Antibody fragments as a possible therapeutic treatment for infectious bronchitis in poultry

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Abstract

Infectious bronchitis virus (IBV), a coronavirus, is the etiological agent for infectious bronchitis (IB), an acute respiratory disease of poultry. Infectious bronchitis is a notifiable disease, and taking in consideration that poultry is the second most consumed meat within South Africa, it highlights the importance of monitoring IBV outbreaks. Recombinant single chain variable fragments (scFv) have been used in therapeutic treatments for various human and veterinarian viruses, including other coronaviruses, such as SARS-CoV and MERS-CoV. The study aims to select scFv against the IBV antigen, with the use of phage display technology and commercial ELISA plates coated with the most prevalent IBV strains namely, H120 and M41. It has been proven that the S1 protein induces the binding of neutralising antibodies which provides protection against lethal CoV infections, thus, indicating a possible application for a therapeutic treatment. In this study, phage clones were selected from a human domain (dAb) library (Source BioScience, Australia). Panning was repeated three times using commercial ELISA plates coated with M41 and H120 IBV strains. Positive monoclonal phage clones were retrieved from the polyclonal mix by a sandwich ELISA and sequenced. The selected scFvs were expressed by Isopropyl β-D-1 thiogalactopyranoside induction and purified by immunoprecipitation with the Pierce Anti-c-Myc Agarose kit (Thermo Scientific, USA). After purification, binding ability of the scFvs were determined by means of a direct competitive ELISA. The neutralising ability of the scFvs was then determined by a virus neutralisation assay *in ovo* with the Avipro IBV H120 strain (Lohmann Animal Health Gmbh, Germany). This was performed in 9-day old SPF eggs over a time-period of six days. One set of eggs were injected with a dilution range of the IBV H120 strain and another set by a mixture of 2 µg/ml scFv with the IBV H120 strain. The end-point titres were determined and compared by the Spearman-Karber method (Spearman, 1908). Statistical analysis was performed using the student t-test with a *p*-value of 0.05. Round 1 of panning resulted in a total of 1.2×10^6 phages/ml and after round 3 a total of 3.0×10^{10} phages/ml were obtained. A total of 96 phage clones were manually selected from which only 12.5% showed a positive result during the sandwich ELISA. The 12 positive clones were sequenced and analysed based on nucleotide and amino acid composition. A total of five scFvs contained a complete variable heavy (VH) chain sequence, two of which was identical. This resulted in four unique and complete scFv sequences. These sequences showed a high variance in the nucleotide composition through-out the sequence. However, variation in the amino acid composition was only observed in the third complementary determining region. The scFvs were expressed and resulted in concentrations ranging from 204.38 µg/ml to 265.07 µg/ml. Detection of IBV antigen binding ability of the purified scFvs was conducted by a direct competitive ELISA. However, no statistical difference in absorbance values were observed, indicating insufficient binding of the

scFvs. The *in ovo* virus neutralisation assay resulted in a one log reduction of the end-point titres. Statistical analysis proved one of the reductions to be statistically significant with a *p*-value less than 0.05, resulting in a partial neutralisation effect from the scFv. In conclusion, the selection process showed a progressive enrichment of antigen specific clones from the dAb library. The scFvs were successfully expressed, purified and characterised in terms of binding ability.

Keywords: Infectious bronchitis virus; phage display; single chain variable fragments; virus neutralisation; therapeutic treatment

Declaration

I declare that the dissertation hereby submitted for the qualification Magister Scientiae *in Microbiology* at the University of the Free State is my own independent work and has not been previously submitted by me for a qualification at/in another University/faculty.

Furthermore, I concede copyright to the University of the Free State.

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JM Coetzee

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I dedicate this thesis to my grandmother (Nettie Coetzee) for her undying love and encouragement throughout the whole process even though she had no idea what I was doing.

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Abbreviations

BCA - Bicinchoninic Acid

BLAST – Basic Local Alignment Search Tool

BSA – Bovine Serum Albumin

BTV - Bluetongue Disease Virus

$CaCl_2$ - Calcium chloride

CDR - Complementarity determining region

dAb - Domain antibody

DEPC - Diethyl pyrocarbonate - treated water

E. coli - *Escherichia coli*

EDTA - Ethylenediaminetetraacetic acid

EID_{50} - Embryo-Infectious-Dose-50 %

ELD_{50} - Embryo-Letal-Dose-50 %

ELISA - Enzyme-linked immunosorbent assays

ERGIC – Endoplasmic reticulum Golgi intermediate compartment

Fab - Antigen binding fragment

HI - Hemagglutination inhibition

HRP - Horse Radish Peroxidase

IB - Infectious bronchitis

IBDV - Infectious bursal disease virus

IBV - Infectious bronchitis virus

IP - Immunoprecipitation

IPTG - Isopropyl β-D-1 thiogalactopyranoside

KSCN - Potassium cyanide

M9 - Minimal Media

Mab - Monoclonal antibody

M-MuLV - Moloney Murine Leukemia Virus

MPBS - Phosphate buffered saline supplemented with marvel milk powder

mRNA - Messenger Ribonucleic acid

NI – Neutralisation Index

PBS - Phosphate buffered saline

PBST – Phosphate buffered saline supplemented with 0.1 % Tween

PEG - Polyethylene glycol

pIII - Gene 3 protein

pVIII - Gene 8 protein

RF - Replicative form

RFLP - Restriction fragment length polymorphism

RNA - Ribonucleic acid

RT-PCR - Reverse transcription polymerase chain reaction

S1 - Subunit 1 protein

SARS-Cov - Severe Acute Respiratory Syndrome Coronavirus

scFv - Single chain variable fragment

SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SOC - Super Optimal Broth

SPF - Specific pathogen free

ssRNA - Single stranded Ribonucleic acid

SUMO - Small ubiquitin-related modifier gene

TEMED – Tetramethylethylenediamine

TMB – Tetramethylbenzidine

TOC - Tracheal organ cultures

TSA – Trypticase soy agar

TY- Tryptone yeast

TYE - Tryptone yeast extract

UTR - Untranslated region

VH - Variable heavy

VH-CDR3 - CDR3 of the heavy chain

VL - Variable light

VN - Virus neutralisation

Chapter 1

Literature review

1. Introduction

1.1. An overview of Infectious bronchitis virus

1.1.1. Virus classification

Infectious bronchitis virus (IBV) forms part of the Nidovirales order. All viruses within this order are enveloped, non-segmented positive-sense RNA viruses with large genomes (Kint *et al.*, 2015). Other features of viruses in the Nidovirales order include conserved genomic organisation, expression of non-structural genes by ribosomal frame shifting, large replicase-transcriptase polyproteins and the expression of downstream genes by synthesis of 3′ nested sub-genomic mRNAs (Kint *et al.*, 2015). IBV is further classified under the family Coronaviridae that contains four genera, of which all represent pathogens of veterinary or human importance (Beach & Schalm, 1936). Infectious bronchitis virus belongs to the *Gammacoronavirus* genus associated with Coronavirus pathogens in poultry and mammalian species (OIE, 2013; Abolnik, 2015). The Coronavirus is further allocated into three distinct groups based on antigenic properties and sequencing data. According to De Wit (2000) the different IBV can be identified and allocated in three different types. Proteotype is used to provide the immune response of the chicken and efficiency of a vaccine. The second and third types are antigenic, which include the serotypes that focus on antigen-antibody reaction, and genotypes which are based on the genome characterisation. The prevalence of IBV is not limited to chickens and recently it has been proven that other poultry species such as geese, ducks, and pigeons also play a role in the spread of avian IBV strains worldwide (Pattison *et al*., 2008; Awad *et al.*, 2014).

1.1.2. Genomic properties

Infectious bronchitis virus is 120 nm in diameter with a pleomorphic envelope and has a helical symmetric nucleocapsid (OIE, 2013; Kint *et al*., 2015). Most genome regions are conserved, non-segmented and codes for four structural proteins namely the spike (S) glycoprotein, nucleocapsid (N) phosphoprotein, membrane (M) glycoprotein and the envelope (E) protein (Al-Beltagi *et al*., 2014). The IBV single-stranded (ssRNA) genome consists of roughly 30 kbp and is arranged as follows: 5’ untranslated region (UTR)–1a/1b–S1/S2–3a/3b–E–M–5a/5b–N– 3’ UTR (Hewson *et al.*, 2012; Abolnik, 2015).

The N protein is known for containing highly conserved amino acid and nucleotide sequences and aids the replication process (Bande *et al.*, 2016). The M protein is the most abundant trans-membrane protein and plays a role in virus particle assembly (Kint *et al*., 2015). The E protein is present in small quantities, within the Golgi complex of infected cells, which creates the incentive for envelope maturation (Kint *et al*., 2015). It is further associated with envelope assembly, budding, ion channel activity, and apoptosis. Another role of the E protein is the promotion of viral release by altering the secretory pathway of cells (Bande *et al.*, 2016; Kint *et al*., 2015).

A unique feature of the IBV coronavirus is the presence of club-shaped S proteins that emanate from the virion surface. This protein contains the most important antigenic and functional regions (Pattison *et al.*, 2008; Wang, 2016). The S protein is post-translationally cleaved into S1 (N-terminal) and S2 (C-terminal) subunits and contains a high variance in nucleotide sequences (Al-Beltagi *et al*., 2014). The S2 subunit is anchored in the viral membrane by a small hydrophobic trans-membrane stalk, while the S1 forms the rounded part of the spike (Al-Beltagi *et al*., 2014). The S1 subunit plays an important role in the attachment and entry of the virus and has been considered as the determinant for viral diversity and immune protection (Kant *et al.*, 1992; Bande *et al.*, 2016). The S2 subunit drives virus-cell fusion and is more conserved and possesses an immuno-dominant region located in the N-terminal which can induce neutralising, but not serotype-specific antibodies (Abolnik, 2015). The S1 subunit consists of about 520 amino acids, including serotype specific hypervariable regions that induce neutralising and haemagglutinating antibodies (Cavanagh *et al.*, 1986).

The IBV life cycle within a host starts by the attachment and entry initiated by the interactions between the S1 subunit protein and sialic acid receptor (Abolnik, 2015; Wang, 2016). This interaction determines if a coronavirus can infect the specific species and also directs the tropism of the virus. After attachment, the virus enters the cytosol of the host cell by proteolytic cleavage of the S1 followed by the fusion of the viral and cellular membranes resulting in the release of the viral genome into the cytoplasm. Translation of the replicase gene and assembly of the viral replicase complexes occurs after the entrance of the viral genome, followed by synthesis of both genomic and sub-genomic viral RNA (Bande *et al.*, 2016). The viral structural proteins are translated and inserted into the endoplasmic reticulum that moves along the secretory pathway into the endoplasmic reticulum – Golgi intermediate compartment (ERGIC) (Bande *et al.*, 2016). The viral genomes are then encapsulated, forming mature virions that are transported and released by exocytosis (Kint *et al*, 2015; Bande *et al.*, 2016).

