038	
P(T<=t) two-tail	1	
t Critical two-tail	2.306004135	
However:	p=1.000	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 1**

	Variable 1	*Variable 2*
Mean	12.49082912	2.935408133
Variance	118.3860105	4.87194586
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	1.924541793	
P(T<=t) one-tail	0.063303958	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.126607915	
t Critical two-tail	2.776445105	
However:	p=0.127	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 3**

	Variable 1	*Variable 2*
Mean	12.49082912	12.46560658
Variance	118.3860105	16.52569409
Observations	5	5
Hypothesized Mean Difference	0	
df	5	
t Stat	0.00485567	
P(T<=t) one-tail	0.498156764	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	0.996313528	
t Critical two-tail	2.570581836	
However:	p=0.996	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 4**

	Variable 1	*Variable 2*
Mean	12.49082912	3.096531323
Variance	118.3860105	2.642038323
Observations	5	7
Hypothesized Mean Difference	0	
df	4	
t Stat	1.915424011	
P(T<=t) one-tail	0.063971955	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.12794391	
t Critical two-tail	2.776445105	
However:	p=0.128	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 7**

	Variable 1	*Variable 2*
Mean	12.49082912	7.912095065
Variance	118.3860105	141.4999874
Observations	5	7
Hypothesized Mean Difference	0	
df	9	
t Stat	0.69112289	
P(T<=t) one-tail	0.253460711	
t Critical one-tail	1.833112933	
P(T<=t) two-tail	0.506921422	
t Critical two-tail	2.262157163	
However:	p=0.507	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 10**

	Variable 1	*Variable 2*
Mean	12.49082912	8.29654543
Variance	118.3860105	1.760577632
Observations	5	5
Hypothesized Mean Difference	0	
df	4	
t Stat	0.855632127	
P(T<=t) one-tail	0.220213786	
t Critical one-tail	2.13184678 6	
P(T<=t) two-tail	0.440427572	
t Critical two-tail	2.776445105	
However:	p=0.440	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 11**

	Variable 1	*Variable 2*
Mean	14.05201545	7.091066158
Variance	64.92628342	13.05003184
Observations	3	4
Hypothesized Mean Difference	0	
df	3	
t Stat	1.394853714	
P(T<=t) one-tail	0.128697657	
t Critical one-tail	2.353363435	
P(T<=t) two-tail	0.257395313	
t Critical two-tail	3.182446305	
However:	p=0.257	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 15**

	Variable 1	*Variable 2*
Mean	8.347924082	8.42529618
Variance	21.32571717	72.06294129
Observations	3	5
Hypothesized Mean Difference	0	
df	6	
t Stat	-0.01667829	
P(T<=t) one-tail	0.493617017	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.987234034	
t Critical two-tail	2.446911851	
However:	p=0.987	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 18**

	Variable 1	*Variable 2*
Mean	20.52655085	19.48484963
Variance	52.22917637	96.8178662
Observations	3	5
Hypothesized Mean Difference	0	
df	6	
t Stat	0.171781693	
P(T<=t) one-tail	0.434628271	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.869256542	
t Critical two-tail	2.446911851	
However:	p=0.869	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

t-Test: Two-Sample Assuming Unequal Variances — **Day 21**

	Variable 1	*Variable 2*
Mean	7.859758109	9.919146138
Variance	7.184933854	8.39440116
Observations	4	10
Hypothesized Mean Difference	0	
df	6	
t Stat	-1.268505552	
P(T<=t) one-tail	0.125804844	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.251609688	
t Critical two-tail	2.446911851	
However:	p=0.252	
Null Hypothesis is rejected (p>0.05)	**Data is not statistically significant**	

CHAPTER 5

POST-MORTEM EXAMINATION OF CHICKEN LYMPHOID TISSUES AFTER INFECTION WITH *Avibacterium paragallinarum* SEROVAR C-3 INFECTION

Sections of Chapter 5, have been used for manuscript for submission in a peer-reviewed journal, with the title “Omens and Remnants of Infectious Coryza: A macabre tale of necropsy and immunohistopathology of chicken lymphatic tissues after infection with *Av. paragallinarum serovar C-3* infection”.

5.1. Introduction

Infectious coryza (IC) is a contagious poultry disease with the causative agent being *Avibacterium paragallinarum* (Yamamoto, 1984). Infectious coryza affects the upper respiratory tract (URT) of chickens, however in rare cases where the disease is severe or complicated, the infection spreads to the trachea, air sacs and lungs, leading to pneumonia and air sacculitis, even in the absence of other avian pathogens (Droual *et al*. 1990; Hoerr *et al*. 1994; Deshmukh *et al*. 2015). Although, infectious coryza is a disease pertaining to the URT, there are a multitude of clinical signs and symptoms (Blackall and Soriano, 2008). The initial stage of the disease, following an infection starts off with nasal and ocular sero-mucus

secretions which transform into caseous deposits around the para-nasal regions and eyes, which is often accompanied by conjunctivitis (Deshmukh *et al*. 2015). Facial oedema, excessive secretion of tears, swelling of the sinuses, swollen-head syndrome (especially in males), lethargy, anorexia, diarrhoea, poor appetite and water consumption, difficulty in breathing, fetid odour of exudates and poor growth in younger chickens are also observed (Blackall, 1999; Pattison *et al*. 2008). In the lower air tract, rales may be heard due to infection, and lesions can be present leading to acute catarrh (Blackall and Soriano, 2008). In older layer birds, the reproductive organs such as the ovary and salpinx are affected by IC, resulting in poor egg quality and a significant drop in egg production (Deshmukh *et al*. 2015). In broiler birds, due to inanition caused by IC as a result of poor appetite and decreased water consumption, there is a drastic decline in the feed conversion efficiency, resulting in a reduction in flesh growth and extreme culling in juvenile birds (Deshmukh *et al*. 2015).

Gross pathological and histological studies have been documented and conducted on chickens infected with *Av. paragallinarum* (Fujiwara and Konno, 1965; Sawata *et al*. 1985; Droual *et al*. 1990; Hoerr *et al*. 1994; Blackall and Soriano, 2008; Paudel *et al*. 2017). A study by Fujiwara and Konno (1965), showed histopathological responses of chickens from 12 h to 3 months following intranasal inoculation with *Av. paragallinarum*. The chronology in histological changes were recorded, whereby at 20 h pathological changes were first observed, consequently by 7–10 days the severity within tissues were seen and finally tissue repairment occurred within 14–21 days (Fujiwara and Konno, 1965). Furthermore, the study revealed sloughing, tissue disintegration and hyperplasia of mucosal and glandular epithelia of the nasal cavity, infraorbital sinuses, and trachea caused by IC (Fujiwara and Konno, 1965). Additionally, in the tunica propria of the mucous membranes, hyperemia (excess of blood being transported and supplied to organs) with heterophil accumulation were also observed (Fujiwara and Konno, 1965). Furthermore, within the nasal cavity, the mucous membrane of the lamina propria showed mast cells during IC invasion, which shows that the innate immune response was at play (Sawata *et al*. 1985). In contrast, when infections in the lower respiratory

tract of birds occurred, acute catarrhal bronchopneumonia, pneumonia or air sacculitis resulted, whereby the lumen of secondary and tertiary bronchi were congested with heterophils and cell debris making it difficult for the bird to breathe (Blackall and Soriano, 2008). Moreover, epithelial cells of capillaries situated in the lungs displayed swelling and hyperplasia (Blackall and Soriano, 2008). In some birds, catarrhal inflammation of air sacs was observed with swelling, hyperplasia and heterophil infiltration (Blackall and Soriano, 2008). Lesions may also be observed, due to acute catarrhal inflammation of the upper respiratory tract (Akter *et al*. 2013). It is suspected that the immune molecules and responses of mast cells, heterophils, and macrophages may be accountable for the severe vascular alterations and cell damage leading to the clinical signs and symptoms of infectious coryza.

Necropsy is a term used to describe a post-mortem examination performed on an animal species, as opposed to autopsy which is used exclusively for human patients (King, 1989). Necropsy is a valuable tool in furthering our knowledge and providing perception for specific diseases (King, 1989). A study by Sandoval *et al*. (1994), *Av. paragallinarum* was isolated not only from the infraorbital sinuses, but also liver, kidney, lungs, eye, tarsus, heart and ovary, whereby this was the first report of the bacteria spreading to downstream organs other than the site of primary infection which is the URT. This finding suggests that *Av. paragallinarum* may be invasive and is also likely to cause septicaemia in organs, resulting in a systemic inflammatory response eventually leading to shock and perhaps even mortality in chickens (Sandoval *et al*. 1994). Akter *et al*. (2013) reported on necropsy findings following infection of chickens with *Av. paragallinarum*, whereby the mucous membranes of nasal passages and trachea showed haemorrhage. Histopathological findings by Akter *et al*. (2013) of nasal septum collected from samples, showed parakeratosis, congestion of nasal passages, hyperplasia of mucous glandular cells and hyperplasia of nasal sinuses.

Immunohistochemistry (IHC) has an important role in pathology and is a powerful diagnostic tool that uses monoclonal and polyclonal antibodies to detect and determine the distribution of specific antigens within tissues (Duraiyan *et al*. 2012). The availability of biopsies or fragments of tissue is required, which are embedded and then cut with a microtome, whereby the sections are stained and incubated with an appropriate antibody (Duraiyan *et al*. 2012). The antibody present within the tissue, can then be visualized using a light, fluorescent or electron microscope and in some cases autoradiography, using a marker such as a fluorescent dye, enzyme label, radioactive element or colloidal gold that is directly linked to an appropriate primary or secondary antibody (Coons *et al*. 1941; Coons and Kalpan, 1950; Nakane and Pierce, 1966, Faulk and Taylor, 1971; Mason and Sammons, 1978). Conventional analysis using histology techniques of hematoxylin and eosin, performed after an autopsy is necessary, however differential diagnosis cannot be performed and hence there are limitations, therefore immunohistochemistry can provide better insight (Bernardi *et al*. 2005; Roulson *et al*. 2005; de Matos *et al*. 2010). The main goal of IHC is to conduct staining of tissue sections, by causing minimal damage on the cell or tissue, and by using very small amounts of antibody, thereby allowing pathologists to make a diagnosis and prognosis in health and disease (Duraiyan *et al*. 2012). Although, IHC is the standard method for detecting proteins *in situ* on thin sections of formalin-fixed paraffin-embedded (FFPE) tissue followed by an assessment of antibody reactivity using image analysis, it is costly, time-consuming, laborious and prone to human error (Raab, 2000; Prichard, 2014; Kalyazhny, 2016; Guirado, 2018).

The aim of this study is to perform a necropsy on birds infected with infectious coryza (IC), specifically the SA-3 strain (serovar C-3), and conduct immunohistochemical staining on the tissues harvested from both healthy (control birds) and infected chickens (experimental birds) presented with score 1, score 2 and score 3. Necropsy will provide some pathological insight of how the disease and bacterial pathogen affects the tissues of the infected chickens that displayed clinical signs and symptoms. Immunohistochemistry using a selection of monoclonal

antibodies for the detection of T lymphocyte, B lymphocyte and macrophage lineages were used, to understand the distribution and function of these immune cells during the immunological response at the different stages of IC, as the disease progresses from mild (score 1) to moderate (score 2) to severe (score 3).

5.2. Materials and methods

5.2.1. Study design and ethics approval

Ethics approval was obtained as per Section 3.2.1, Chapter 3 and Section 4.2.1, Chapter 4 for conducting necropsy and immunohistochemistry (Chapter 5) as the aftermath of the research study. Following the challenge study conducted in Chapter 4 (Section 4.2.1 – Section 4.2.8) based on the method and scoring described in Section 3.2.4 (Chapter 3), SPF/unvaccinated White Leghorn chickens at 25 weeks were randomly selected from the total population of experimental (N_E= 30) and control (N_C= 8) subjects, where 2-4 chickens showing the same disease score (0, 1, 2 or 3) as well as re-infected chickens (RI) were chosen for sacrifice.

5.2.2. Necropsy, sample collection and formalin fixation

There were no mortalities recorded in the study caused by IC (Chapter 4), however morbidity was high. A total of 12 chickens (N_s=12) were selected for sacrifice from: control (Control 1, Control 2), score 1 (E1, E23), score 2 (E8, E20), score 3 (E11, E19, E22, E24) and RI (E15, E16) chickens. Chickens selected based on the disease score, were then sacrificed by decapitation. Nasal swabs were then collected from the dead chickens, by first making a small and precise incision into the sinus cavity of the heads obtained from both infected, re-infected and control birds using sterile scissors, proceeded by the insertion of a sterile cotton swab into

the sinus cavity. The heads were stored at -80°C and sinuses were dissected and stored in 500µl of RNAlater® RNA Stabilization Solution (Thermo Fisher Scientific) at -80°C for future studies.

The sacrificed chickens were then necropsied and the relevant lymphoid tissues were harvested such as spleen, liver, intestine, uterus (shell gland) and trachea (Figure 5.1). The trachea of chickens Control 1-healthy, E15 RI-score 3, E19-score 3, were swabbed with a sterile cotton swab. The liver of chicken E20 was also swabbed with a sterile cotton swab. The harvested tissues were fixed in 10% buffered formalin immediately after dissection and stored at 4°C or room temperature until further use. Tissues need to be fixed for 6-72 h in 10% buffered formalin prior to processing (Wolff *et al*. 2013). Fixation is crucial as it allows for thin sectioning of tissue by hardening of the tissue, it prevents autolysis of cells and infectious agents and improves cell avidity for efficacious dissection, processing and microscopic examination of histopathology specimens (Grizzle, 2009).

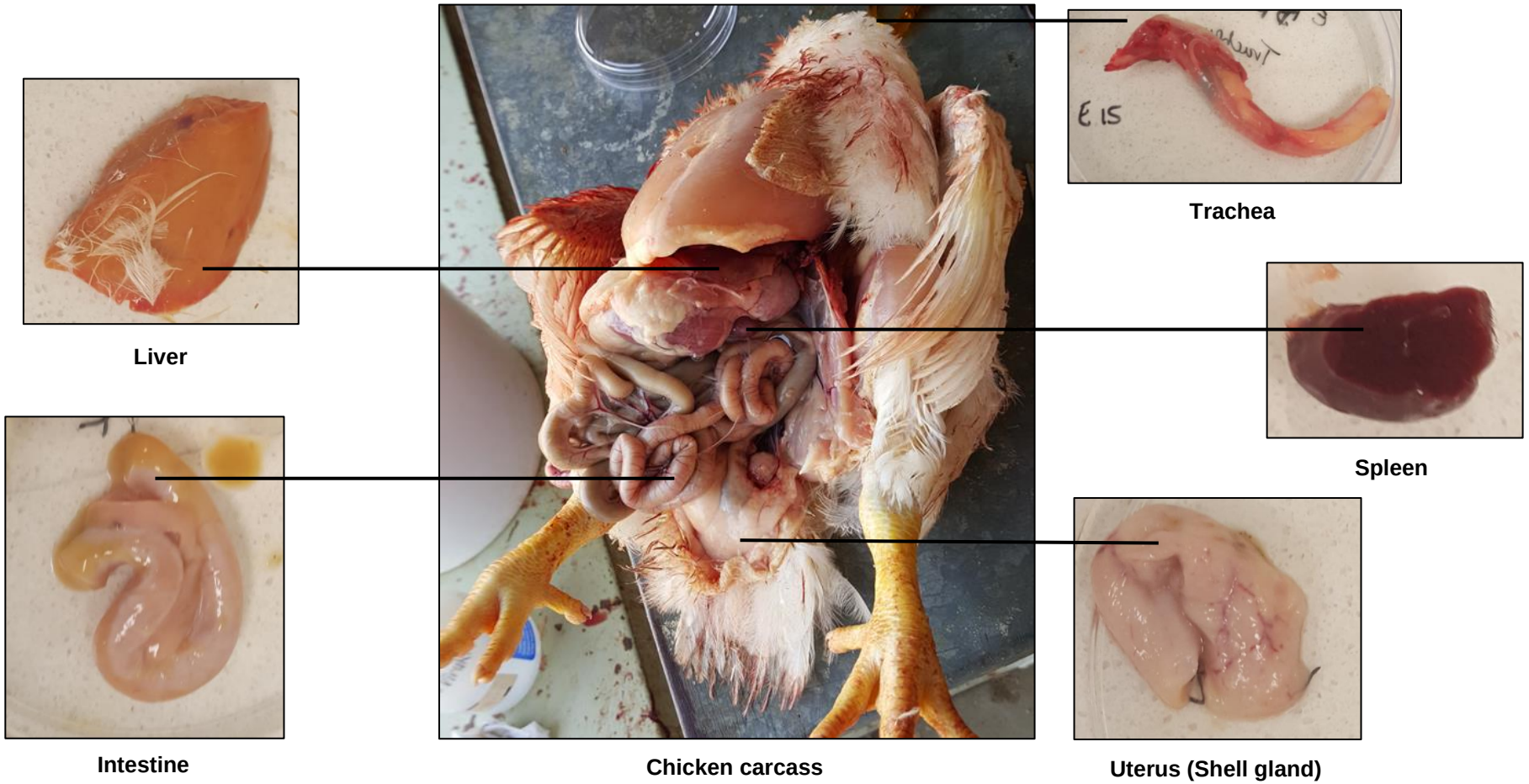


Figure 5.1: Necropsy and gross post-mortem examination was conducted on a chicken carcass. The lymphoid tissues such as the liver, intestine, trachea, uterus (shell gland) and spleen were removed, harvested and fixed in 10% buffered formalin and stored at 4°C or room temperature until further use.

5.2.3. Microbial cultivation, isolation and identification

To investigate which microorganism was the main causative agent of disease and also to prove Koch's postulates, re-isolation of the pathogen from the site of infection and from other suspected areas was attempted (Falkow, 1988). Cotton swabs collected from the nasal cavity, trachea and liver of chickens mentioned in Section 5.2.2 (Chapter 5) were then spread over the surface of blood tryptose agar (BTA) plates containing cattle blood (Onderstepoort Biological Products, Pretoria) and cross-streaked with *Staphylococcus epidermidis* for cultivation as well as TSA plates (supplemented with 0.2% (v/v) NAD^+), as described in Section 3.2.3.1 (Chapter 3) and Section 4.2.3 (Chapter 4). BTA plates were cultured at 37°C for 24 h in a candle jar (due to microaerophilic nature of *Av. paragallinarum*) and TSA plates supplemented with 0.2% NAD^+ (v/v) were incubated at 37°C for 24 h since it is a non-selective media and a variety of micro-organisms obtained from the nasal swabs wanted to be grown. Single colonies of suspected *Av. paragallinarum* displaying satellitic behaviour near the *S. epidermidis* "feeder cultures", were plated onto fresh BTA plates and cross-streaked with *S. epidermidis* and were once again placed in a candle jar and incubated at 37°C for 24 h. The bacterial culture was passaged every 2 days on BTA plates to keep the bacterial culture viable.

A pre-inoculum was prepared from the samples collected as described in Section 3.2.3.1. (Chapter 3), containing a bacterial culture of less than 24 hours of age, which was then inoculated into a 50 ml tube containing 50 ml of TSB supplemented with 0.04% NAD^+ (v/v) and incubated for a further 10-14 h at 37°C (Labwit Scientific), grown to an optical density (OD_{600}) of 1.0. The bacterial culture was then centrifuged at 3000 x *g* for 10 min, to obtain a pellet which was re-suspended in 10 ml of 1X phosphate-buffered saline (PBS) (pH 7.4, Merck) which was kept at 4°C until further use.

A volume of 1-3 ml of the bacterial culture was used for genomic DNA extraction as described in Section 3.2.3.2 (Chapter 3), a species-specific PCR was then performed following the extraction of genomic DNA as described in Section 3.2.3.3 (Chapter 3) and finally agarose gel electrophoresis and visualisation of the correct DNA fragment size was conducted as per Section 3.2.3.4 (Chapter 3).

5.2.4. Immunohistochemistry (IHC) of samples collected

5.2.4.1. Anatomical grossing, tissue processing and paraffin wax impregnation

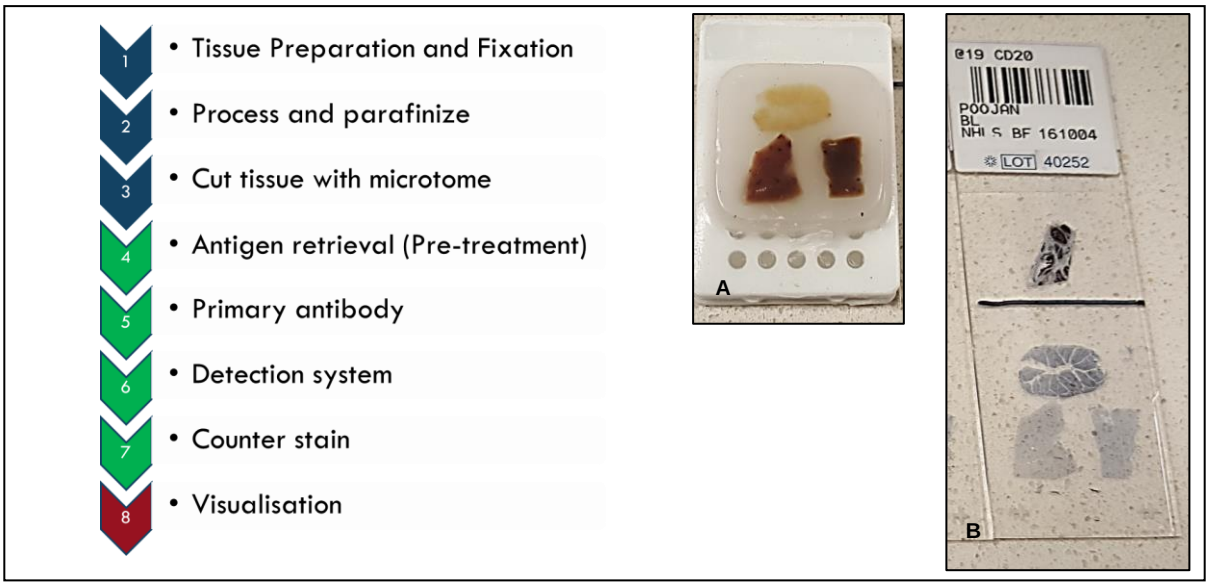


Figure 5.2: Summarized flow diagram of steps followed for IHC staining. (A) Lymphoid tissue fragments embedded in paraffin wax. (B) IHC slide containing sections of FFPE tissues stained with anti-CD20 monoclonal antibody.

The lymphoid tissues fixed in 10% buffered formalin were sent to and conducted at the National Health Laboratory Service (NHLS, Department of Histopathology, Universitas,

Bloemfontein, South Africa) to obtain FFPE tissues stained with monoclonal antibodies such as such as anti-CD3 (T lymphocytes), anti-CD20 (B lymphocytes) and anti-CD68 (macrophages) to detect and determine the distribution and expression of immune cells within tissues. Haematoxylin and eosin staining of lymphoid tissues were also conducted, however the results were not included in this study. The Department of Histopathology (NHLS, Universitas, Bloemfontein, South Africa) is an accredited laboratory by the South African National Accreditation System (SANA), whereby calibration, quality assurance and controls and standard operating procedures (SOPs) are followed and used on a routine basis. A summarized protocol of the different steps and procedures used in immunohistochemical staining is shown (Figure 5.2).

Table 5.1: Reagents and conditions used for processing of tissue samples with Tissue-Tek® VIP5 automated processor (Sakura Finetek) as per the SOP of the NHLS.

Steps	Reagent	Conditions	Time
1	Formalin	10%	2 h
2	Ethanol	50%	15 min
3	Ethanol	70%	1 h
4	Ethanol	96%	1 h
5	Ethanol	100%	30 min
6	Ethanol	100%	1 h
7	Ethanol	100%	1 h
8	Ethanol	100%	1 h
9	Xylene	100%	45 min
10	Xylene	100%	45 min
11	Paraffin Wax	62°C	30 min
12	Paraffin Wax	62°C	1 h
13	Paraffin Wax	62°C	1 h
14	Paraffin Wax	62°C	1 h

Once, at the Department of Histopthology at the NHLS, the samples were prepared for tissue processing, all tissues were processed and embedded using Tissue-Tek® (Sakura Finetek) devices and apparatus. Each tissue was trimmed using an ultra-sharp 130 mm feather blade of the Tissue-Tek® Accu-Edge® trimming knife (Sakura Finetek) for trimming the tissue so that it was no larger than 10 mm x 10 mm x 3 mm and placed into a Tissue-Tek® processing/embedding cassette (Sakura Finetek), whereby details of the specimen on the block was labelled using a pencil. We allowed for 2 samples per block to prevent overcrowding of samples on one block and for cost and time effectiveness. The tissue cassette was then completely submerged in 10% buffered formalin for 16-24 h at room temperature. The tissue cassette was then processed overnight using a Tissue-Tek® VIP5 automated processor (Sakura Finetek) to impregnate the tissues with paraffin wax, which included steps such as dehydration, clearing, and paraffin embedding, using fresh solutions other than paraffin was performed. The program used for the lymphoid tissues containing the reagents used and respective processing conditions are depicted in Table 5.1.