RNA viruses possess an inherent ability to quickly adapt to environmental changes by introducing mutations during replication (Hewson *et al*., 2012). Variation is due to genome

mutations and recombination of different IBV strains that occur when the viral RNA-dependent RNA-polymerase, without proofreading ability, replicates the viral genome (Mckinley *et al.*, 2008). The mutation rate of IBV has been estimated to range between 10^{-3} and 10^{-5} substitutions per nucleotide (Lee & Jackwood, 2001). Various studies around the world have been conducted on the variations on the S1 subunit, since variation within strains explains the prevalence of IBV in vaccinated flocks (OIE, 2013). Variations of the amino acid sequence cause a decrease in cross protection of vaccines between strains that leads to the development of endemic vaccines for specific strains (Al-Beltagi *et al*., 2014). A study conducted by Pohuang and co-workers (2014) showed variation in a QX strain in Thailand. This specific serotype was first identified in 1996 in China and has since spread to other countries (Wang *et al*., 1998). The strain isolated in Thailand shows close relations to that of other countries but has a definite variation in pathogenicity. As mentioned, the diversity on the S1 subunit region is the major cause of variation. Therefore, characterization of the sequence of S1 subunit is necessary to identify the virus strains and to compare similarities with vaccine strains (Bakhshesh *et al.*, 2016). Pohuang and co-workers (2014) showed that the QX IBV strain arose due to recombinant genetic variation. A heterologous IBV vaccine against the virus showed little cross protection, which is expected as cross protection decreases as the degree of amino acid identity between S1 subunits decreases. The vaccine strains of IBV used in Thailand consist of H120, Massachusetts, Massachusetts and Connecticut, Massachusetts 5 and Massachusetts 4-91 strains, which differ from the QX IBV strain identified (Pohuang *et al*., 2014). Due to the different strains, the vaccine protection may be insufficient, but a protectotype can be used to provide a broader scope of protection against the heterologous serotypes (Sarueng *et al.*, 2014).

1.1.3. Epidemiology and World Organisation for Animal Health (OIE) status

Infectious bronchitis virus is distributed worldwide as the etiological agent of infectious bronchitis (IB) within poultry. The first incident of IB was reported in 1931 in the United States where the Beaudette strain was isolated from young chicks and it was then defined as an acute, highly infectious, respiratory disease of chickens (Beach & Schalm, 1936). Following this, the M41 strain was isolated in 1937. These two strains were serologically similar and the first live IBV vaccine was created based on the M41 strain (Cavanagh *et al.*, 1992). Avian IBV has been isolated in broilers, layers and breeder chickens and can infect chickens of all ages (Butcher *et al*., 2015). The virus can be transmitted by direct and indirect methods. Indirect methods of infection include mechanical spread by contaminated equipment, manure fertilisers, egg packing materials and the contamination of egg surfaces (OIE, 2013). Wind can

also contribute to the spread of IBV between farms, while direct transmission occur directly between chickens within susceptible flocks (Ignjatovic & Sapats, 2000).

Initially, IBV replicates in the upper respiratory tract but replication may also occur in the oesophagus, proventriculus, duodenum, jejunum, bursa of Fabricius, rectum and cloaca (Awad *et al*., 2014). The virus is highly contagious and is shed by both the respiratory tract and the faecal route (Awad *et al*., 2014; Chacón *et al.*, 2014). Infectious bronchitis virus is temperature sensitive, unstable at a high pH and easily inactivated by common disinfectants. However, IBV can remain viable for several weeks within the faeces of infected chickens (Greenacre, 2013; Butcher *et al.*, 2015).

The main effects of this virus on chickens include respiratory tract infection, egg laying problems and renal damage (Pattison *et al.*, 2008). The adverse effects of IBV are not just on poultry but on the poultry industry as well. In layers and breeders production losses occur due to delayed maturity, decline of egg quantity and quality, poor weight gain, downgrading of carcasses, and mortality (Ignjatovic & Sapats, 2000). The mortality rate of IBV depends on the presence of secondary infections, flock age, immune status, management, environmental factors and strain type. Infectious bronchitis virus can infect chickens of all ages but younger chickens are more susceptible (Awad *et al.*, 2014).

The incidence of IB varies in different geographic regions. In some regions it is a consistent problem where in other parts, periodic outbreaks occurs, which may lead to epidemics. Colder environmental temperatures usually correlate with an increase in the incidence of IB due to the containment protocols to protect the flock from colder temperatures (Ignjatovic & Sapats, 2000). However, the occurrence of inadequate vaccination and antigenic variants also play a role in the difference between geographic regions (Ignjatovic & Sapats, 2000). According to the OIE (2017), IB is classified as a List B notifiable disease. This means that IBV is considered to be of socio-economic and/or public health importance and is important in the international trade of animals and animal products. Thus, poultry being transported between countries should be tested for IBV. IBV cannot be transmitted from hens to eggs and therefore hatching eggs pose no risk (OIE, 2017). Chickens that tested negative should be kept in quarantine conditions for at least four weeks as well as live chickens that tested positive as the virus can be spread by faeces (OIE, 2017).

1.1.4. Clinical signs and symptoms

As mentioned, the most common symptoms of IB are of respiratory tract descent, although other clinical forms include reproductive disorders and nephritis (Awad *et al.*, 2014).

Respiratory signs include tracheal rales, gasping, sneezing, watery nasal discharge, lacrimation and facial swelling (OIE, 2013). The respiratory disease symptoms are mainly based on the age of chicks and the virus strain and is the most common cause of reproductive tract damage within chickens (Ignjatovic & Sapats, 2000). Oviduct damage leads to a decrease in egg quantity and quality (Awad *et al.*, 2014). Eggs are usually rough-shelled or possess watery yolk, which leads to a decrease in egg production of 5% to 10% for about 10 to 14 days (Greenacre, 2013). Examples of the decrease in egg quality can be seen in Figure 1 (The Chicken Vet, 2018).

Figure 1: Representation of eggs produced by chickens that were infected with IBV.

Some strains may also be nephropathogenic and cause mortality at a higher rate than strains infecting the respiratory or reproductive systems (Bande *et al.*, 2016). Nephropathogenic strains are usually characterised by mild and transient respiratory signs followed by depression, ruffled feathers, rapid weight loss and diarrhoea. The nephropathogenic strains first replicate within the trachea and then spreads to the kidney tissue, although the route of dispersal is not known (Ignjatovic & Sapats, 2000). Clinical signs will develop within 36 to 48 hours and will last for approximately 7 days in the absence of secondary infections (Greenacre, 2013). The clinical signs are influenced by the field virus strain, the host, environment, and the management and biosecurity (Ganapathy, 2009). Initial infections are aggravated by secondary infections and co-infection with immunosuppressive viruses, such as infectious bursal disease virus (IBDV). Although no treatment for IBV exist, secondary infections can be treated with antibiotics (Pattison *et al.,* 2008; OIE, 2013; Bande *et al.*, 2016)

Clinical diagnosis requires virus isolation from infected flocks by detection of the IBV virus itself or a specific antibody response (De Wit, 2000). Virus isolation is done by the use of embryonated specific pathogen free (SPF) eggs, cell cultures, and tracheal organ cultures

(TOC), which is followed by genotype testing (Bande *et al.*, 2016). The IBV genotypes are mainly identified by reverse-transcription polymerase chain reaction (RT-PCR) and the serotypes are determined by haemagglutination inhibition (HI) tests and enzyme-linked immunosorbent assays (ELISA), although it should be taken into account that ELISA tests are more sensitive and easier applied (Pattison *et al*., 2008; Bande *et al.*, 2016). Additional detection tests include electron microscopy, monoclonal antibodies, virus neutralisation (VN), immunohistochemical or immunofluorescence tests, and immunisation challenge trials in chickens (OIE, 2013). Variant strains of IBV can be detected by antigenic variation, which is determined by VN and genotyping (Pattison *et al*., 2008).

1.1.5. Control and prevention

There is currently no treatment available for IBV infections. However, prevention methods are in place such as disinfection, biosafety protocols and vaccination (Cavanagh, 2007; Kint *et al*., 2015). Infectious bronchitis influences the economics of the poultry industry in terms of egg production and quality and performance in broilers (Pattison *et al*., 2008). Infectious bronchitis virus variants are of economic importance since a unique lineage can be linked to different geographical areas worldwide (Pattison *et al*., 2008). Examples include the XQ strain identified in Thailand (Pohuang *et al*., 2014), various strains from Egypt (Al-Beltagi *et al*., 2014) and IBV isolates from Canada that were clustered into nine different genotypes belonging to four groups (Martin *et al*., 2014). Variants of IBV are becoming more prominent as simpler detection methods are developed. Variant IBV influences virus isolation and serology findings and can also lead to the variant strains overcoming the immunity induced by vaccination for non-variant strains (OIE, 2013). The Massachusetts strains are most commonly used for vaccines, because of the provided cross protection and the fact that the strain matches the most commonly encountered strains in the field (CEVA, 2005). The epidemiology and the control of the IB disease worldwide are therefore influenced by the occurrence of variant strains (Pattison *et al*., 2008).

1.1.5.1. Vaccination

The only practical means of controlling IB is vaccination, which is routinely used throughout the intensive poultry industry. According to Cavanagh (2007) vaccination against IB has been practiced for an extended period, but the following features should be taken into account during the vaccination: First, protection is not long-lasting and revaccination is required. Secondly, the correct antigenic type vaccine should be used and thirdly, the timing and method of vaccination differs due to circumstances (Ignjatovic & Sapats, 2000). Vaccination should be

performed as early as possible, thus a live attenuated vaccine is usually given to chicks at one day of age in the hatchery by spray, and later by drinking water or spray (Tarpey *et al.*, 2006).

Live vaccines are produced by the passaging of IBV strains in embryonated SPF chicken eggs to achieve reduced virulence. This type of vaccine is used worldwide and can be classified as a mild or a virulent vaccine. The vaccine is administered by spray or in drinking water (Ignjatovic & Sapats, 2000). Day-old chicks are exposed to a vaccine with low virulence that does not cause retarded growth as with more virulent vaccines, which are given as a booster vaccine (Cavanagh, 2007). A disadvantage of low virulence vaccines is the low level of immunity provided, as it only protects the respiratory tract and not kidney or oviduct tissues. Areas with a high density of chickens require multi-vaccinations to prevent back-passaging, while broilers will be sufficiently protected by a single vaccination. The most frequently used vaccines are based on the Massachusetts strains such as M41, Ma5 and H120 (Ignjatovic & Sapats, 2000). Due to the occurrence of variant strains, two or more antigenic types of vaccines are usually required, as cross-protection decreases as the similarity between amino acids of the S1 subunit of strains decreases (Cavanagh, 2007).

Inactivated vaccines are produced from IBV infected allantoic fluid, which is inactivated and prepared as an oil emulsion vaccine (Ignjatovic & Sapats, 2000). The goal of inactivated vaccines are to produce long-lasting immunity (Cavanagh, 2007). It is commonly used in layers and breeders, as it is administered by inoculations at thirteen to eighteen weeks of age which lead to the assumption that the chickens have already been vaccinated with live attenuated vaccines. An advantage of the use of inactivated vaccines is the high and uniform levels of antibodies maintained for an extended period of time while a disadvantage is the expensive nature of production (Ignjatovic & Sapats, 2000).

1.2. IBV detection and isolation

There is a need for diagnostic tools that can identify IB when clinical symptoms are identified in the field. These tools have to be able to detect IBV in a rapid manner and differentiate between the IBV strains (Villarreal, 2010). The detection and differentiation of the virus is important due to the occurrence of variant strains and the fact that respiratory symptoms and poor egg production in poultry are not specific to IBV (De Wit, 2000). If the specific IBV strains are identified this information can be used with the selection of a vaccine program to provide the most efficient protection for the flock.

Although there is a list of different diagnostic techniques, these techniques all have advantages and disadvantages. In addition to the disadvantages of the techniques, the

detection of IBV can also be problematic (Villarreal, 2010). There are factors that influence the IBV detection but the biggest influencing factor is the method of sampling. The time between infection and sampling is a determinant factor as the highest concentration can be found during the first three to five days post infection, the chickens' immunity during infection and number of chickens sampled should also be taken into account (Villarreal, 2010). Furthermore, the selection of organs and the quality and storage of samples are also important as IBV samples should not be stored on dry ice due to CO_2 being virucidal to IBV (National Research Council, 1959; OIE, 2013).

Diagnosis of IBV is currently done by both direct and indirect techniques. The most commonly used techniques are used in combination. Infectious bronchitis virus can be diagnosed by virus cultivation and isolation alongside the use of RT-PCR (De Wit, 2000; Wang, 2016). The steps rely on the replication of the virus in embryonated SPF eggs or TOCs, followed by reverse transcribing genomic RNA into cDNA and amplification through RT-PCR. After amplification, restriction fragment length polymorphism (RFLP) can be used to determine the specificity of the RT-PCR product (De Wit, 2000). Reverse transcriptase PCR is increasingly used to identify the S protein genotypes of IBV field strains. A major disadvantage of using RT-PCR is the fact that it identifies IBV genotypes but does not distinguish between infectious and non-infectious virus particles and cannot successfully differentiate between genetically different strains classified as the same serovar (Okino *et al*., 2005).

A serovar can be defined as strains which possess similar antigens that can be detected by immunodiffusion but differs in cross neutralisation tests (Cowen & Hitchner, 1973). Serological tests can be performed by commercial ELISA kits, VN and HI tests (OIE, 2013). A major disadvantage of serotyping is the lack of standardisation between the different systems. Consistency can be achieved by the standardisation of antisera and the increase in accuracy of serology tests (De Wit, 2000).

Indirect methods for IBV diagnosis include the detection of antibodies by ELISA, VN, Immunodiffusion in agar gel and HI tests (Villarreal, 2010). The detection of IBV antigens is performed through the use of IBV antibodies, which can either be anti-sera or monoclonal antibodies (Mabs). Anti-sera has problems in terms of standardisation caused by biological variation while using Mabs has the disadvantage of variation occurring in an epitope which can prevent binding (De Wit, 2000). Prevention methods can be implemented by using antibodies which bind to conserved regions of the virus and by using a mixture of Mabs (De Wit, 2000). Enzyme-linked immunosorbent assays monitor antigen-antibody responses and allow the detection and titration of antibodies from a pool of serum samples (Villarreal, 2010). Enzyme-linked immunosorbent assay was originally developed by both Engvall and Perlmann

together with van Weeman and Schuurs in 1971 (Mockett & Darbyshire, 1981). In addition, the commercial kits are cross reactive allowing for the general serological monitoring of vaccinal responses and field challenges (OIE, 2013). Enzyme-linked immunosorbent assay has a higher degree of sensitivity than VN or HI methods (Mockett & Darbyshire, 1981; OIE, 2013). When comparing ELISA and HI tests, ELISA has been used to detect antibodies in chickens with high sensitivity while HI test results cannot be used with a high degree of confidence due to cross-reactive antibodies from multiple infections and vaccinations (Mockett & Darbyshire, 1981). An ELISA can detect early produced IgG antibodies and an antigen ELISA can be used to detect IBV antigens in allantoic fluid of inoculated eggs (Mockett & Darbyshire, 1981). Although the principle seems straight forward, there are reported limitations that include the low sensitivity for detecting IBV antigens directly from chicken organs and ELISA is more limited to research purposes than other methods such as HI (Mockett & Darbyshire, 1981; De Wit, 2000).

1.2.1. Infectious bronchitis virus cultivation

Specific pathogen free (SPF) embryonated eggs are used for the cultivation and propagation of a vast range of viruses including mumps virus (Lundbäck, 1955), Newcastle disease virus (Yilma & Mekonnen, 2015), Blue tongue virus, rabies virus and Infectious bronchitis virus (Shittu *et al*., 2016; Yu *et al*., 2017). This is due to the fact that the mentioned virussed and IBV are epitheliotropic virusses that replicate in a variety of epithelial tissues (Kint *et al*., 2015). Specific pathogen free eggs are used for the cultivation as eggs that are non-SPF could possibly possess antibodies that may interfere with the replication of IBV (Kint *et al*., 2015). Embryos that are 9-11 days of age are usually inoculated through the allantoic route, as it is the simplest method that provides the highest titres (Kint *et al*., 2015). Alternatively, the amniotic, yolk sac or chorioallantoic membrane inoculation routes can be used. These techniques are successful due to the fact that IBV can propagate within biological systems such as the embryonated eggs (Kint *et al*., 2015). As the virus propagates within the eggs, morphological changes are observed. Common changes include: curling of embryos with wry neck and deformed feet compressed over the head; retardation of growth; thickened, dried, fibrotic amniotic membrane; less amniotic fluid and weak embryos (National Research Council, 1959).

The amount of virus particles can be determined by an end-point virus titration assay. During a titration assay, embryonated SPF eggs are incubated with a ten-fold serial dilution of a virus and monitored. The virus titre can then be calculated using the Reed and Muench (1938) method or the Spearman and Karber method (Spearman, 1908). The virus titre is defined as the reciprocal of the dilution at which 50 % of the inoculated embryos die (Reed and Muench,

1938).The EID_{50} is the amount of virus that will infect 50 % of virus-inoculated embryonated SPF eggs while the LD_{50} is termed as the amount of virus that will kill 50 % of virus-inoculated embryonated eggs (Kint *et al*., 2015).

1.2.2. Virus neutralisation assay

A virus neutralisation assay can be used to detect antibodies with the ability to neutralise virus infectivity (Hitchner, 1973). Neutralising antibodies are highly specific and target important antigens of the viral surface that play a role in the process of cell absorption (Villareal, 2010). These assays are serovar-specific and require sera with no cross reaction with other serovars. Virus neutralisation assays can be carried out in SPF embryonated eggs, cell cultures or in tracheal ring cultures (De wit, 2000; Bande *et al.*, 2016). To perform these assays the virus concentration should first be standardised and the fixed amount required should be determined. Further, two-fold or ten-fold serial dilutions are needed of unknown sera which is then mixed with a fixed amount of infective virus. The reaction is allowed for a specific time and temperature, depending on the virus type. The virus-serum mixture is then assayed for virus infectivity using end-point titration methods to determine the highest dilution of sera that possesses detectable neutralising antibodies (Hitchner, 1973).

The lack of standardisation for VN assays make comparison very difficult and therefore a VN assay should be decided on by sensitivity, reproducibility and titre detection (Wooley *et al.*, 1976; De wit, 2000). Injecting chicken embryos with Embryo-Infectious-Dose-50 % (EID_{50}) can be used to determine the fraction of surviving virus particles which can then be used to determine the sero-groups. This procedure can be altered to determine if recombinant antibody fragments are efficient in neutralising virus particles (Guo *et al.*, 2010). A study conducted in 1984 by Mockett and co-workers on the Massachusetts M41 strain of IBV determined that the virus was neutralised by the majority of monoclonal antibodies to the spike glycoprotein and some anti-membrane protein monoclonal antibodies can neutralise the virus if complement is present with the use of VN assays.

1.3. Phage display technology

1.3.1. Phage display technology overview

The use of phage display has proven to be an influential technique to display peptides or proteins within libraries and is very well suited for high-throughput generation of antibodies and antibody fragments for research purposes such as massive target identification (Kretzschmar & von Rüden, 2002). The isolation of monoclonal antibodies using large phage antibody libraries is considered to be the most successful application of this technology

(Kretzschmar & von Rüden, 2002). Additionally, techniques have been developed to build large libraries of antibody fragments containing at least 10^8 individual fragments (Hengerer *et al.*, 1999). The technology provides a versatile method, for not only screening for antigen specific antibodies, but also screening for protein interactions which can be further applied in therapeutic areas (Dale *et al.*, 2013).

The basic principle of phage display technology exist in three steps namely, the generation of a gene library, inserting genes into phage particles that are expressed on the phage surface and the selection of genes for binding antibody fragments by affinity purification (Azzazy & Highsmith, 2002). These three steps will result in antibody clones that can be used for further analysis (Hengerer *et al.*, 1999). The first ligand that was successfully expressed on a phage surface was antibodies in 1989 (Rader & Barbas, 1997). The procedure was done by fusing the coding sequence of the antibody variable (V) regions that encodes for a single chain variable fragment (scFv) to the amino terminus of the phage minor coat protein (Azzazy & Highsmith, 2002).

The most widely used antibody library methodologies are based on the use of a filamentous bacteriophage that infects *Escherichia coli*. The phage genome consists of 11 genes that express about 2700 copies of gene 8 protein (pVIII), which is a 50 amino acid residue protein also known as the major capsid protein, and 3 to 5 copies of the gene 3-encoded adsorption protein (pIII) that is one of three minor coat proteins and consists of 406 amino acids (Azzazy & Highsmith, 2002). The non-lytic filamentous bacteriophage M13 induces a state in which the infected bacteria produce and secrete phage particles without undergoing lysis and infect strains of *E. coli* by the attachment of the phage pIII to the F-pilus (Azzazy & Highsmith, 2002). This is then followed by the entering of circular ssDNA into the bacterium where it converts into the double-stranded plasmid like replicative form (RF) (Azzazy & Highsmith, 2002). The RF then undergoes rolling circle replication to create ssDNA, which serves as a template for expression of the phage surface proteins (Azzazy & Highsmith, 2002).