5.2.4.2. Paraffin embedding and tissue sectioning

Prior to embedding the tissues in paraffin wax, the tissues needed to be placed facing downwards and as flat as possible against the mould. Multiple pieces of tissue needed to be placed in the middle of the mould to ensure that adequate wax surrounded the tissue fragments, although overcrowding of the blocks should be avoided. For tubular structures such as the trachea needed to be embedded with the lumen facing downwards, to allow for a cross-section of the lumen could be obtained. Large and hard tissue fragments should be embedded at an angle. The correct orientation of the tissues needs to be conducted prior to embedding, so that there would be an even amount of wax that surrounds the section within the mould as well as to facilitate the sectioning of the tissues. There are different types of moulds, however the most commonly used are metal moulds available in an assorted range of sizes and depths.

The tissues were embedded using the Tissue-Tek® Embedding Center (Sakura Finetek). Before embedding tissues, the temperature indicators of each compartment of the console needed to be checked such as the paraffin chamber (62°C), hot plate (62°C), forceps chamber (65°C) and, the left and right thermal chambers (62°C); the paraffin volume, base mould and paraffin tray. Both cryo console (cold plate) and light key (work light) were switched on. In the first step, heated forceps were used to remove one cassette from the paraffin bath and was placed on either the left or right hot plate. The cassette cover was removed. One of the base moulds was selected from the heated chamber that would best fit the tissues in the cassette. While holding the base mould under the paraffin dispenser, the fingerplate was pressed to dispense just enough paraffin to half-fill the base mould. The base mould was then moved to the cold plate so that the base mould cooled rapidly and a thin layer of paraffin solidified. However, should the tissues not sink to the bottom of the mould, return the base mould back to the hot plate and then once the paraffin has melted use the forceps to lightly push the tissues down into a proper position onto the bottom of the base mould, the base mould should be returned to the cold plate and the procedure repeated until the tissues are properly orientated. During this process the paraffin should never completely solidify. Once the paraffin layer had cooled, a cassette was placed over properly positioned tissues in the base mould, whereby the embedded tissues adhere to the cassette. The base mould was then moved to the hot plate under the paraffin dispenser and while holding the cassette under the paraffin dispenser, the fingerplate was pressed to dispense paraffin into the base mould until the cassette was filled with paraffin, whereby care was taken to not overfill the base mould. The embedded tissues were then placed onto the cold plate. The paraffin block was then checked to ensure that it had completely solidified and was then released from the base mould and stored on the cold plate until sectioning with a microtome.

Once the tissues embedded in paraffin were ready, the universal cassette clamp and knife holder were correctly secured. The hand wheel was checked if locked and the blade was

inserted and secured. The paraffin embedded block was then placed in the block holder, whereby the block was trimmed to expose the tissue for large tissues at 10-20 µm, 5 µm for small tissues and no trimming was needed for minute biopsies. Once trimming was completed, the blocks were placed on an ice tray to cool. The microtome (Leica Biosystems) was set to 3-4 µm for cutting. Before proceeding to cut, the block was polished and then cut. The cut sections were then stretched up in a floatation bath at a temperature of 45-56°C. The sections were then picked up onto labelled frosted slides. The slides were then placed in an oven (EcoTherm Labotec) at 45-60°C for ± 20 minutes to dissolve the excess wax, for drying and to ensure the cut section adheres to the slide. The slides were then ready to be stained.

5.2.4.3. Antibody staining and microscopy

The following monoclonal antibodies of mouse origin: CONFIRM anti-CD20 (L26 clone, 760-2531) and CONFIRM anti-CD68 (KP-1 clone, 790-2931), and of rabbit origin CONFIRM anti-CD3 (2GV6 clone, 790-4341) were used for IHC staining (Ventana Medical Systems Inc.). CD20 is a non-glycosylated phosphoprotein expressed on the cell surface of all mature B lineage cells, CD3 is a pan T cell marker and CD68 is a glycosylated glycoprotein expressed in macrophage and monocyte lineages (Maloney *et al*. 1994; Dorfman *et al*. 2006; Chistiakov *et al*. 2017). Immunohistochemical studies were performed on paraffin sections using the BenchMark XT fully automated slide staining system (Ventana Medical Systems Inc.) and OptiView DAB IHC detection kit (Ventana Medical Systems Inc.) which is an indirect, biotin-free system and multimer kit based on the chromagen 3,3′-diaminobenzidine (DAB) which in the presence of peroxidase activity is oxidized forming an insoluble brown product which can be visualized for immunohistological staining. The antigen retrieval step was conducted by the BenchMark XT fully automated slide staining system using Cell Conditioning Solution (CC1) at 100°C for 30 min (Ventana Medical Systems Inc.). Anti-CD20 and anti-CD3 were used to stain all lymphoid tissues such as liver, spleen, intestine, uterus (shell gland) and trachea,

whereas anti-CD68 was used to stain the trachea for macrophages and monocytes. Tissues known to express anti-CD20, anti-CD3 and anti-CD68 of interest were used as positive controls. The antibody-stained slides were then rinsed with EZ Prep (X1) (Ventana Medical Systems Inc.) working solution. This was followed by a washing step with tap water. The slides were counterstained with Mayer's haematoxylin (for staining of the nucleus and cytoplasm) and washed in Scott's tap water substitute; a blue alkaline solution that helps with colour development of blue tissue stains (Leica Biosystems). Finally, the slides were rinsed several times with 100% ethanol until clear to dehydrate and were then coverslipped. Visualisation of the IHC slides was conducted with the Eclipse 50i microscope, DS-Fi1 digital microscope camera and NIS-Elements F 4.00.06 Build 786 microscope imaging software (Nikon), whereby images were taken at 100X magnification.

5.3. Results and discussion

5.3.1. Necropsy, sample collection and formalin fixation

Gross post-mortem examination was conducted on the dead chickens from control and experimental birds. During the dissection, a purulent green fluid in the abdominal cavity was observed most likely due to infection in one of the experimental chickens at score 1 (Figure 5.3A). Splenomegaly (enlargement of the spleen) was observed with chicken E20, attributed to an infection present in the chicken as the spleen has a role in haematopoiesis and immunity by removal of abnormal erythrocytes, clearance of pathogens/antigens and the synthesis of immunoglobulin G (IgG) (Figure 5.3B) (Chapman and Azevedo, 2018). Jaundice of the liver but not hepatomegaly, in 10 out of 12 chickens was observed which was due to high levels of bilirubin formed due to haemolysis of red blood cells (Figure 5.3C). Liver lesions were observed in chicken E20 (Figure 5.3D). The liver lesions might have been due to *Av. paragallinarum* spreading to other organs other than the upper respiratory tract, as reported

(Figure 5.3D) (Sandoval *et al*. 1994). Additionally, liver lesions may have been caused by the systemic effect caused by the immune response due to IC infection. The liver is the main organ of detoxification; and LPS (lipopolysaccharide) present in the outer membrane of Gram-negative bacteria *Av. paragallinarum* is an endotoxin, hence liver disease may develop as a result of IC, causing jaundice and in some cases liver lesions. There was mild to moderate inflammation and mucosal secretions in the nasal passages of score 2 and 3 chickens. Moreover, in the nasal passage of chicken E15 RI with score 3, there was haemorrhage (Figure 5.4A). The trachea of E15 RI (Figure 5.4C) and E19, were congested with mucous and also displayed haemorrhage. The trachea of the control chicken was not congested with mucous, nor was there haemorrhage (Figure 5.4 B).

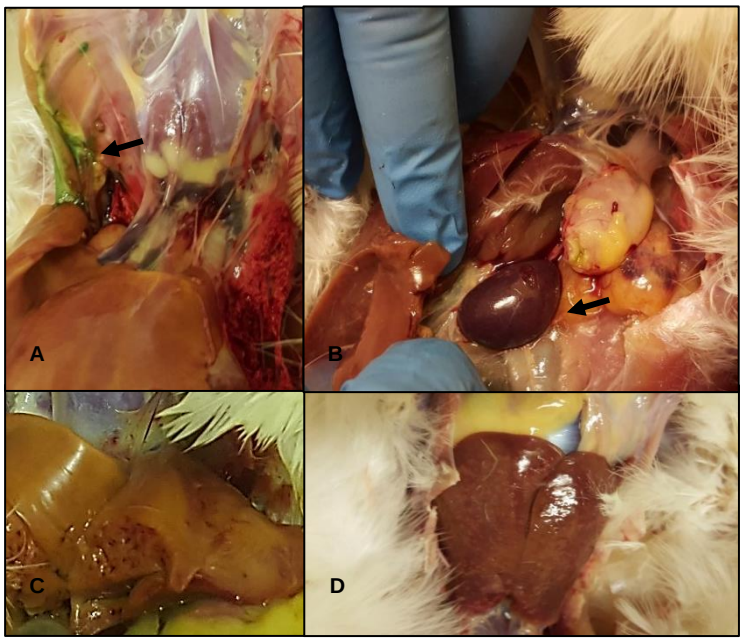


Figure 5.3: Following the dissection, post-mortem examination of internal lymphoid organs. (A) A purulent green fluid in the abdominal cavity was observed most likely due to infection. Unfortunately, samples swabs were not taken of the fluid. (B) Splenomegaly was observed with chicken E20, attributed to an infection present in the chicken. (C) Liver lesions were observed in chicken E20, due to *Av. paragallinarum* spreading to other organs other than the upper respiratory tract. (D) Jaundice of the liver but not hepatomegaly, in 10 out of 12 chickens was observed which was due to liver disease .

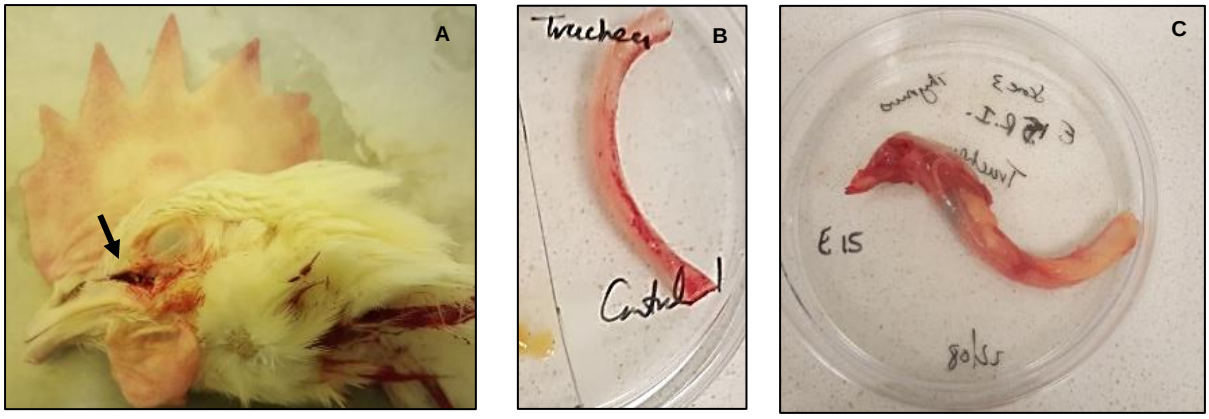


Figure 5.4: Necropsy of trachea. (A) The nasal passage of chicken E15 RI with score 3, there was haemorrhage. (B) The trachea of Control 1 was clear without congestion with mucous. (C) The trachea of E15 RI were congested with mucous and also displayed haemorrhage.

5.3.2. Microbial cultivation and identification

The present investigation was carried out for the isolation and identification of *Av. paragallinarum*, the causative agent of IC, from layer chickens by cultural and morphological examinations as well as molecular techniques. For this study, a total of 12 nasal swab samples were collected from the sinus cavity of dead birds (N_s= 12). None of the swab samples cultured on TSA plates supplemented with 0.2% NAD^+ (v/v) displayed any growth (Figure 5.5). The colony characteristics of *Av. paragallinarum* observed on the BTA plate containing *S. epidermidis* were seen as tiny dewdrops, mucoid, smooth iridescent colonies with no haemolysis, similar to the findings of other authors (Blackall, 1989; Page *et al.* 1963) (Figure 5.6). It was also observed that as the disease score progressed from score 1 to score 2 to score 3, the microbial growth from the nasal exudates on the BTA plates increased and as such it became more difficult to re-isolate *Av. paragallinarum*, due to the chickens being

immunocompromised during the infection (Figure 5.6). However, it was possible that these microorganisms present in the sinus cavity are commensals, and thus have a role to play in the immunity of the host and can induce T cell regulatory responses similar to the gut which also has a mucosal environment (Belkaid and Hand, 2014.). Unfortunately, we could only successfully culture and re-isolate *Av. paragallinarum* from 6 out of 12 nasal swab samples from the chickens (E1, E8, E20, E22, E23, E24), as a result of the cultures not surviving too long following the transfer from the host environment and also due to the bacteria not being able to survive or adapt after passaging. Nasal swabs from chicken Control 2 did not display any growth on the BTA plate and Control 1 did display growth, however the microorganism did not have any morphological characteristics similar to *Av. paragallinarum* (Figure 5.6). Nonetheless, DNA extraction was conducted on Control 1 just to ensure the microorganism did not mask *Av. paragallinarum* on the BTA plate.

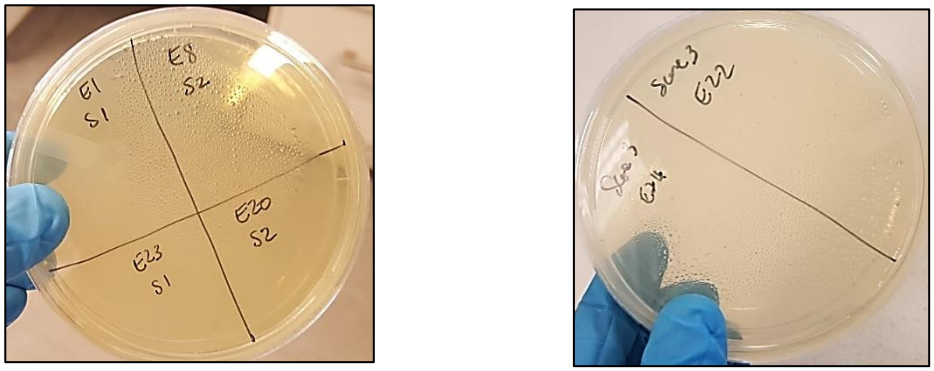


Figure 5.5: No growth observed on TSA supplemented with 0.2% NAD^+ (v/v) plates for nasal swabs obtained from chickens E1-score 1, E23-score 1, E8-score 2, E20-score 2, E22-score 3 and E24-score 3.

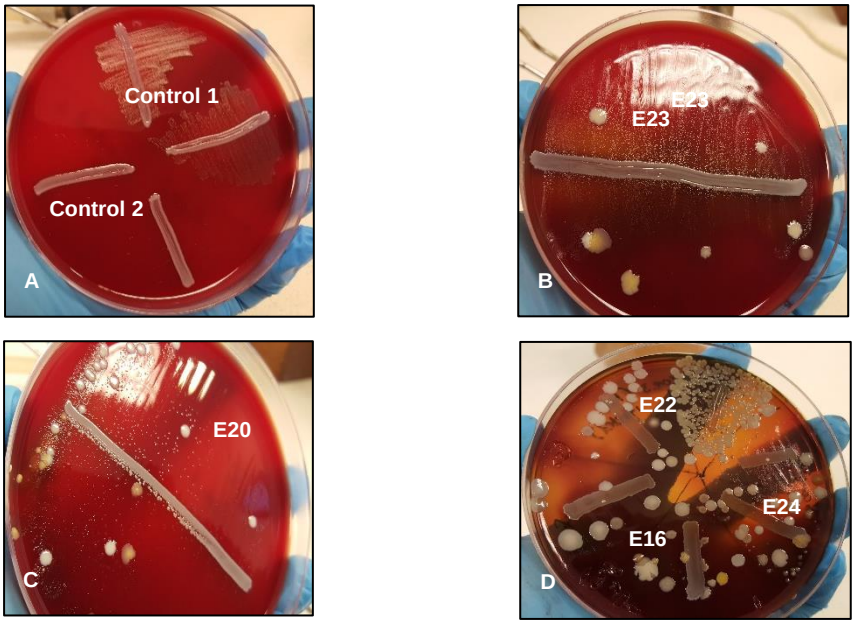


Figure 5.6: Nasal swab samples cultured on BTA plates. (A) Control chickens at score 0: Control 1 showing growth not pertaining to *Av. paragallinarum*. No growth was seen for Control 2. (B) E23 at score 1 showing growth of *Av. paragallinarum* seen as tiny dew drop colonies with no haemolysis. (C) E20 at score 2 showing a mixed culture of *Av. paragallinarum* and other microorganisms which appear to be haemolytic, colonies of *Av. paragallinarum* were still clearly visible. (D) Cultures of 48 h of E22, E24 and E16 at score 3 showing an overgrowth of microorganisms which appear to be haemolytic, we could not isolate *Av. paragallinarum* from E16 even at 24 h growth, but were successful with E22 and E24.

The liver of chicken E20 which was also swabbed with a sterile cotton swab, showed no growth on BTA or TSA supplemented with 0.2% NAD^+ (v/v) plates (Figure 5.7). The tracheal swabs of chickens (Control 1, E15 RI and E19) were cultured on BTA plates and TSA supplemented with 0.2% NAD^+ (v/v) plates. None of the tracheal swabs cultured on TSA supplemented with 0.2% NAD^+ (v/v) plates had any growth (Figure 5.8A). *Av. paragallinarum* was not found in the tracheal swab of the control chicken, as there was no growth on the BTA plate, implying that no cross contamination occurred between the control and experimental groups during the challenge study, as IC is contagious and spreads across cages (Figure 5.8B). The tracheal swabs of both chicken E15 RI (Figure 5.8C) and E19 (both at score 3) were cultured and *Av. paragallinarum* was successfully isolated.

Figure 5.7: Liver swabs of chicken E20 showed that there was no growth observed on (A) BTA or (B) TSA supplemented with 0.2% NAD+ (v/v) plates. Indicating that the lesions could be caused as a result of a systemic reaction caused by the avian immune response to IC.

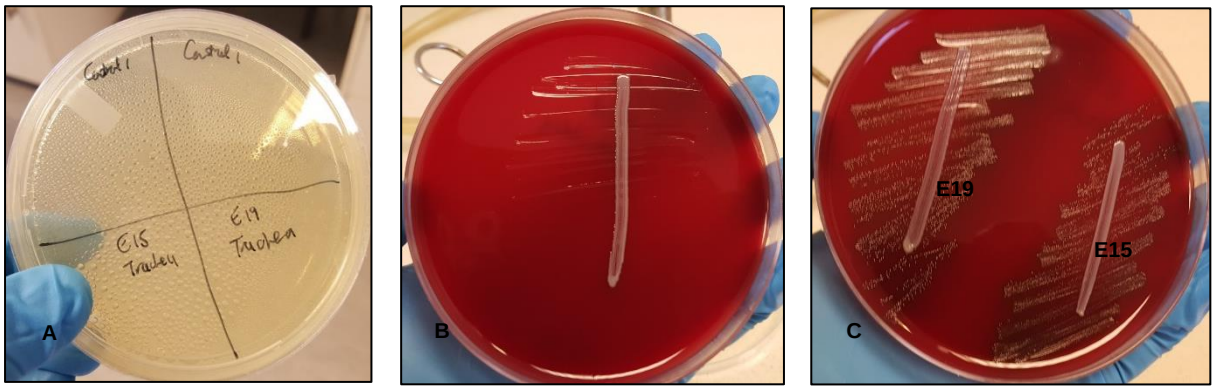


Figure 5.8: Tracheal swabs of chickens Control 1, E15 RI and E19. (A) Tracheal swab samples of Control 1, E15 RI and E19 that showed no growth. (B) Tracheal swab from Control 1 showing no growth, indicating there was no cross contamination between Control 1 and experimental birds. (C) Tracheal swab samples of E15 RI and E19 showed growth, however the culture was a mixed culture and aseptic techniques had to be performed before achieving a pure culture of *Av. paragallinarum*, which was successfully re-isolated.

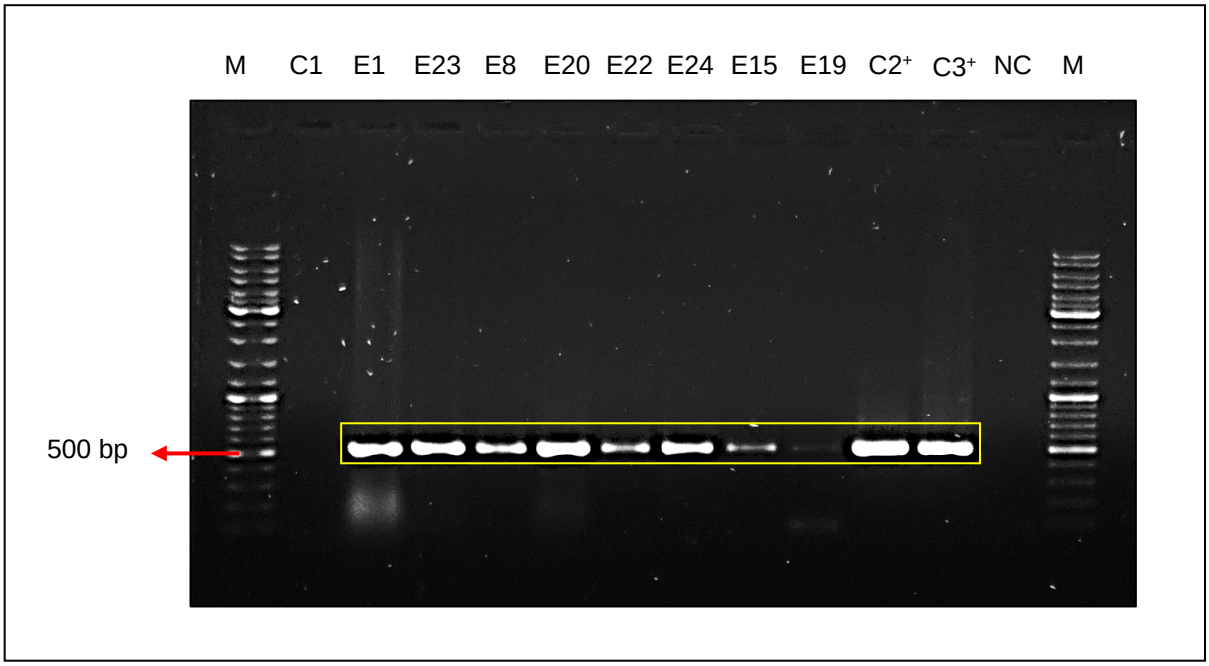


Figure 5.9: HPG2-PCR conducted from nasal and tracheal swab samples, whereby amplification was observed for all samples, with an expected band size of 500 bp, confirming the presence of *Av. paragallinarum* DNA. Lane M- molecular marker O'GeneRuler™ DNA Ladder; lane 1: Control 1 (C1)- score 0 (control bird) ; lane 2: E1- score 1; lane 3: E23- score 1; lane 4: E8- score 2; lane 5: E20-score 2; lane 6: E22-score 3; lane 7: E24- score 3; lane 8: E15 RI- score 3 (tracheal swab); lane 9: E19- score 3 (tracheal swab); lane 10: Modesto (C-2) positive control; lane 11: SA-3 (C-3) positive control; lane 12: negative control; Lane M molecular marker O'GeneRuler™ DNA Ladder.

The nasal swab sample from Control 1 was also cultured and DNA extraction was performed, to verify if *Av. paragallinarum* was present, where no colonies from *Av. paragallinarum* was found. A species-specific/ HPG2-PCR was performed, using the DNA extracted from the re-isolated bacterial culture obtained from nasal swabs of chickens Control 1 (C1), E1, E8, E20, E22, E23, E24 and the tracheal swab samples from E15 RI and E19. Following the species-specific PCR/ HPG2-PCR an expected amplicon size of 500 bp was obtained indicative that *Av. paragallinarum* was successfully re-isolated and cultured from the nasal and tracheal

swabs sampled (Figure 5.9). Re-isolation and identification of *Av. paragallinarum* from the tracheal swabs was successfully conducted, this suggests that *Av. paragallinarum* can spread further down the respiratory tract (Droual *et al*. 1990; Hoerr *et al*. 1994; Deshmukh *et al*. 2015).