The process of sub-cloning of phage genomes into expression vectors can influence transformation efficiency of scFvs. To eradicate the sub-cloning process, phagemid vectors can be implemented. According to Griffiths & Duncan (1998) phagemids are hybrids of phage and plasmid vectors with a high transformation efficiency and can undergo direct secretion of soluble antibody fragments without sub-cloning and is therefore preferred above other phage vectors (Hoogenboom *et al.*, 1991). Phagemids not only contain the origins of replications for both M13 phage and *E. coli*, but also the *pIII* gene cloning sites and an antibiotic resistance gene (Azzazy & Highsmith, 2002). The scFv is fused at the N-terminus on truncated or mature pIII proteins and utilise the lacZ promotor to drive expression of the antibody-pIII fusion by

removing the catabolic repressor (glucose) that leads to expression of monovalent phage particles (Hoogenboom *et al.*, 1991; Lee *et al.*, 2007; Tikunova & Morozova, 2009). Phagemids can either be grown as plasmids or as recombinant M13 phages by "phage rescue" where a helper phage that contains a slightly defective origin of replication supplies all structural proteins required (Azzazy & Highsmith, 2002). The phage particles may incorporate either pIII derived from the helper phage or the polypeptide-pIII fusion protein and the ratio depends on the phagemid type, growth conditions, the nature of the polypeptide fused to pIII, and proteolytic cleavage of antibody-pIII fusions (Azzazy & Highsmith, 2002; Lee *et al*., 2007). The phagemid vector system has a few limitations such as gene deletion and plasmid instability, nevertheless it has been successfully used to isolate antibody fragments by conditional display on phages or the secretion of the antibody in the periplasmic space of *E. coli* (Azzazy & Highsmith, 2002).

Antibody libraries for single chain variable fragments (scFv) or antibody binding fragments (Fab) are created by genes that are cloned into engineered phage or phagemid vectors to be displayed on the bacteriophage surface (Kretzschmar & von Rüden, 2002). The library quality depends on the functional size and antibody sequence diversity for affinity and expression levels. The library design should also allow the rapid engineering of antibodies for optimization, and low immunogenicity of the antibodies for therapeutic applications (Hoogenboom *et al.,* 1991). The quality control can be achieved by determining the number of recombinant clones and if the phagemids contain the inserted fragment by PCR screening and blot analysis (Azzazy & Highsmith, 2002). Advantages of phage libraries include high throughput and easy engineering of antibodies. An additional benefit include the determination of antibody specificity which would have otherwise been eliminated in other methods (Kretzschmar & von Rüden, 2002).

1.3.2. Overview of phage display libraries

There are different types of antibody phage libraries including naïve, synthetic, immune, and universal libraries, but universal libraries differ from immune-derived libraries by being antigen-independent (Kretzschmar & von Rüden, 2002). Naïve repertoires are also known as "single pot libraries" and are made up from the V-genes from the IgM mRNA of B-cells isolated from unimmunised human donors' peripheral blood lymphocytes, bone marrow, spleen cells, or from animal sources (Mai *et al.*, 2006). Key advantages of single-pot repertoires include the isolation of human antibodies to self, non-immunogenic or toxic antigens, usability for all antigens, short antibody generation time, and direct isolation of high affinity antibodies (Griffiths & Duncan, 1998). The disadvantages are the low affinity of antibodies isolated from small sized libraries; time consuming when constructing large libraries, and the content and

quality which are influenced by unequal expression of the V-genes repertoire, unknown history of the B-cell donor, and potential limited diversity of the IgM repertoire (Azzazy & Highsmith, 2002).

The V-genes from immune libraries are derived from the IgG mRNA of B-cells from an immunised animal or human (Mai *et al.*, 2006). The immune antibody library will be enriched in antigen-specific antibodies, and some of these antibodies will have undergone affinity maturation by the immune system (Griffiths & Duncan, 1998). High-affinity antibodies were reportedly derived from mice, chickens, and rabbits but disadvantages of immune libraries include extended time periods required for immunization, lack of immune response to self or toxic antigens, unpredictability of the immune response to the antigen of interest, and that a new library must be constructed for each antigen (Azzazy & Highsmith, 2002).

Synthetic antibody libraries are built artificially by the *in vitro* assembly of V-gene segments and D/J segments. The specificity of an antibody resides in the six complement determining regions (CDRs) (Griffiths & Duncan, 1998; Lin *et al.*, 2011). The CDR3 of the heavy chain (VH-CDR3) is the most diverse loop in terms of composition and length and is most central to the epitope (Azzazy & Highsmith, 2002). Randomising the VH-CDR3 region using oligonucleotide directed mutagenesis or PCR-based techniques will provide several synthetic repertoires. These synthetic antibody repertoires increase the overall performance of the library and the functional library size and have the potential to control and define contents, local variability, and overall diversity (Hoogenboom *et al.,* 1991).

1.3.3. Antibody fragments as therapeutic treatment

The increased knowledge of recombinant technology, protein expression and immunoglobulin structures enable the alteration of these molecules with the aim of producing functional antigen binding molecules for therapeutic treatments (Miller *et al.*, 2005). According to Chadd & Chamow (2001), the use of antibodies for therapeutic treatment range from autoimmune disorders to cancer and viral or bacterial infections.

There are currently two main types of recombinant antibodies used for therapeutic treatment. The first type is based on the whole immunoglobulin molecule with a long half-life period (Andersen & Reilly, 2004), while the second type consists only of antibody molecules fragments (Azzazy & Highsmith, 2002). All these fragments are susceptible to proteolysis. The Fab fragment consists of variable and constant segments as well as light and heavy chains, linked by disulphide bonds and is larger than the fragment which is composed of only variable light (VL) and variable heavy (VH) regions (Monnier *et al*., 2013) (Figure 2). The fragment is

artificially bound by a single peptide chain allowing the formation of an antigen-binding site. The fragment is then referred to as a scFv fragment (Azzazy & Highsmith, 2002).

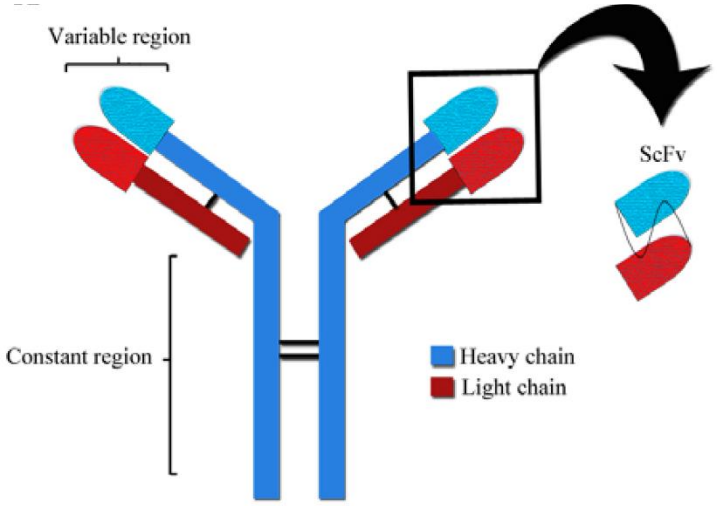

Figure 2: Schematic illustration of a single chain variable fragment (scFv) with variable light (VL) and variable heavy (VH) regions from a full length monoclonal antibody (Mab).

The Fab and scFv antibody fragments have more advantages in comparison to immunoglobulins for therapeutic applications (Kretzschmar & von Rüden, 2002). Advantages include higher penetration rate and rapid production in prokaryotes. Although antibody fragments seem to be more advantageous, complete immunoglobulins have longer serum half-lives and have the Fc-receptor that allows binding and removal by phagocytosis (Persic *et al.*, 1997). Formats for antibody fragment display are scFv fragments, Fab fragments, variable fragments with an engineered intermolecular disulphide bond to stabilise the VL-VH chains (dsFvs), and diabody fragments. Single chain variable fragments are more stable compared to Fab due to its small size and has lower affinity than the parent antibody (Hoogenboom *et al.*, 1991). Fab lack the tendency to dimerise which is considered as an advantage. Dimerization is frequently observed in scFv but scFv has a higher molecular weight which complicates selection and characterisation (Hoogenboom *et al.*, 1998). The scFv format has several other problems including the PCR primers used to amplify immunoglobulin genes that generally lack the efficient assembly of a linker sequence (Imai *et al.*, 2006). Antibody fragments, in general, have the advantage of protein domain separation by protease digestion which can then be cloned and displayed by the use of phage display technology (Azzazy & Highsmith, 2002).

1.3.3.1. Antibody selection

Unlike the laborious process of selecting conventional monoclonal antibodies, recombinant antibodies with the use of phage display can be directly selected during the panning process, allowing rapid isolation of highly specific antibodies (Sapats *et al.*, 2003). Panning and screening are the two consecutive steps for antibody selection with the use of phage libraries (Kretzschmar & von Rüden, 2002). Panning describes the process where the library phages are incubated with the selected antigen, unbound phages are discarded and remaining phages recovered after several washing steps by disrupting the phage-antigen interaction without compromising the phage infectivity. The recovered phages are amplified by infecting *E. coli* and the process is repeated (Kretzschmar & von Rüden, 2002).

Several panning strategies exist, such as selection using immobilised antigens where libraries are selected by flowing through an affinity column. Enzyme-linked immunosorbent assay is an excellent example of antigen adsorbed onto plastic surfaces (Kretzschmar & von Rüden, 2002). This method must take the conformational integrity of the immobilised antigen into account as some phage antibodies may not be able to recognise the native form. Another method includes selection using antigens in a labelled solution which overcomes issues with conformational changes and it allows for more accurate quantification with the use of lower concentrations (Azzazy & Highsmith, 2002).

Direct selection on cell surfaces can be carried out on either monolayers of adherent cells or on cells in suspension (Hoogenboom *et al.*, 1998). Simultaneous positive and negative selection may be applied to optimise the isolation of antigen-specific binders and minimise the binding of irrelevant binders. This is done by setting up a competition by adding a small number of antigen-positive target cells and an excess of antigen-negative “absorber” cells to serve as a sink for the nonspecific adherence of irrelevant binders. Alternatively, *in vivo* selection can also be done by directly inserting phage repertoires into animals followed by the collection of tissue-specific endothelial cell markers (Azzazy & Highsmith, 2002).

After a successful panning process, the screening process follows, which entails the selection of monoclonal antibodies from a polyclonal mixture. The infected *E. coli* cells are plated on a selective agar medium followed by the selection of single colonies allowing highly specific, monoclonal antibody clones to be obtained and which can then be further analysed (Kretzschmar & von Rüden, 2002).

The most efficient screening assays are fast, robust, and should be closely linked to the functional ligand requirements. Screening methods vary from as simple as ELISA plates to a

bioassay (Hoogenboom *et al.*, 1998). Phagemid vectors have been developed to speed up the screening process. Common problems that occur with the screening process are the antibody fragment expression levels in *E. coli* which are dependent on the primary sequence of the individual antibody and can vary to a large extent (from 10 mg/L to 100 mg/L). If the expression levels are insufficient, consideration should be given to using an alternative expression system (Kretzschmar & von Rüden, 2002). Enzyme-linked immunosorbent assay, Western blots, or immunofluorescence do not always provide a high enough affinity to be used as sensitive diagnostic reagents. This requires the improvement of phage antibody fragments affinity by multimer formation, affinity maturation by site-directed mutagenesis, and affinity maturation by chain shuffling (Azzazy & Highsmith, 2002). With these alterations, screening efficiencies can be increased, resulting in more specific antibody selection.