The re-isolation and culture of *Av. paragallinarum* from the nasal and tracheal samples is in accordance with the four criteria that was established by Robert Koch and is proof of Koch's postulates in identifying the causative agent of IC, that (1) the pathogen was present in all cases of the disease (2) the pathogen could be isolated from the diseased host and cultivated as a pure culture (3) the pathogen from the pure culture must have been able to cause disease when inoculated into healthy hosts (4) the pathogen must be re-isolated from the current host and shown to be similar as the originally inoculated pathogen. There was no other strain of *Av. paragallinarum* apart from the SA-3 strain (serovar C-3) inoculated into the infra-orbital cavity of the SPF chickens, hence only that specific strain could be re-isolated and cultured. Moreover, *Av. paragallinarum* serovar could not be re-isolated from the control chickens, hence we can assume that the strains are similar. This assumption can be validated, as challenge chickens were injected with *Av. paragallinarum* serovar C-3 (SA-3 strain) shown in previous studies (Chapter 3 and Chapter 4) supported with data from BLASTn that shows that the strain used throughout the experimental studies shares 100% correlation with the *Av. paragallinarum* strain SA-3.

5.3.3. Immunohistochemistry (IHC) of samples collected

Immunohistochemistry (IHC) was conducted and following visualization as described in Section 5.2.4.3 (Chapter 5), analysis of all lymphoid images was performed and the results reported. Initially we had intended to study the bursa of Fabricius and thymus that are the major lymphoid organs in the avian immune system, the sites of B and T cell maturation, that

actively play a role in the innate and adaptive immune system. However the chickens used in the study were already mature at 25 weeks of age, hence both organs had undergone involution, had completely disappeared and could no longer be found/obtained.

After IHC was performed, a brown precipitate could be observed on the tissue sections when visualized with a light microscope at a magnification of 100X when stained with monoclonal antibody. This gave a qualitative indication of the level of expression of the protein of interest (CD3, CD20 and CD68) within the tissue during disease progression. The intensity or level of expression of each antibody marker that bound to antigen was classified as negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++).

The anti-CD3 marker is a pan- T lymphocyte marker, whereby the results for the liver, spleen and intestines were compiled in Figure 5.10 and Table 5.2. The anti-CD20 marker is a pan- B lymphocyte marker, whereby the results for the liver, spleen and intestines were compiled in Figure 5.11 and Table 5.3. The liver is a unique organ that is supplied with antigen-rich blood from the gastrointestinal system which then passes a network of sinusoids and screened by antigen presenting cells and lymphocytes (Crispe, 2003). Moreover, the liver is a non-lymphoid organ, whereby 90% of the reticuloendothelial system (also known as the monocyte phagocyte system) is found in the liver (Baas *et al.* 1994). However, the liver consists of both conventional and unconventional subpopulations of lymphocytes from the innate immune system, comprising of natural killer cells (NK) and natural killer T cells (NKT), and the B and T lymphocytes involved in adaptive immunity (Racanelli and Rehermann, 2006). T lymphocytes are categorized as conventional ($CD4^+$ and $CD8^+$ cells) and unconventional ($\gamma\delta$ T cells) (Racanelli and Rehermann, 2006). The liver also has Kupffer cells which are macrophages found specifically in the liver, are primarily involved in phagocytosis and constitute for approximately 20% of parenchymal cells in the liver (Mackay, 2002).

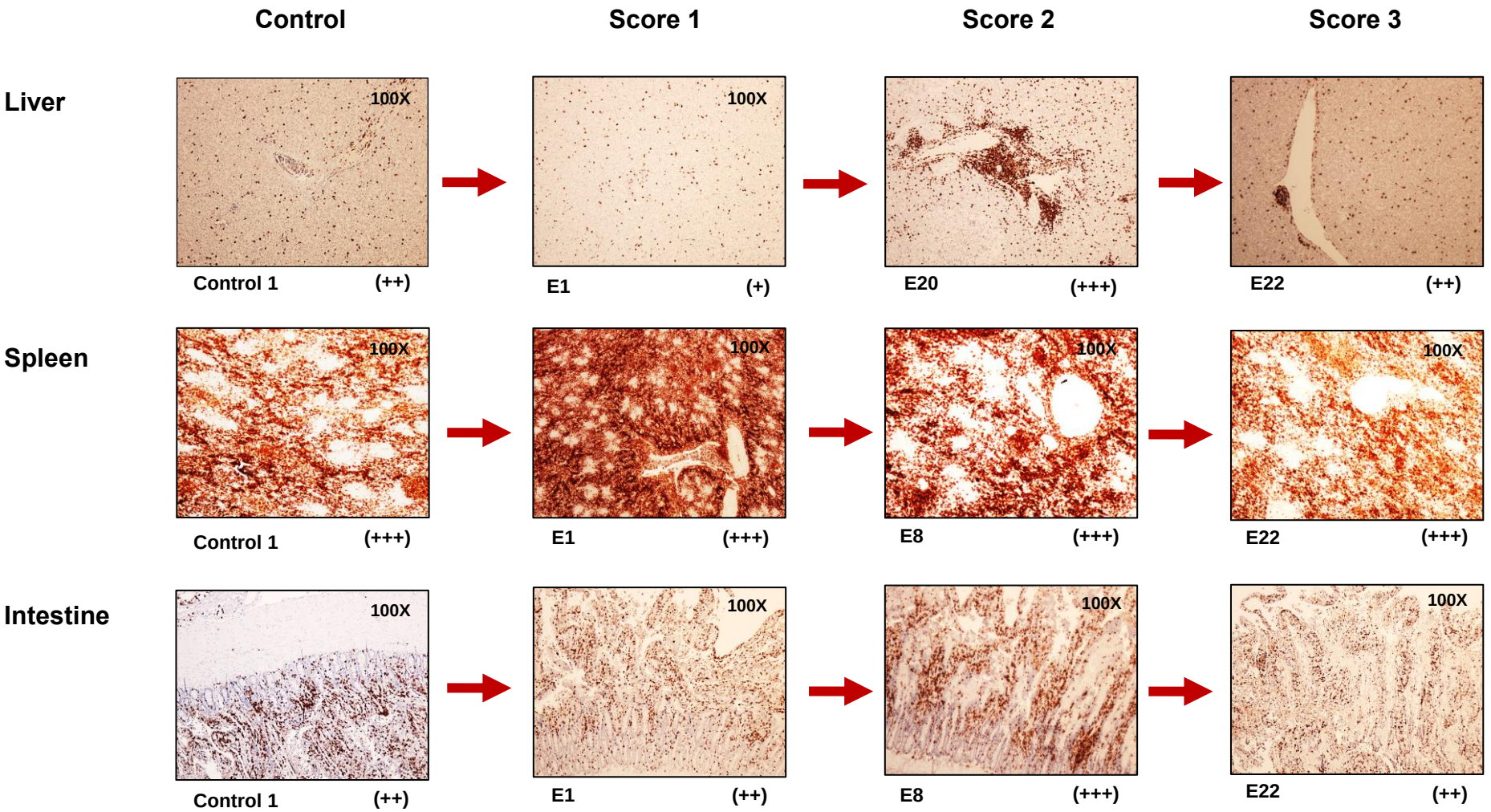


Figure 5.10: Results for immunohistochemical staining with anti-CD3 marker for the liver, spleen and intestine of control and infected birds. Anti-CD3 was used to determine the expression and distribution of T lymphocytes on the cells of lymphoid tissues harvested.

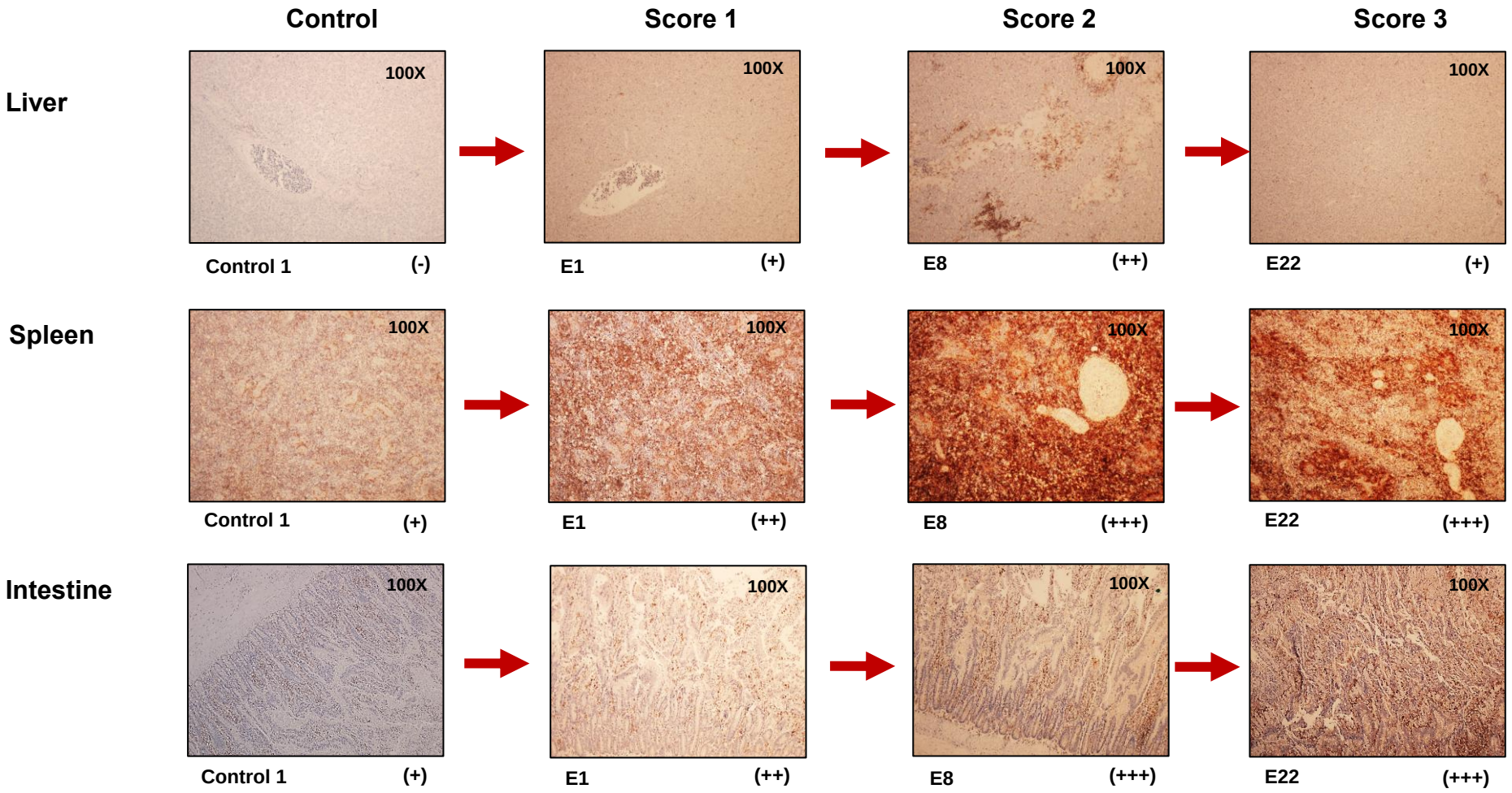


Figure 5.11: Results for immunohistochemical staining with anti-CD20 marker for the liver, spleen and intestine of control and infected birds. Anti-CD20 was used to determine the expression and distribution of B lymphocytes on the cells of lymphoid tissues harvested.

Table 5.2: IHC results for the detection of cells expressing CD3, which is the T lymphocyte population in the liver, spleen and intestine for control, score 1, score 2 and score 3 chickens.