1.3.3.2. Single chain variable fragment

Single chain variable fragments are small polypeptides (25-30 kDa) that can recognise antigens by fusing the variable (V) domains of the heavy (H) and light (L) chains of immunoglobulins through a linker short peptide of which the orientation of the VH and VL chains is reported to affect the expression efficiency (Malpiedi *et al.*, 2013). The most common peptide linker is the flexible linker, $(Gly_4Ser)_3$, and that varies from 10 to 25 amino acids in length and typically include hydrophilic amino acids to avoid intercalation of the peptide between the V domains throughout protein folding (Ye *et al.*, 2008; Weisser & Hall, 2009; Ahmad *et al*., 2012). The V domains contain two conserved cysteine residues forming a disulphide bridge that is required for the stability of the folded polypeptide. Thus, scFvs contain two disulphide bridges to maintain its folding and antigen-binding properties (Jurado *et al.*, 2002). According to Zhang and co-workers (2002), the correct formation of disulphide bonds in scFvs is the crucial factor responsible for solubility of scFvs as the formation of disulphide bonds are considered a rate-limiting step for protein folding.

1.3.3.3. Single chain variable fragment and phage display library

Using phage display with scFv has advantages including the fact that phages are more stable, are better immunogens, and can reproduce rapidly and cost-effectively. Genes from phages can also be easily altered and higher affinity scFv mutants can be obtained by site-directed mutagenesis (Ahmad *et al.*, 2012). Leong and Chen (2008) stated that the scFv fragments becoming part of protein therapy has the advantage of synthesising proteins that can possibly be used as treatment. Usually monovalent antibody fragments, including the scFv fragments, have a low affinity, and a short half-life but due to cost-effectiveness and ease of expression and the additional benefit of being easily genetically engineered for improving specificity and

affinity make these fragments an attractive tool for therapeutic uses (Weisser & Hall, 2009; Wang *et al.*, 2013). scFv fragments have the ability to retain the original antigen-binding site allowing it to maintain its specific affinity for the antigen (Ye *et al.*, 2008; Weisser & Hall, 2009). The application of scFv fragments can be seen in proteomic research as it contributes to therapeutic treatments and can be applied in laboratory assays that includes immunological diagnoses of viruses, effectively neutralising viruses and inhibiting viral infection or replication (Malpiedi *et al*., 2013). Single chain variable fragments have been used against bluetongue virus, severe acute respiratory syndrome coronavirus and infectious bursal disease virus (Lin *et al.*, 2015).

To produce soluble scFv, antigen-positive phages are used to infect a specific strain of *E. coli* (e.g. *E. coli* HB2151) that will direct production of soluble scFv. Such *E. coli* strains are termed "non-suppressor" strains as they recognise an amber stop codon, engineered between the scFv gene and pIII in the phagemid, and only express the scFv without the pIII protein. The phagemid is also designed to introduce a tag fused to the expressed scFv, thereby permitting rapid and simple protein purification by immobilised metal affinity chromatography. Depending on the isolated clone, soluble antibodies may be present in the culture supernatant, the bacterial periplasm, and/or inside the bacterial cells. All three fractions must be isolated and analysed in a Western blot, using a commercially available conjugated anti-tag antibody, to determine the location of the soluble antibodies. Soluble scFvs are relatively simple to isolate, can be economically produced in bacteria in very large quantities, and do not require complex refolding procedures as insoluble scFv molecules (Azzazy & Highsmith, 2002).

1.4. Expression of recombinant antibody fragments

Each protein varies in terms of expression and is influenced by a range of factors such as solubility, stability and size. To express recombinant proteins successfully, the cloned gene must be transcribed and translated efficiently and therefore these factors should be considered. Antibodies differ due to amino acid sequence and expression efficiency depends on the type of molecule, required quantity and quality as well as the purity of the final product (Verma *et al.*, 1998).

According to Andersen and Krummen (2002), *E. coli* is the most commonly used organism for recombinant antibody expression. Reasons for this include various advantages provided by this system, one of which being the ability to produce large quantities in a shorter time due to fast reproduction times, small quantity recombinant DNA needed for transformation and the inexpensive nature of the process. To ensure successful expression, the desired gene should be inserted in the correct frame. To control expression and prevent mutation and gene loss,

an inducible promotor is used. The ribosome binding site also plays a role as it consists of an initiation codon and Shine-Dalgarno sequence (Verma *et al*., 1998). Other than the promotor and binding site, the transcription terminator is also of high importance as it prevents transcription beyond the desired gene and adds stability to the DNA (Verma *et al*., 1998).

Yin and co-workers (2007) state that proteins can be expressed within *E. coli* in three forms. The first form is fusion proteins where the gene of interest is fused with a sequence and poly-histidine tag that can then be used for further protein monitoring. The second form is secreted proteins that can accumulate in the extracellular medium or in the periplasm. The third form is inclusion bodies which contains insoluble molecules and mis-folded proteins that require refolding processes (Verma *et al*., 1998). Refolding inclusion bodies can be successfully achieved by on-column refolding method and folding can be achieved by cytoplasmic expression that promote proper folding and oxidation *in vivo*. Alternatively, expression can be directed to the periplasmic space and antibody fragments can be obtained through osmotic lysis. High concentrations of reasonably pure scFv fragments can be easily obtained by directing expression into the periplasmic space (Weisser & Hall, 2009).

E. coli cannot express all genes as expression depends on the structural features of the gene, stability and translational efficiency of mRNA, degree of protein folding, degradation by proteases, codon usage, and toxicity of the protein to the host (Sivashanmugam *et al.*, 2009). Since *E. coli* is a prokaryote, no post-translational modifications occur, which is a disadvantage of this expression system. *E. coli* also tends to retain the amino terminal methionine which affects the stability and the immunogenicity (Yin *et al.*, 2007). The product can also be affected by the lack of chaperones (Yin *et al.*, 2007). *E. coli* strain BL21 (DE3) is considered to be the best candidate to bypass the intracellular proteases which prevent the expression of some bioactive proteins. This is possible due to the lack of the lon protease as well as proteases of the outer membrane (Yin *et al.*, 2007). It has become apparent that problems with *E. coli* expression of antibodies are reduced with the use of phage libraries as phage selection operates not only to isolate ‘good binders’ but also ‘good expressors’. In addition to the good yield by use of phage libraries, changing the sequence and length of the linker peptide between the scFvs can be used to increase the yield (Verma *et al*., 1998). Alternative to *E. coli*, *Bacillus subtilis* can also be utilised with the advantage of no lipopolysaccharide production, easy recombination and the secretion directly into surrounding medium (Yin *et al.*, 2007).

1.4.1. Single chain variable fragment expression

Before expressing a recombinant protein, a few important factors should be considered such as the origin of the protein (Kornberg, 2006). A protein from prokaryotic origin will most likely be expressed within *E. coli,* while expression of a protein from eukaryotic origin, such as scFv, may be less successful. According to Jurado and co-workers (2002) *E. coli* has been utilised in the expression of recombinant antibody fragments such as scFv. In addition to the type of protein, the solubility, post-translational modifications and codon usage also plays a major role. As stated by Ye and collegues (2008), high-level expression of scFv has not been successful due to the fragment containing disulphide bonds, which are difficult to form in an intracellular environment and the consequent formation of inclusion bodies. Inclusion bodies are a frequent occurrence as a result of over-expression of recombinant antibody fragments without a leader sequence (Zhang *et al*., 2002). The antibodies must then again be denatured *in vitro* for correct protein folding to occur. This problem can be prevented by the use of a single peptide which directs secretion into the periplasmic space that lies between inner and outer membranes of Gram-negative bacteria (Ahmad *et al*., 2012). Other possible solutions include reducing the cultivation temperature or Isopropyl β-D-1 thiogalactopyranoside (IPTG) concentration, as well as reducing cultivation time which allows recombinant proteins time to fold properly (Rosano and Ceccarelli, 2009). Alternatively, as a study conducted by Ye and co-workers (2008) proved, the attachment of a highly stable and compact small ubiquitin-related modifier gene (SUMO) structure to the N-terminus of the scFv fragment assists with protein folding and enhance solubility and expression. This is based on SUMO being an ubiquitin-like protein, which has proven chaperone activity. It was also reported that the solubility and affinity of scFv was improved by co-expression of molecular chaperones such as Skp, Dsbc, and FkpA (Wang *et al.*, 2013).

The process of heterologous expression is done by collecting proteins followed by precipitation and purification. Precipitation methods are based on the stability of proteins and interactions with temperature, pH, salts, lipophilic agents, cross-linking agents and water-extracting agents. However, as mentioned with expression, the precipitation conditions of proteins differ with each type of protein (Kornberg, 2006).

Ammonium sulphate precipitation is most widely used due to affordability, prevention of microbial growth, protection against protein denaturation, and application to produce a bulk amount of precipitate (Kornberg, 2006). The basic principle resides in protein solubility influenced by salt concentrations. At a low salt concentration, protein charged groups are stabilised and moves into the solution. This process is referred to as salting-in. Salting-out occurs as the concentration of salt increases and water becomes less available resulting in

precipitation of the proteins. After precipitation, the salt should be removed as it may impact protein assays and purification methods (Wingfield, 2016). Dialysis is the most common method to remove salts as it makes use of semi-permeable membranes, which allows small salt ions to move freely past the membrane while larger molecules, such as proteins, are retained. The protein and salt mixture is placed within a dialysis membrane followed by the placing of the tube in a low salt buffer. This allows the salt ions to move out to the buffer and water to move into the tube through passive diffusion. As water moves into the tube, the protein concentration will decrease but the proteins can be concentrated by various methods including, the use of ultrafiltration spin-columns (Kornberg, 2006).

1.4.2. Purification of single chain variable fragments

Purification and first extraction of scFv is mostly conducted by affinity-based chromatography, which is based on the specific interaction between the antibody molecule and a complementary ligand which binds after passing a solution containing it through the chromatographic column under favourable conditions (Jungbauer *et al.*, 2004). This method reduces non-specific interactions, increasing yields and facilitates the removal of contaminants. Alternatively, non-affinity chromatography, immunoprecipitation, liquid–liquid extraction, and cryogels can also be implemented (Leong & Chen, 2008). Refolding of scFv fragments located within inclusion bodies are most commonly done by immobilised metal affinity chromatography (Jungbauer *et al*., 2004). According to Willey and co-workers (2011), immunoprecipitation is a reaction that involves soluble antigens that react with antibodies to from a large aggregate that can precipitate out of a solution. This process occurs as antibodies become attached to antigens and settle out of a solution due to sufficient size but only if there is an optimal ratio of antigen to antibody (Willey *et al*., 2011).

The most common method of purification of recombinant scFvs is by histidine-tagging proteins. This works due to a sequence of six or more histidine residues attached to the C-or N-terminus of the protein. Due to histidine possessing a high affinity for metal-ions a protein bearing material will be able to bind to a solid material, referred to as resin, which is covered in nickel or cobalt atoms. This allows the separation of his-tagged proteins from other cellular proteins (Willey *et al*., 2011). Similar methods with the same principle exist for Myc-c tagged proteins (Thermo Scientific, Undated *a*).

1.4.3. Quantification detection of single chain variable fragments

Protein concentration can be determined using a vast range of assays, including the Bradford assay and the bicinchoninic acid (BCA) assay. The BCA assay is based on a similar principle

as the Bradford assay, with a few alterations to increase sensitivity. The principle of the Bradford assay is based on the binding of a protein to a dye under acidic conditions which results in a colour change which is then measured and used with a standard curve to determine the protein quantification (Willey *et al*., 2011). The BCA assay makes use of bicinchoninic acid for the colorimetric detection and quantification of proteins. This assay combines the reduction of Cu^{+2} to Cu^{+1} in an alkaline medium with the detection of Cu^{+1} by BCA (Thermo Scientific, Undated *b*). The reaction product is a highly soluble complex with an increasing absorbance at A_{564} as the protein concentration increases. Factors such as molecular structure, peptide bonds, and amino acids are responsible for the colorimetric detection that takes place with BCA. The protein concentrations are determined by a standard curve of bovine serum albumin (BSA). The assay has two protocols that can be followed, namely the test tube method and the microplate. The microplate is utilised if a limited amount of protein sample is available while the test tube method provides more sensitive results but requires more protein sample (Thermo Scientific, Undated *b*).

Further analyses of an expressed scFv involves the binding ability of the fragments that can be determined by the use of a competitive ELISA. The principle of competitive ELISA is based on the competitive binding activity between the added antibody that is unlabelled and a secondary conjugated antibody. First, the unlabelled antibody is added and allowed to bind to the antigen coated onto the plate. Following this, the unbounded antibodies are removed by consecutive washing steps. The second conjugated antibody is then added to bind to the remaining antigens. The substrate of the conjugated enzyme is then added and the absorbance of the signal is measured. It is expected that the higher concentration of unlabelled antibody bound to the antigen, the weaker the observed signal will be. If more unlabelled antibodies bind to the antigen, less of the secondary conjugated antibody can bind and that causes the signal measured to decrease. The advantage of using a competitive ELISA includes the use of impure sample that will still introduce binding activity, high specificity, sensitivity and flexibility since both direct and indirect methods of ELISA can be applied. After a scFv has been successfully expressed and the binding ability has been determined, further investigation can be done for the therapeutic treatment and application as scFv has been identified to neutralise toxins and antigens of a vast range of viruses.

1.5. Introduction to the current study

The occurrence of Infectious bronchitis virus (IBV) is worldwide and is currently only controlled by vaccination, but vaccination is not always as efficient as one may think (Pattison *et al.*, 2008; Bande *et al.*, 2015). Vaccine breaks that occur not only affects individual poultry farms, but with the increase of vaccine outbreaks it influence the poultry industry as a whole.

Infectious bronchitis virus is seen as a notifiable disease and this is a problem for the poultry industry as well as food production for consumers worldwide (OIE, 2013).

Infectious bronchitis virus serotypes are becoming more prevalent as more sophisticated detection techniques are developed and this causes reason for concern as only a narrow range of cross-protection between serovars are provided by the current vaccines used (Abolnik, 2015). Therefore, a treatment option for IBV is proposed. Not only will this decrease the influence of IBV in the poultry industry, but it will be used as a secondary fall-back on the control of IBV in addition to vaccines. The treatment is based on the neutralisation of the virus particle by neutralising antibodies (Awad *et al.*, 2015). This treatment can thus possibly be used post-infection and prevent and decrease the influence of IBV on the chickens and poultry industry.

The main aim of this project is to express neutralising antibodies against the IBV particle. This is achieved by the use of different technologies combining recombinant phage display technology and the immunoglobin structure with the expression of a functional protein. The human domain antibody (dAb) phage display library provides a phagemid based format library to select for scFv antibody fragments. With the combination of commercialised ELISA plates, scFv can be selected and identified against IBV. Although, with the use of commercialised ELISA plates, this process only selects for specific strains of IBV which is considered as the most prevalent strains. If this technique is considered sufficient, more variant strains can be investigated to further the application of this treatment. Further, *in ovo* studies in SPF eggs were carried out to determine if the expressed scFv antibody fragments can neutralise the vaccine strain H120 as well as a competitive ELISA to confirm the binding ability of the expressed fragments *in vitro*.

This project does not pose an alternative method to control IBV but should rather be viewed as an additional safety protocol that can be implemented in the case of vaccine breaks. This study provides both educational value to increase the knowledge on virus neutralisation, recombination of immunoglobins, phage display, and expression of proteins, as well as the practical application and implementation in the poultry industry. Further, knowledge obtained during this study can potentially be used as a treatment option for other diseases.

1.6. Problem statement

In an ideal poultry production setting, chickens will be vaccinated against IBV by a live attenuated vaccine with the advantage of high and uniform levels of antibodies for an extended period of time. However, this is not the case and the reality is that IBV is distributed worldwide

with a vast number of serotypes which are associated with specific geographical regions caused by variant strains. IBV is the etiological agent for infectious bronchitis within poultry flocks and causes respiratory symptoms, egg laying problems and mortality within the chickens. Due to the symptoms caused by IBV within chickens, the poultry production industry is affected by loss in production due to delayed maturity, decline of egg quantity and quality, poor weight gain, downgrading of carcasses, and mortality. This is a concerning issue because poultry is considered as one of the most consumed meat world-wide, especially in developing countries. As the occurrence and vaccine breaks of IBV become more frequent, a problem can therefore be expected with the poultry production for consumers.

The main manner in which IBV is currently controlled is by prevention methods such as vaccination. An additional control measurement can be implemented, namely the treatment of post-infected chickens by neutralisation of the virus with scFv. The IBV particle has a stalk like structure, emanating from the surface called the S protein, with a S1 subunit that induce neutralisation antibodies. Single chain variable fragments (scFv) that can be expressed as neutralising antibodies are theoretically able to bind to the S1 subunit and neutralise the IBV particle preventing the replication of the virus particle and prevents the spread of the virus particles within the infected chickens, thereby decreasing the symptoms of IB. If this treatment can be implemented successfully, the control of IBV is not only reliant on prevention by vaccines and if vaccine breaks do occur, the variant strain can be determined and a treatment can be given to the infected flocks to prevent symptoms of IB to worsen and prevent the decrease of poultry production within the infected flocks. Additionally, subunit vaccines are also an efficient option, but a treatment by virus neutralisation will be more cost effective than the creation of sub vaccines for each strain.

In conclusion, IBV is a reoccurring problem due to variant strains and vaccine breaks. This influence poultry health as well as a decrease in poultry production and which causes a dent in food production for consumers. An alternative treatment of IBV can possibly be implemented by the neutralisation of the specific strain causing IB within a flock which is more cost effective and provides a secondary option in the control of IBV additionally to prevention methods.

1.7. Aims and objectives

The aims of this study were to detect and express single chain variable fragments (scFvs) with the ability to bind to IBV antigens and to determine the neutralisation ability of these scFvs against the H120 IBV antigen.

These aims were achieved by detecting scFvs with the human domain antibody (dAb) library and phage display technology. Expression of the IBV binding scFvs was achieved by using an *E. coli* expression system and purified by immunopurification. The binding efficiency of the expressed scFv was determined by the use of a competitive ELISA and the neutralisation ability of the expressed scFvs were done through an *in ovo* neutralisation assay with the use of end-point titres and the Spearman and Karber method (Spearman, 1908).

1.8. Hypothesis

Recombinant scFvs can be selected and expressed that will possess virus neutralisation ability against the vaccine strain H120 of Infectious bronchitis virus (IBV).

Chapter 2

The screening for phage clones with the ability to bind IBV antigens

Introduction

The production of antibodies and use thereof have increased over time as technology development provides ease of production and specificity so that the designed molecules can easily be applied to research, diagnostics and therapeutic treatments (Almdni, 2013). Phage display has formed a big part of the production of antibodies and antibody fragments (Kretzschmar & von Rüden, 2002; Almdni, 2013). Phage display relies on the introduction of a foreign genetic sequence into a bacteriophage genome. The sequence is then expressed on the surface of the bacteriophage, which creates the opportunity for selection of specific antibodies or antibody fragments from a diverse pool through two consecutive steps, namely screening and selection (Kretzschmar & von Rüden, 2002; Almdni, 2013). The selected antibodies or antibody fragments can be further analysed to ensure antigen-specific antibodies (Almdni, 2013).

2.1 Phage display technology

Phage display technology is a versatile method for the detection of antigen-specific antibodies as well as the detection of protein interactions that can possibly be implemented as therapeutic treatments (Dale *et al.*, 2013). This technology has been applied in proteomic research and the diagnostics of various viruses such as severe acute respiratory syndrome (SARS-CoV), bluetongue disease virus (BTV) and infectious bursal disease virus (IBDV) (Miller *et al.*, 2005; Dimitrova *et al.*, 2009). Phage display technology has also been used in the development of snake anti-toxins, which indicates a promise in delivering biotechnological-based therapies with improved efficacy and potentially lower production costs (Laustsen *et al*., 2017). The technology allows for the selection of antibodies against any biological and non-biological antigens by bypassing the use of animals in the process.

The expression of the antibody on the surface protein provides the opportunity for selection where phage clones bind to the introduced antigen followed by the removal of non-binding phage clones during several washing steps. The bound phage clones are then recovered by elution and amplified by reinfection within a host, such as *E. coli* in the case of a bacteriophage M13 display system (Sapats *et al.*, 2003). The infected *E. coli* then releases phage particles that contain the coding sequence of the specific antibody or antibody fragments and can therefore be further analysed (Almdni, 2013). Selected clones with the expressed antibodies have a high binding affinity that allows screening to take place by a monoclonal ELISA

(Enzyme-linked immune-sorbent assays). A monoclonal ELISA is whereby bacteriophage clones are isolated from a polyclonal mixture with the use of an anti-bacteriophage horseradish peroxide (HRP) conjugated antibody as the primary antibody (Kretzschmar & von Rüden, 2002).

2.2 Recombinant antibody libraries

According to Nelson, 2010 recombinant antibodies are the key to develop and customise therapeutics. Recombinant antibodies can be characterised to determine certain properties such as half-life, distribution, affinity, penetration and bioactivity to allow for the selection of safe and efficient treatments. Antibody fragments appear to be more advantageous than whole antibody molecules (Nelson, 2010). Antibody fragments have higher penetration rates, are less costly to manufacture, and more time efficient to produce in prokaryotes (Kretzschmar & von Rüden, 2002; Nelson, 2010). Combining phage display technology with recombinant antibody fragments creates a platform for the development of high antigen-specific recombinant antibody fragments with the prior mentioned advantages (Nelson, 2010; Dale *et al*., 2013).