CD3 (T Lymphocyte population)			
Sample number	**Liver**	**Spleen**	**Intestine**
Control 1	++	++	++
Control 2	++	+++	+++
Score 1- E1	+	++	++
Score 1- E23	+	++	++
Score 2- E8	+++	+++	+++
Score 2- E20	+++	+++	+++
Score 3- E11	+	+++	++
Score 3- E15 RI	+	+++	++
Score 3- E16 RI	+	+++	++
Score 3- E19	+	+++	++
Score 3- E22	++	++	++
Score 3- E24	++	++	++

The liver contains a very small population of $CD3^+$ cells of $CD4^+$ (T helper) cells, $CD8^+$ (cytotoxic) cells and NKT (natural killer T) cells involved in cytolytic activity, as seen in Control 1 bird that was not infected with the *Av. paragallinarum* SA-3 strain (serovar C-3) (Figure 5.10) (Table 5.2). For immunohistochemical staining of the liver with anti-CD3 we observed an increase in the T lymphocyte population from a score 1 (E1) that was weakly positive (+), to a score 2 (E20) that was strongly positive (+++) and then a decrease at a score 3 (E22) that gave a weakly positive (+) expression of the marker (Figure 5.10) (Table 5.2). At score 1 (E1 and E23), the innate immune system is activated, whereby Kupffer cells are activated by

various bacterial stimuli such as lipopolysaccharides (LPS) and in turn secrete cytokines such as IL-1β, IL-6 and TNF-α to promote the infiltration and antimicrobial activity of neutrophils (Figure 5.10) (Table 5.2) (Gregory and Wing, 1998). In addition, the neutrophils eliminate bacteria that are attached to Kupffer cells and hepatocytes via surface phagocytosis, whereby there is stimulation of other innate immune cells by the secretion of cytokines that promotes the influx and activation of $CD4^+$ and $CD8^+$ T cells (Gregory and Wing, 1998), which was why there was an efflux of $CD3^+$ cells at score 2 (E8 and E20) (Figure 5.10) (Table 5.2). However, when the adaptive immune system becomes activated, T cell mediation against bacteria relies solely on the constant supply of activated effector $CD8^+$ T cells that have a role in cytotoxicity for the maintenance of the immune responses and in controlling the spread and expansion of bacterial pathogens (Racanelli and Rehermann, 2006). During bacterial invasion, Kupffer cells, dendritic cells (DC) and liver sinusoidal endothelial cells (LSEC) present antigens to naïve $CD4^+$ T cells whereby IL-4 and IL-10 are secreted leading to immunological consequences, naïve $CD8^+$ T cells whereby they become effector $CD8^+$ T cells involved in cytotoxic activity. The decrease observed at score 3 (E11, E15 RI, E16 RI, E19, E22 and E24) may be due to $CD8^+$ T cells becoming activated and once bound to endocytosed antigens presented by major histocompatibility complex (MHC) class II undergoes apoptosis, leading to a reduction in the T cell population observed (Figure 5.10) (Table 5.2).

The results for the IHC staining of the liver with anti-CD20, were similar to that of the anti-CD3, whereby we see an increase in the expression of the B lymphocyte population at a score 2 (E8) giving a moderately positive (++) result (Figure 5.11) (Table 5.3). In Control 1, we observe that there were no B lymphocytes present, as none of the cells were stained positive (Figure 5.11) (Table 5.3). For immunohistochemical staining of the liver with anti-CD20 we observed an increase in the T lymphocyte population from a score 1 (E1) that was weakly positive (+), to a score 2 (E8) that was strongly positive (++) and then a decrease at a score 3 (E22) that gave a weakly positive (+) expression of the marker (Figure 5.11) (Table 5.3). The slight increase in the B cell population at score 2 (E8), was due to the adaptive immunity that “kicks

in" from score 1 (E1) to score 2 (E8), hence there was an increase in the peripheral B cell response as the humoral response came into play. At score 3 (E22), there was a decrease in the humoral response due to the feedback mechanism of deterring immune cells and molecules from damaging the host's tissues (Chapter 2). Although B cells function in the humoral response by secretion of antibodies such as IgA, IgM and IgY, the exact function of B cells in the liver is still unknown, especially since the liver is neither a lymphoid or ectopic organ. Moreover, from the results in Table 5.3 it was observed that there was negative (-) to very low expression (+) of CD20 in the liver, except with chicken E20 whereby there was moderate expression of the B lymphocytes. The liver obtained from E20 had both high CD3 and CD20 expression (+++), implying that both the humoral and cell-mediated responses occurred leading to the release inflammatory cytokines and antibodies leading to the liver lesions that were observed. Hence, from our results obtained it is apparent that the liver has a role to play in B cell proliferation due to high expression (+++) of the CD20 at score 2 (E8 and E20) (Figure 5.11) (Table 5.3).

In the spleen, CD3 and CD20 were constantly highly expressed (++/+++) in all scores and in the control bird (Figure 5.10 and Figure 5.11) (Table 5.2 and Table 5.3). Initially, lymphomyeloid tissues develop from either epithelial (bursa of Fabricius and thymus) or mesenchymal (spleen, lymph nodes and bone marrow) anlages which harbour haematopoietic cells. Haematopoietic stem cells enter the bursal or thymic anlages which are the central lymphoid organs and develop to become immunologically competent B and T cells (Sturkie, 1943). However, upon maturation the mature T and B cells enter the circulation and colonize the peripheral lymphoid organs also known as ectopic organs such as the spleen, lymph node and gut-, bronchus- and skin-associated lymphoid tissues (Davison *et al*. 2011). Thus, in these organs B and T lymphocytes, are compartmentalized into B and T dependent zones (Schat *et al*. 2014). In the avian spleen, the T dependent zone includes the peri-arteriolar lymphatic sheath (PALS) that surrounds the splenic central artery and the

interfollicular region, whereas germinal centres (GC) and the peri-ellipsoidal white pulp (PWP) of the spleen are B dependent zones (Davison *et al*. 2011). In avian anatomy, the spleen becomes the secondary lymphoid organ after the bursa of Fabricius and thymus undergo involution. Hence, there is an abundance of T and B lymphocytes in the spleen, which is why B and T lymphocytes were constantly and highly expressed (++/+++) throughout all scores and in the control bird with IHC anti-CD3 and anti-CD20 staining of the spleen (Figure 5.10 and Figure 5.11) (Table 5.2 and Table 5.3). In the avian immune system, the spleen has a closed circulatory system and is not a reservoir of erythrocytes for immediate release into the bloodstream (Sturkie, 1943). Moreover, there are capillaries that connect the red pulp of the spleen to the sinuses, whereby the sinuses are drained by collecting veins that will eventually leave the spleen. Hence, it is possible that the injected SA-3 strain of *Av. paragallinarum* via infra-orbital injection directly into the sinus cavity circulated to the spleen, leading to an increased inflammatory and adaptive responses from the T and B lymphocytes respectively, whereby the T and B lymphocytes also migrated to peripheral tissues leading to systemic immunological responses in the host organism. However, the hypothesis proposed has not been proven.

One of the symptoms of IC is diarrhoea, which occurs primarily because some of the exudates containing *Av. paragallinarum* spreads to the water and feed in cages. Eventually, the chickens ingest the contaminated water and feed, thus causing infection and an inflammatory response in the gut. The gut-associated lymphoid tissue (GALT) consists of an assorted range of cell subsets of unique and representative cell populations from systemic tissues (Davison *et al*. 2011). The avian GALT commences in the lamina propria of the villus (Schat *et al*. 2014). Each single villus is composed of connective tissue fibres, smooth muscle fibres, nerves and blood and lymph vessels, whereby macrophages amalgamate with lymphocytes in the lamina propria (Davison *et al*. 2011). Additionally, B and T cells in the intestine form distinct regions when more lymphocytes infiltrate the villus (Schat *et al*. 2014). The T cells are found at the

centre of the villus, whereas B cells and GC are located in the lymphoid follicles entwined with dendritic cells, $CD4^+$ cells and macrophages, situated at the deep and mid-section of the lamina propria (Hoshi and Mori, 1973; Jeurissen *et al*. 1994). Furthermore, the avian GALT is populated with heterophils, macrophages, dendritic cells, natural killer (NK) cells, and B and T lymphocytes, however the proportions of each cell type depends on the locality and age of the bird (Davison *et al*. 2011). The gut epithelial layers also consist of highly specialized lymphocytes called the intraepithelial lymphocytes (IEL) (Davison *et al*. 2011).

Table 5.3: IHC results for the detection of cells expressing CD20, which is the B lymphocyte population in the liver, spleen and intestine for control, score 1, score 2 and score 3 chickens.

CD20 (B Lymphocyte population)			
Sample number	**Liver**	**Spleen**	**Intestine**
Control 1	-	+	+
Control 2	-	+++	+++
Score 1-E1	+	++	+
Score 1- E23	+	+++	+
Score 2- E8	+	+++	++
Score 2- E20	++	+++	++
Score 3- E11	-	++	++
Score 3- E15 RI	-	++	++
Score 3- E16 RI	-	++	++
Score 3- E19	-	++	+
Score 3- E22	+	++	+++
Score 3- E24	+	++	+++

The IHC results for anti-CD3 for the small intestine showed moderate expression (++) of the T lymphocyte population in control bird (Control 1), score 1 birds (E1 and E23) and score 3 birds (E11, E15 RI, E16 RI, E19, E22 and E24) with strongly positive expression (+++) of T lymphocytes at score 2 in chickens E8 and E20. Initially, the small intestinal IEL population is populated with NK cells and T cells (with γδ or αβ T cell receptor (TCR)), whereby most of the T cells express the CD8 co-receptor with smaller populations of $TCR\alpha\beta^{+}CD4^{+}$ and $CD4^{+}CD8^{+}$ cells (Vervelde and Jeurissen, 1993; Lillehoj, 1994; Göbel *et al*. 2001; Lillehoj *et al*. 2004). In contrast to the IEL population, the T cell population of the lamina propria is sparsely populated with γδ T cells, with the majority of the T population being the αβ T cells dominated by $CD4^{+}$ cells and a minority of $CD8^{+}$ cells (Davison *et al*. 2011). Thus, due to the presence of distinct T cell populations in the IEL and lamina propria, we observed moderate expression (++) of $CD3^{+}$ cells in control and score 1 birds (Figure 5.10) (Table 5.2). However, during the first few days of infection the innate immune response plays a crucial role with macrophages, NK cells and $TCR\gamma\delta^{+}$ T cells, which are active during the transition from the non-infectious to score 1 phase, which is perhaps the reason we saw small clusters of T cells consisting of $CD4^{+}$ T helper cells and naïve $CD8^{+}$ cells (Figure 5.10). During the adaptive immune response, from score 1 to score 2, the naïve $CD8^{+}$ cells become activated and are involved in cytotoxic activity, whereby $CD4^{+}$ cells are T helper cells involved in the activation of B cells via the MHC class II (Vainio *et al*. 1984). The influx in $CD3^{+}$ effector T cells at score 2 could also be attributed to the migration of T cells from peripheral lymphoid tissues. We then, observed a slight decrease in the T lymphocyte population (Figure 5.10) (Table 5.2), which might be due to $CD8^{+}$ cytotoxic T cells that underwent apoptosis after antigen presentation and display via macrophages complexed to MHC class I leading to the release of cytotoxins causing cell death of the $CD8^{+}$ cell-MHC class I- antigen complex formed.

The IHC results for anti-CD20 of the small intestine showed low expression (-/+) of the B lymphocyte population in control bird (Control 1) and score 1 birds (E1 and E23), with

moderate expression (++) in score 2 birds (E8 and E20) and moderate (++) to high expression (+++) in score 3 birds (E11, E15 RI, E16 RI, E22 and E24) (Figure 5.11) (Table 5.3). Chicken E19 had low expression of the marker. Unlike other tissues, B cells are almost absent in the IEL, which is why we observed very low expression (-/+) of CD20 in the control and score 1 birds. During the early to late adaptive responses as seen with score 2 and 3 chickens (Figure 5.11) (Table 5.3), the B lymphocyte population increases from moderate (++) to high (+++) expression with anti-CD20 marker, due to potential B cell migration from ectopic B cell rich lymphoid tissues to the gut periphery and also due to clonal expansion of B cells when they become activated mainly because B cells have a role in the humoral response in the gut. Following clonal expansion, the B cells become plasma cells that secrete immunoglobulins and memory cells that develop and build immunological memory until the next encounter with the same antigen leading to more rapid immunity against the pathogen. Moreover, B cells secrete IgA antibody at high concentrations in intestinal fluids (Lebacq-Verheyden *et al*. 1972). Immunoglobulin (Ig) A is mainly found in its monomeric form, however it is secreted in a polymeric configuration (Bienenstock *et al*. 1973). It has been proposed that bacterial and viral targets coated in IgA followed by ligation with FcαR in humans leads to a cascade of immunological responses such as phagocytosis, respiratory burst and release of cytokines, in the annihilation of invading microorganisms (Monteiro and Van De Winkel, 2003). However, in chickens the function of IgA and its immune mechanisms have yet to be elucidated.

For bird Control 2, we observed very high expression (+++) of both CD3 and CD20 in the spleen and intestine. The spleen is a major secondary lymphoid tissue and constantly expresses T and B lymphocytes which was why we observed high expression (+++) of T and B lymphocytes regardless of whether an infection was present or not, however the high expression (+++) of CD3 and CD20 in the GALT indicated infection. This indicated T and B lymphocyte infiltration into these lymphoid organs and increased adaptive immune responses. Therefore, Control 2 could have been exposed to *Av. paragallinarum* or other pathogens,

however the bacterial load as mentioned in Chapter 4 was not sufficient to cause IC. The liver of Control 2 with anti-CD20 stained negative and with anti-CD3 there was moderate expression similar to Control 1, which indicated there were small clusters of naïve B and T lymphocytes present.