2.3 Human domain antibody (dAb) library

The human domain antibody (dAb) library kit (Source BioScience, Australia) makes use of single chain variable fragments (scFvs) and is based on the expression of scFvs on the pIII minor surface protein of the M13 filamentous phage. The library makes use of phagemid display that utilises a phagemid vector. The advantages of using phagemid display includes high transformation efficiency and direct secretion of soluble scFv without sub-cloning (Lee *et al.*, 2007). In addition to the phagemid advantages, the use of the scFvs also provide advantages as the original antigen binding sites being present, good immunogenic nature, ease of gene altering and the fact that high affinity of mutants can be obtained by site-directed mutagenesis (Almdni, 2013). Combining scFvs and the phagemid vector has the advantage of high affinity selection, good quantifiable expression within the periplasm of prokaryotic cells and the ease of tissue penetration during therapeutic uses (Lee *et al.*, 2007). The library is based on a single human hypervariable region (VH) framework with synthetic diversity introduced into the three complementary determining regions (CDRs), where binding of the scFv and antigen takes place, by PCR mutagenesis. Diversity is introduced at specific sites in the CDR1, CDR2, and CDR3 namely the H27-H33, H35, H50, H52-H54, H94, H95-H100 (a-k), H101, and H102. The pR2 vector is utilised and the library size of this specific kit is estimated to be 3×10^9 (Lee *et al.*, 2007). The scFvs are fused to the minor coat protein pIII of the filamentous phage M13 that can infect the TG1 TR *E. coli* strain. It should be noted that

this infection does not cause lysis of the infected bacterial cell and phages are continuously produced and released by secretion (Lee *et al.*, 2007; Rakonjac *et al*., 2011).

The human domain antibody (dAb) display library kit (Source BioScience, Australia) provides two *E. coli* strains. The TG1 TR *E. coli* strain is a suppressor strain that allows the scFv to remain fused to the pIII gene by suppressing the TGA amber stop codon located between the terminal Myc-c tag of the scFv insert and the pIII gene (Lee *et al.*, 2007). In addition to the TG1 TG *E. coli* strain, the HB2151 *E. coli* strain is a non-suppressor strain without the ability to suppress the TGA amber stop codon. The HB2151 *E. coli* strain therefore allows for the expression of soluble scFvs within the periplasm by not suppressing the TGA amber stop codon located between the terminal Myc-c tag of the scFv insert and the pIII gene (Lee *et al*., 2007). According to Lee and co-workers (2007), some antibody fragments may contain additional amber stop codons within the CDRs allowing a low expression efficiency to be observed within the TG1 TR *E. coli* strain, and no expression in the HB2151 *E. coli* strain.

To ensure the most monovalent display of scFvs fused to the pIII gene of the phage clone, the dAb library makes use of the phagemid display that utilises a helper-phage (Lee *et al*., 2007; Tikunova & Morozova, 2009). The helper-phage is trypsin sensitive and ensures that elution between the terminal Myc-c tag on the scFv and the pIII gene can occur during the selection process as elution occurs by trypsin cleavage (Lee *et al*., 2007). If the helper-phages are not trypsin sensitive, insufficient elution, and high background sensitivity is reported to be observed during selection. The KM13 helper-phage is used to superinfect the *E. coli* strain to produce phage particles that carry the selected scFv. Selection of superinfected cells occur because the KM13 helper-phage contains a kanamycin resistant gene. The helper-phage maximises the production of scFvs and limits its own ssDNA packaging to a minimum to allow for the most monovalent display of the scFvs (Enea *et al*., 1982).

2.4 Aim and objective

The aim of this chapter was to use a dAb phage display library (Source BioScience, Australia) to select for scFvs that can bind to IBV antigens in a highly specific manner, and to assess the amino acid diversity within the scFv sequences obtained from selected monoclonal phage clones through Sanger sequencing (Sanger *et al*., 1977). This entails the detection and selection of phage clones against common IBV strains namely M41 and H120 with the use of a dAb phage display library (Source BioScience, Australia) and commercialised IBV coated ELISA plates (BioChek, UK) to result in the retrieval of phage clones potentially expressing specific scFvs that could bind IBV antigens.

2.5 Materials and methods

The human dAb phage display kit (Source BioScience, Australia), provided glycerol stocks of the following: TG1 TR *E. coli* strain, helper-phage KM13, domain antibody library, and the expression strain *E. coli* HB2151. Panning and selection were conducted followed by a monoclonal sandwich ELISA to confirm binding of phage clones expressing scFvs that have the ability to bind IBV antigens. Commercial ELISA plates (BioChek, UK) coated with a mixture of M41 and H120 IBV strains were utilised during the panning, selection and monoclonal sandwich ELISA. The monoclonal sandwich ELISA made use of a mouse anti M13 bacteriophage antibody (Bio-Rad Laboratories Inc., UK) and anti-mouse horseradish peroxidase (HPR)-conjugated antibodies (Bio-Rad Laboratories Inc., UK).

2.5.1 Preparation of KM13 helper-phage stock

The helper-phage stock (10^7) was prepared by growing the TG1 TR *E. coli* strain on M9 minimal plates [1.3 % w/v disodium phosphate (Na_2HPO_4-$7H_20$), 0.3 % w/v monopotassium phosphate (KH_2PO_4), 0.05 % w/v sodium chloride (NaCl), 0.1 % w/v ammonium chloride (NH_4Cl), 0.4 % v/v glucose, one mg/ml VitB1, 2.0 mM Magnesium sulphate ($MgSO_4$-$7H_2O$), 0.1 mM calcium chloride ($CaCl_2$) and 1.5 % w/v agar] for 36 hours at 37 °C. A single colony was then inoculated into 5 ml 2x tryptone yeast (TY) medium [1.6% (w/v) tryptone, 1% (w/v) yeast extract and 5% (w/v) NaCl] and incubated at 37 °C, 250 rpm for 24 hours. The culture was then diluted 100-fold into 5 ml 2x TY medium and grown at 37 °C with a rotation of 250 rpm until an OD_{600} equal to 0.5 was reached. A dilution series of the originally provided helper-phage was prepared in phosphate buffered saline (PBS) (10^{12} PFU/ml-10^4 PFU/ml). Ten microliters of each dilution were mixed with 200 µl of the diluted culture in order to infect cells with the helper-phage. The infected cells were incubated in a 37 °C water bath for 30 minutes without agitation. Thereafter, the infected cells were mixed with H-top agar [1% (w/v) tryptone, 0.8% (w/v) NaCl and 8% (w/v) agar] and poured onto pre-warmed tryptone yeast extract (TYE) plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.8% (w/v) NaCl and 1.5% (w/v) agar], without the supplementation of antibiotics. The plates were incubated overnight at 37 °C.

An overnight culture of the TG1 TR *E. coli* strain was grown as described above. A single plaque from the incubated plates was selected. This plaque was inoculated into 5 ml TG1 TR *E. coli* culture with an OD_{600} of 0.5, and incubated at 37 °C, 250 rpm for two hours, followed by 100-fold dilution into 500 ml 2x TY medium in a two litre flask. The two litre flask was incubated at 37 °C, 250 rpm for two hours followed by the addition of kanamycin to a final concentration of 50 µg/ml. The flask was incubated at 30 °C, 250 rpm for 24 hours.

Following this process, polyethylene glycol (PEG) purification was performed as follows; an overnight culture of 500 ml was centrifuged at 10 800 x *g* for 15 minutes. The cell pellet was discarded and the helper-phages present within the supernatant were precipitated. A total of 100 ml PEG solution [20% (w/v) PEG 6000, 2.5 M NaCl] was added to 400 ml of the supernatant and incubated on ice for one hour. The solution was centrifuged at 7000 x *g* for 30 minutes and the supernatant was discarded as the helper-phages are to be present within the precipitate. The pellet was resuspended in 5 ml of PBS. One millilitre of PEG solution was added to the 5 ml PBS resuspended pellets followed by incubation on ice for 10 minutes and centrifugation at 3200 x *g* for 10 minutes, after which the supernatant was discarded. The pellet was again resuspended in one ml PBS, followed by centrifugation at 3200 x *g* for 10 minutes and the supernatant was filtered through a 0.45 µM cellulose acetate membrane syringe filter (GVS Life Science, USA). The phage titre was determined with a Biowave II (Biochrom LTD., England) spectrophotometer at a wavelength of 260 nm. The concentration of phages per millimetre was determined using Equation 1 (Lee *et al*., 2007).

$$\text{Phage/ml} = OD_{260} * 100 * 22.14 * 10^{10} \quad \text{Equation 1}$$

The OD_{260} value is the measured value of the pVIII major surface protein of the filamentous bacteriophage, the 100 represents the volume (µl) measured, 22.14 is a constant while, 10^{10} was the dilution factor used. After phage determination, a trypsin-cleavage test was performed to determine if the selected helper-phage is trypsin sensitive and sufficient for use during the experiment. First, an overnight culture of TG1 TR *E. coli* strain was grown and diluted 100-fold in 5 ml 2x TY medium [supplemented with 0.1 % (v/v) glucose and 50 µg/ml kanamycin] and cultivated until an OD_{600} of 0.5 was reached. Five microliter trypsin solution was added to 45 µl of purified phage and incubated at 30 °C for 30 minutes. After incubation, 0.001 % (v/v) of trypsin treated phage was diluted into one ml PBS from where five 100-fold dilution series were made and a total of 200 µl of TG1 TR *E. coli* culture was added to each dilution and mixed with 3 ml H-top agar (temperature of 50 °C) and poured onto pre-warmed TYE plates (supplemented with 50 µg/ml kanamycin) and incubated at 37 °C for 24 hours. The process was repeated excluding the addition of trypsin. The trypsin treated phages are expected to be 10^5 - 10^8 fold lower than non-trypsin treated phages, due to the helper-phage being trypsin sensitive.

2.5.2 Growth of original phage antibody library

A total of 25 µl of the original provided library was spread onto TYE plates [supplemented with 4 % (v/v) glucose and 100 µg/ml ampicillin]. A total of four TYE plates were used. The plates were incubated at 37 °C for 24 hours. The cells were scraped and resuspended in 20 ml 2x TY

medium. Aliquots of two millilitre 20 % glycerol stocks, containing cells of the original library, were made and stored at -80 °C.