For the re-infected chickens E15 RI and E16 RI, there was not much of a difference in the IHC staining of the anti-CD3 and anti-CD20 of the liver, spleen and intestine results compared to other infected chickens (Table 5.2 and Table 5.3). This implies that the second injection given when the chickens were at score 2, did not have any significant systemic effect on the tissues of infected chickens as immunological memory had already been established prior to the second injection via infra-orbital injection. Therefore, the chickens E15 RI and E16 RI developed score 3, not as a result of the second injection containing the SA-3 strain (serovar C-3) of *Av. paragallinarum* , but because of previous exposure from the first injection. From the necropsy and IHC results the immunological and systemic responses from IC infection, there were no severe lesions found nor extensive internal damage caused, as described in the literature for the re-infected chickens (Sawata *et al*. 1985; Blackall and Soriano, 2008). The sinus responses of re-infected chickens when compared to score 3 chickens had similar symptoms such as haemorrhage and congestion.

We performed IHC on the cross-sections (CS) and longitudinal sections(LS) of the trachea using anti-CD3, anti-CD20 and anti-CD68. The results were recorded in Table 5.4 and as Figure 5.12. From the results we observed moderate expression (++) of CD3 from Control 1 and E15 RI- score 3, with low expression (+) in E19- score 3 (Figure 5.12) in the mucosa, submucosa and cartilaginous layers of the trachea. In Section 5.3.2, it was found that *Av. paragallinarum* was present in E19 and E15 RI both at score 3, hence the decreased expression of CD3 seen in E19 was expected as T cells undergo apoptosis after antigen

presentation following the cell-mediated response, which is the reason for the diminished T cell population. However, there was no change in E15 RI, due to T cells still actively playing a role in the cell-mediated response consisting of naïve T cells and effector T cells in the trachea during IC infection.

Table 5.4: IHC results for the detection of cells expressing CD3, CD20 and CD68; which represent the T lymphocyte, B lymphocyte and macrophage/monocyte population within the trachea of control, score 3 and score 3 RI birds.

Trachea			
Sample number	**CD3**	**CD20**	**CD68**
Control 1	++	++	++
Score 3- E19	+	++	-
Score 3- E15 RI	++	-	-

Both Control 1 and E19 at score 3 moderately expressed (++) CD20 in tracheal tissue, indicating the presence of B lymphocytes involved in the mucosal humoral response by the release of antibody IgA. There was no expression (-) of CD20 in E15 RI-score 3, which might be because the humoral response had already occurred in the trachea before the tissue was harvested, there was no intrinsic expression of CD20 or the antigen could not be retrieved from the trachea during the antigen retrieval phase of IHC. However, since T and B lymphocytes were expressed in E19 having the same score, this indicated that similar results also had to be obtained for E15. As such, the results for E15 showed that once the adaptive

immune responses occurred, it would be difficult to obtain the expressed antigens in the tissues if not harvested at the correct period of expression.

CD68 was used to detect the presence of macrophages or monocytes in the trachea. Only Control bird 1 expressed CD68, which was moderately expressed (++). Macrophages and monocytes play a role in innate immunity, as personal scavengers of the host, involved in phagocytosis. At score 3 in chickens E19 and E15 RI, the macrophages were absent due to adaptive immunity that took over the immune response of the host, which was the reason CD68 stained negative in both score 3 chickens. Moreover, macrophages in the presence of *Av. paragallinarum* would have ingested the antigen leading to the cascade in immunological responses and adaptive immunity, which is perhaps why no macrophages were present even at score 3, as most possibly they were destroyed or cleared during the pathogenic attack from the upper to the lower respiratory tract. There are numerous suicide programmes employed by macrophages to prevent the spread of microbial replication (Chow *et al*. 2016). Pro-death factors can be activated which can trigger apoptotic cell death, resulting in the release of caspases (caspase-1 and caspase-11 in humans) leading to pyroptosis, which is a fast and lytic form of macrophage suicide (Czabotar *et al*. 2014; Chow *et al*. 2016). Pyroptosis is often a highly inflammatory response due to the release of pro-inflammatory cytokines, whereby there is activation of neutrophils that function in the clearance of the intracellularly ingested pathogens by macrophages (Chow *et al*. 2016).

Infectious coryza (IC) causes a decrease in egg production, hence it was investigated to see whether the uterus (shell gland) had a specific type of T or B lymphocyte distribution and expression. Surprisingly, Control 1, E11-score 3 and E15 RI-score 3 expressed low and sparse levels of CD3, related to T lymphocyte expression. Implying that the major adaptive response was the cell-mediated response, involving naïve and effector T lymphocytes within

the uterus. There were very few cells that expressed CD3 in the uterus, but nonetheless there was some expression. However, the CD20 antigen was not expressed at all in any of the birds, perhaps this might be because the uterus (shell gland) is not a B lymphocyte-rich tissue. The shell gland has a vital role in egg production as it secretes albumin and the egg shell, during egg formation in the hen. It is also possible that only specific parts of the reproductive tract express B lymphocytes, whereby the uterus is not one of these B lymphocyte-rich tissues. From the results obtained (Table 5.5 and Figure 5.13), it was observed that the uterus of chickens at different clinical scores, had very low numbers B lymphocytes, thus this could be the reason why *Av. paragallinarum* could easily and systemically affect the reproductive organs as some of these regions have lowered B and T cell populations, resulting in a delayed humoral immune response and more favourable spread of the pathogen within the uterus. However, decreased appetite caused by IC also affects the production and formation of eggs, as there is a decrease in the percentage of calcium needed for egg formation, especially the egg shell. Hence, there is definitely a correlation between the chicken's diet and egg formation (Surai and Sparks, 2001). However, IHC needs to be conducted in future studies on other tissues of the reproductive tract such as the ovary, magnum and isthmus to determine the distribution of T and B lymphocytes.

Table 5.5: IHC results for the detection of cells expressing CD3 and CD20; which represent the T lymphocyte and B lymphocyte population within the uterus (shell gland) of control, score 3 and score 3 RI birds.

Uterus (Shell gland)		
Sample number	**CD3**	**CD20**
Control 1	+	-
Score 3- E11	+	-
Score 3- E15 RI	+	-

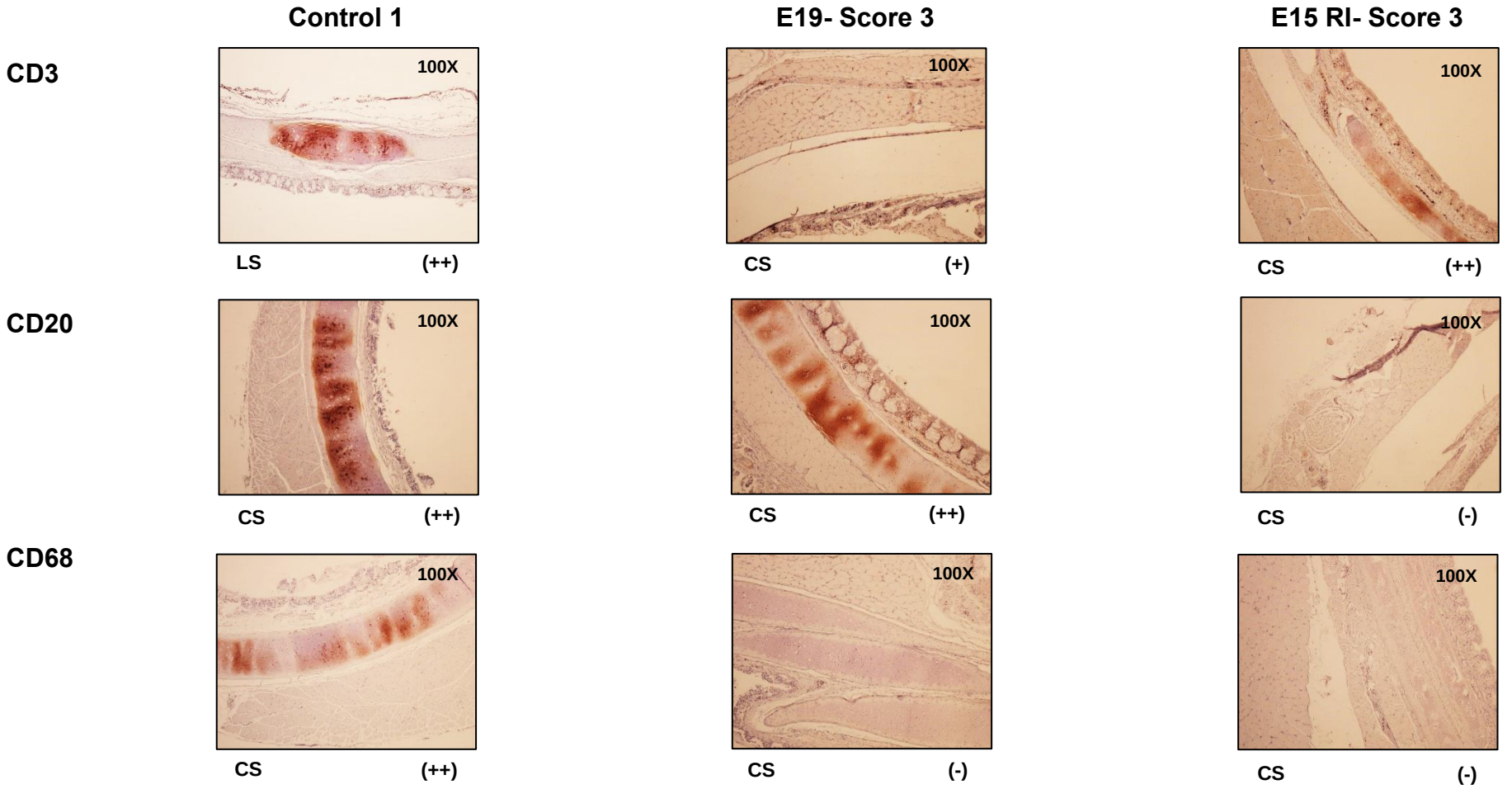


Figure 5.12: Results for immunohistochemical staining of the trachea with anti-CD3, anti-CD20 and anti-CD68 marker for control, score 3 and score 3 RI birds. We analysed both cross-sections (CS) and longitudinal sections (LS) at a 100X magnification.

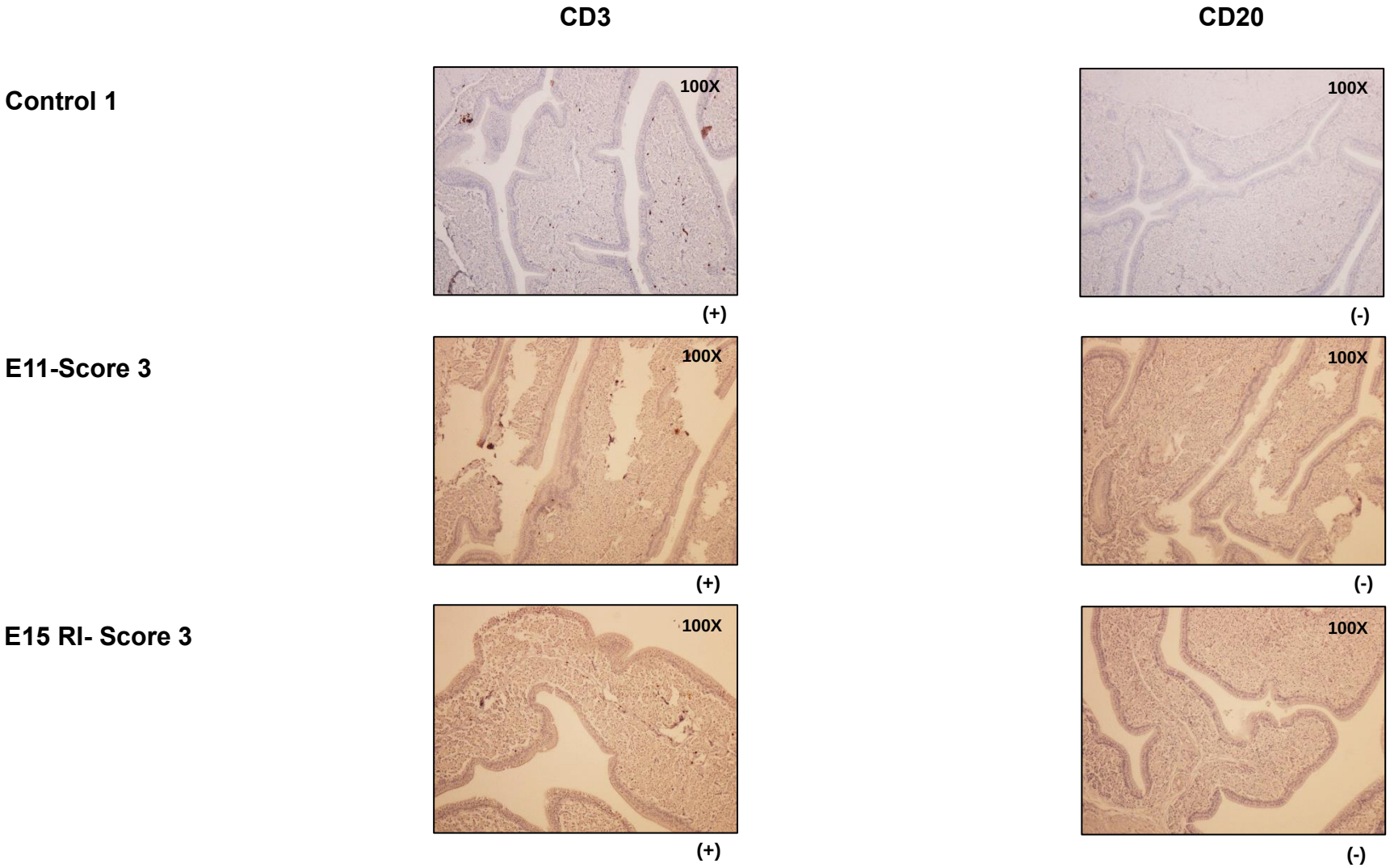


Figure 5.13: Results for immunohistochemical staining of the uterus (shell gland) with anti-CD3 and anti-CD20 marker for control, score 3 and score 3 RI birds. Surprisingly, it was observed that only CD3 was expressed across all three chickens at a control, score 3 and score 3 RI; and not CD20.

5.4. Conclusions

In this chapter (Chapter 5) we tried to conduct necropsy on control, score 1, score 2 and score 3 chickens and perform post-mortem examination of the tissues. Additionally, we tried to determine the distribution and expression of T and B lymphocytes, via the use of IHC and specific antibody markers CD3, CD20 and CD68 respectively. We were successful in determining the expression and distribution of T and B lymphocytes in the liver, spleen and intestine, as well as the trachea and uterus (shell gland). Liver lesions were also obtained, however we were unsuccessful at isolating *Av. paragallinarum* from the liver. In addition, it is still uncertain whether lesions and internal damage caused by IC, as stated in literature was as a result of secondary infection or solely the result of the *Av. paragallinarum*. During cultivation of the nasal swabs on the BTA plates, there was growth of other non-*Av. paragallinarum* microorganisms for infected and control chickens (Figure 5.6), but no growth was observed on TSA plates supplemented with NAD^+ from the chickens, hence in future studies these microorganisms should be identified. Perhaps the interactions between the microorganisms growing alongside *Av. paragallinarum* can provide insight whether there is a symbiotic relationship leading to aggravation of IC or whether these microorganisms have a role in the immunity of the host by providing mucosal protection in the upper respiratory tract. The non-*Av. paragallinarum* microorganisms growing on the BTA plates but not TSA plates supplemented with NAD^+, appeared to be haemolytic in nature due to lysis of BTA plates and could be identified as staphylococci/micrococci which can also grow in basic medium like TSA, however no growth was seen on the TSA plates supplemented with NAD^+. A possible explanation for this set-back could have been that the media used was problematic and was not suitable for bacterial cultivation during the study. Thus, in future experiments more suitable media for *Av. paragallinarum* should be used. Moreover, it was found that *Av. paragallinarum* does spread to the lower respiratory tract into the trachea, as we were able to isolate the bacteria. It was also found that the trachea expressed T and B lymphocytes, primarily involved in the adaptive immune response as a mechanism to defend against *Av. paragallinarum* as

per IHC results. Furthermore, from the uterus, it was found that CD20 was not expressed in Control 1, score 3 and score 3 RI birds, which suggests that this organ has no humoral defences on standby. Hence during *Av. paragallinarum* or pathogenic infection the uterus can potentially be vulnerable to systemic infection, since it has been documented that *Av. paragallinarum* can spread to other organs other than the upper respiratory tract (Sandoval *et al*. 1994). Therefore, this might have an effect on egg production in hens, leading to the decline in egg production. Finally, it was discovered that T and B lymphocytes are not constantly expressed during an infection in the lymphoid tissue, except if the lymphoid tissue becomes a major secondary lymphoid organ like the spleen. The expression of the T and B lymphocytes depends on the timing of when the immune responses occurred and the time of harvest, therefore if the tissues were harvested after an immune response or after clearance, none of the T or B lymphocytes would be observed. Since we could not isolate *Av. paragallinarum* from the tissues, we could only conclude that the effect of IC was systemic leading to the inflammatory and adaptive immune responses as well as B and T lymphocyte distributions observed within the tissue sections following IHC. It should be taken into consideration that during an infectious challenge there is an induction of multiple immune responses whereby some of these responses are unsuccessful at controlling infection and at times, these responses can also be damaging to the tissues of the host. Regrettably, detecting an immune response does not indicate effectiveness or involvement in controlling infection and the "response" data obtained must be interpreted with considerable care and experiments repeated for quality control and comparative purposes.

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CHAPTER 6

CONCLUDING REMARKS AND FUTURE STUDIES

The chicken is a well-studied model in biology, immunology and physiology, however to date there are still gaps in the knowledge pertaining to immunological signalling pathways related to avian diseases, whereby infectious coryza (IC) is one of them. *Av. paragallinarum* serovar C-3 (SA-3 strain), is the most virulent in South Africa, suggesting that it is of economic and veterinary importance. Moreover, failure of commercial vaccination attempts developed for IC, is becoming a serious problem, with the emergence of new serovar variants. Vaccines can no longer provide cross protection against IC. Since prevention is better than cure, it becomes imperative that we understand host-pathogen interactions, disease progression and the immune mechanisms employed by the host's immune defence system against *Av. paragallinarum* strain SA-3 (serovar C-3) as the knowledge obtained from studying the avian immune response is a powerful tool that can be used to combat IC.

From the study, bioinformatics tools for the analysis of differentially expressed genes was conducted. The *in silico* results obtained for the generation of immune signalling pathway maps, results for biological processes through gene enrichment, functional annotations and the generation of functional correlating networks of genes assisted in predicting *in vivo* immune responses during IC infection, in not only blood but also lymphoid and non-lymphoid organs of the avian host such as humoral immune responses, cell-mediated responses, the effect of IL-8, the functions of innate immune cells and the role of leukocytes, as seen in later chapters. The pathogenesis of IC infection with serovar C-3, was found to be similar to that of influenza A virus, suggesting that the presence of HA, prophages and prophage remnants

may have a role to play in virulence. The role of prophages and prophage remnants in the virulence of *Av. paragallinarum* serogroups needs further investigation.

During the pilot study chickens infected with *Av. paragallinarum* serovar C-3 (SA-3 strain) did not yield any IC related signs or symptoms. This suggested that the SPF chickens used in the pilot study obtained had an already well-established immune system based on existing antibodies present in Day 0 chicken blood. Hence, it should be a prerequisite that antibody testing be conducted prior to animal experimentation, as a precautionary measure, so that the immune effects present in the host organism stimulated by previous exposure to pathogens, vaccinations or drugs be known and appropriate measures be taken that would not jeopardise the study. It is also important that during an infection model, the correct dosage/concentration of bacterial culture is administered to achieve the disease threshold, whereby for this study 10^8 CFU/ml was found to be the appropriate concentration for IC infection.

In the infection model, different cell morphologies from blood smears together with leukocyte, CD4 and CD8 flow cytometry profiles of chicken blood samples at different scores were evaluated, providing insight into the innate, humoral and cell-mediated immune responses involved in pathogen elimination. The Th cell response had a major role to play in both innate and adaptive responses of IC infection. No statistically significant decline in egg production was observed in the study, which might be due to a small sample size in the study. Additionally, IL-8 was found to be highly expressed as the symptoms of IC became more aggressive, which suggests that IL-8 could potentially be used as a biomarker for IC. The study also showed that only a minority of chickens reached a score of 3, whereas the rest of the chickens recovered. Moreover, the flow cytometry profiles showing high immune-related activity and the ELISA results displaying high expression of IL-8, for control birds,

indicated that they had an already well-established immune response suspected due to previous exposure to microorganisms before or during the study. However, this did not lead to pathology as the chickens were asymptomatic for IC, thus the study was not jeopardised.

The expression and distribution of T and B lymphocytes in the liver, spleen, intestine, trachea and uterus (shell gland), was determined, via the use of necropsy, IHC and specific antibody markers CD3, CD20 and CD68 respectively. Liver lesions were also observed, however there was no success at isolating *Av. paragallinarum* or any other microorganism from the liver. The role of commensals growing alongside *Av. paragallinarum* can provide insight whether there is a symbiotic relationship leading to aggravation of IC or whether these microorganisms have a role in the immunity of the host by providing mucosal protection in the upper respiratory tract. *Av. paragallinarum* serovar C-3 (SA-3 strain) does spread to the lower respiratory tract into the trachea, as we were able to isolate the bacteria. Furthermore, CD20 was not found to be expressed in the uterus of Control 1, score 3 and score 3 RI birds, which suggests that this organ has no active humoral defences. Hence the uterus can potentially be vulnerable to systemic infection by pathogens, which could have an effect on egg production. *Av. paragallinarum* serovar C-3 (SA-3) could not be isolated from the tissues, we could only conclude that the effect of IC was systemic leading to the inflammatory and adaptive immune responses as well as B and T lymphocyte distributions observed within the tissue sections following IHC. In the disease profiles, flow cytometry profiles and IHC results, it was observed that following the adaptive immune responses and during score 3, towards more severe symptoms, there was a decline in immune activity, which might have resulted due to the inhibition of immune cells and molecules from damaging the host's tissues, which acts as a protective mechanism for the host. This assumption, was also found using bioinformatics tools, whereby it was shown that a Th2 shift occurs which provides protection to the host from hyperinflammation by countering the tissue-damaging effects of macrophages, Th1 cells and proinflammatory cytokines.

This research was novel and very unique since pathogenesis of IC and the monitoring of the avian immune responses was conducted, encompassing disease progression at a genetic, cellular, tissue, and clinical level as a whole. Focus on functional pathways and interaction networks was conducted to gain comprehensive insights into biological processes relevant to protection and pathogenesis in IC infection, which in future can be harnessed for relevant biomarker signatures. The *in vivo* results obtained from the experimental studies in combination with the *in silico* results obtained from bioinformatics tools may provide powerful insight and a birds-eye view into the immune mechanisms between host-pathogen interactions for this disease. Finally, results from this project will also further improve diagnostic tests and vaccination practices for *Av. paragallinarum* serovar C-3 (SA-3 strain) in the veterinary field, through the knowledge gained and the development of diagnostic products such as IC specific ELISA kits, as well as point-of-care testing and haematological avian devices.

Future Research

The next measures from the knowledge gained from this study, would be to conduct RNA-Seq (RNA sequencing) using next-generation sequencing (NGS) on tissue and sinus samples collected, to uncover the presence and quantity of RNA in the biological samples at continuously shifting time intervals and stages of disease progression of IC infection. Hence, the transitioning genes that are up- or downregulated during the progressive disease scores will be unravelled, which would enable more in depth understanding of immune regulatory and immune signalling pathways of IC. The metabolic regulation of immune responses in the avian host during IC infection, is also an area that needs attention. In our study, the gut was found to be an important lymphoid organ in mucosal immunity. In future studies, the interactions of microorganisms in the gut during *Av. paragallinarum* infection needs to be investigated, as the gut microbiota impacts many areas of animal health from innate

immunity to appetite and energy metabolism. Furthermore, challenge studies with other serovars or serogroups needs to be conducted for comparative purposes of immune-related responses and in disease monitoring to *Av. paragallinarum* serovar C-3 (SA-3 strain) infection, for standardisation purposes. The role of prophages and prophage remnants in the virulence of *Av. paragallinarum* needs to be studied, as well as similar pathogenesis to influenza A virus. From the ELISA results obtained from antibody screening and sandwich ELISA of IL-8, the development of an IC-based ELISA to assist in the diagnosis and prognosis of IC, should be conducted from all serogroups and serovars.