2.5.3 Growth of phage antibody library

Three, two millilitre glycerol stock aliquots were thawed on ice and cautiously added into 500 ml of 2x TY medium. The 2x TY medium was supplemented with 4 % (w/v) glucose and 100 µg/ml ampicillin, as the library is ampicillin resistant and the presence of the glucose suppress the expression of the scFvs during bacterial growth. The Biowave II (Biochrom LTD., England) spectrophotometer was blanked with the supplemented 2x TY media and the aliquots were added until the OD_{600} reached 0.1. The flask was incubated at 37 °C with rotation at 250 rpm until an OD_{600} of 0.5 was reached. Thereafter, a total of $1x10^{12}$ helper-phages were added to 250 ml of the culture and incubated in a 37 °C water bath for 45 minutes. The culture was centrifuged at 3200 *x g* for 10 minutes and the supernatant was discarded. The pellet was resuspended in 500 ml 2x TY medium [supplemented with 0.1 % (v/v) glucose, 100 µg/ml ampicillin and 50 µg/ml kanamycin] and incubated at 25 °C with a rotation of 250 rpm for 16 hours. Upon growth of the library, PEG purification was done (as described in section 2.5.1) followed by screening with a monoclonal sandwich ELISA.

2.5.4 Selection by panning

An amount of $5x\ 10^{12}$ purified phages was added to 10 ml PBS supplemented with 5 % (w/v) milk powder (MPBS) and a total of 100 µl was added to each well of a commercial IBV coated 96-well ELISA plate. The ELISA plate was incubated at room temperature for an hour with gentle agitation. Washing then commenced with PBS supplemented with 0.1 % Tween (PBST) for a maximum of 10 washes, using 0.1 ml/well PBST for each wash. Thereafter, 0.2 ml/well PBS was used as final wash, this was repeated twice. A total of 100 µl/well trypsin solution was added to the ELISA plate followed by incubation at room temperature for one hour with gentle agitation. The trypsin solution was then pooled and a total of 30 ml TG1 TR *E. coli* culture (cultivated until an OD_{600} of 0.5, as previously described in section 2.5.1) was added to the eluted phage and incubated in a 37 °C water bath for one hour. Thereafter, the mixture was centrifuged for 5 minutes at 3200 *x g*. The supernatant was discarded and the pellet was resuspended in one ml 2x TY medium. Six TYE plates [supplemented with 4 % (v/v) glucose and 100 µg/ml ampicillin] was used and 166 µl of the resuspended pellet was added on each plate. A 10-fold dilution series was made to determine the titre with the remaining 4 µl diluted into PBS. The plates were incubated at 37 °C for 16 hours. The following day, cells from the TYE plates were scraped and resuspended in 30 ml 2x TY medium and used for the growth of the antibody library for the second round of panning. The process was repeated and a total

of three panning rounds were completed. The titre plates from all three rounds of panning were kept at 4 °C until individual colonies were used for the monoclonal sandwich ELISA.

2.5.5 Screening selection by monoclonal sandwich ELISA

Individual colonies were selected from the titre plates from all three selection rounds. Four wells served as positive controls whilst another four wells served as negative controls. The positive control was an IgG anti-chicken IBV HRP conjugated antibody and negative control was specific pathogenic free serum provided by the commercialised ELISA plate (Biochek, UK). A total of 24 colonies were selected from the selection titre plates, 24 from the second selection titre plates, and 24 from the third selection titre plates. The selected colonies were inoculated into a 96-well plate with 200 µl 2x TY [supplemented with 4 % (v/v) glucose and 100 µg/ml ampicillin]. The plate was then incubated at 37 °C for 24 hours with gentle agitation. A volume of 5 µl overnight culture of the selected colonies were added to a new 96-well plate with 200 µl 2x TY [supplemented with 4 % (v/v) glucose and 100 µg/ml ampicillin] and incubated for three hours at 37 °C with gentle agitation. The rest of the overnight culture was stored at -80 °C after adding 20 % (v/v) glycerol to each well. A total of $4x10^{10}$ helper-phages were added to 5 ml 2x TY medium and 50 µl was then added to the cells in the 96-well plate after the three-hour incubation. The 96-well plate was briefly mixed by gentle agitation and incubated at 37 °C without agitation for one hour. The 96-well plate was then centrifuged at 3200 *x g* for 10 minutes and the supernatant was discarded. The pellets were resuspended in 200 µl 2x TY medium [supplemented with 0.1 % (v/v) glucose, 50 µg/ml kanamycin and 100 µg/ml ampicillin] and incubated for 16 hours at 25 °C.

A sandwich ELISA was performed by centrifuging the 96-well plate for 10 minutes at 3200 x *g* followed by transferring the phage containing supernatant to a new 96-well plate. A total of 25 µl supernatant was added and mixed with 75 µl MPBS. The mixture was then added to a commercial IBV coated ELISA plate. The ELISA plate was incubated for one hour with agitation followed by the washing of the wells with PBS. The mouse anti-M13 bacteriophage antibody (Bio-Rad Laboratories Inc., UK) was added to the wells. The plate was then incubated for one hour without agitation at 37 °C, followed by the washing of the wells with PBST (0.1 % Tween) repeated three times and once with PBS without 0.1 % Tween. The anti-mouse HPR conjugated antibody was added to the wells followed by the washing steps. A total of 100 µl tetramethylbenzidine (TMB) was then added and the plate was incubated at room temperature (25 °C) for 30 minutes for the blue colour to appear, where after 1 M sulphuric acid was added to stop the reaction. The plate was then placed in a plate reader (BioTek, USA) and the absorbance was read at a wavelength of 405 nm. Positive clones were detected with a cut-off absorbance value of 0.4. This was determined by the absorbance of

the positive and negative controls used in the commercialised ELISA plate at a wavelength of 405 nm. The positive clones underwent DNA sequencing to determine the integrity of the scFv sequence and diversity.

2.5.6 Sequencing of positive clones

2.5.6.1 Growth of selected colonies

The positive clones from the selection rounds were selected and were subject to plasmid extraction. Along with the positive clones, a non-binding clone referred to as scFv 15 underwent the same experiments to serve as a non-binding control clone. The concentration and purity of the plasmid DNA was determined by using spectrophotometry. Thereafter, sequencing was performed with the provided reverse primer 5'-CCCTCATAGTTAGCGTAACGA-3' from the dAb library (Source BioScience, Australia) followed by Sanger sequencing (Sanger *et al*., 1977).

2.5.6.2 Plasmid extraction

The plasmids were extracted by the use of the PureYield™ Plasmid Miniprep System (Promega, USA) using the protocol provided by the manufacturer. Briefly, 600 µl overnight culture was added to a microcentrifuge tube and lysis buffer was added to break open the cells. This step was followed by the addition of an ice cold (4 °C-8 °C) acidic neutralisation solution. The microcentrifuge tube was inverted and then centrifuged at 12 000 x *g* for three minutes. The clear supernatant was added to a PureYield™ minicolumn and centrifuged for 15 seconds at 12 000 x *g*. The minicolumn was washed with Endotoxin Removal Wash and again centrifuged for 15 seconds at 12 000 *x g*. The Column Wash Solution was added to the PureYield™ minicolumn and centrifuged for 30 seconds at 12 000 *x g*. An elution buffer (nuclease-free water) was added to the minicolumn and left at room temperature for one minute. The plasmid DNA was eluted in a clean tube by centrifugation at 12 000 *x g* for 15 seconds and stored at -20 °C. The plasmid DNA quality was determined using the Nanodrop ND-1000 UV-spectrophotometer (Nanodrop Inc., USA).

2.5.6.3 Sequencing

Direct sequencing commenced as only a reverse primer sequence (5'- CCCTCATAGTTAGCGTAACGA- 3') was provided by the dAb library. Plasmid DNA was added to the sequencing mix to a final concentration of 200 ng/µl. Sequencing PCR was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as per manufacturer's instructions. The kit provided pre-mixed

reagents for Sanger sequencing namely, di-deoxynucleotidetriphosphates (ddNTPs) and deoxynucleotidetriphosphates (dNTPs), and DNA polymerase. The kit also provided a sequencing buffer that is optimised for the use of the kit.

The reaction was incubated at 96 °C for one minute, followed by an initial denaturation step at 96 °C for 10 seconds, annealing at 50 °C for five seconds and elongation at 60 °C for four minutes (Applied Biosystems, USA). These steps were cycled 25 times. After the sequencing PCR was completed, sample clean-up commenced using the EDTA/ethanol precipitation method. A total of 10 µl sequencing PCR product was adjusted to 20 µl with ultrapure H_2O. To this, a total 5 µl (125 mM) ethylenediaminetetraacetic acid (EDTA) and 60 µl absolute ethanol was then added to a final concentration of 70.5% (v/v), followed by centrifugation at 20 000 *x g* for five minutes at 4 °C. The supernatant was then aspirated and 60 µl ice cold 70 % ethanol was added to the pellet. Centrifugation was repeated, followed by aspiration of the supernatant and drying of pellet. The pellet was stored in a dark room at 4 °C until Sanger sequencing commenced at the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State.

2.5.6.4 Sequence analysis

After the sequences were obtained, analysis was conducted using Geneious V 11.1.4 (http://www.geneious.com, Kearse *et al*., 2012) and the abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017). The human domain (dAb) library kit (Source, BioScience, Australia) is based on a single human variable heavy (VH) framework (V3-23/D47). The abYsis software provides a list of features including automated antibody numbering, alignments, basic local alignment search tool (BLAST) function, numbering schemes, and detection of unusual residues that is all based on the human VH and VL framework abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017). According to Swindells and co-workers (2017) the web-based software, referred to as abYsis includes sequencing and structural data of antibodies that can be analysed in several ways. The numbering schemes used by this software is one of the features provided as well as annotation of the data. The sequences were searched using BLAST against the database and analysed based on the Chothia numbering scheme to identify unusual amino acid residues (residues only being present at that specific position less than 1% of all sequences within the database) (http://www.abysis.org/, Swindells *et al*., 2017).

2.6 Results

2.6.1 Selection and Screening

2.6.1.1 Titration of KM13 helper-phage stock

The human domain (dAb) library utilised a phagemid display and therefore the KM13 helper-phage stock was needed to ensure a large production of phage clones throughout the screening process. A KM13 helper-phage titration was performed to obtain well separated plaques for selection on the agar-overlaid agar plates. A single plaque was selected and purified (Figure 3). In addition, the selected KM13 helper-phage underwent trypsin cleavage to ensure that the selected helper-phage was trypsin sensitive (Figure 4).

The dAb library provided the KM13 helper-phage to a concentration of 10^7 x PFU/ml and after the helper-phage stock was amplified and the phages purified, the stock contained 9.0 x 10^{12} PFU/ml phages (Table A1, Appendix A) correlating with the expected results of the library. Upon comparing the PFU's (Table A2 in Appendix A) of trypsin-treated and non-trypsin treated phages (Figure 4), it is clear that the selected helper-phage plaques resulted in successful KM13 helper-phage stock amplification as no colonies were observed on the TYE plates of the trypsin treated helper-phages, while the amount of phages present on the TYE plate of the non-trypsin treated helper-phages were too numerous to count.

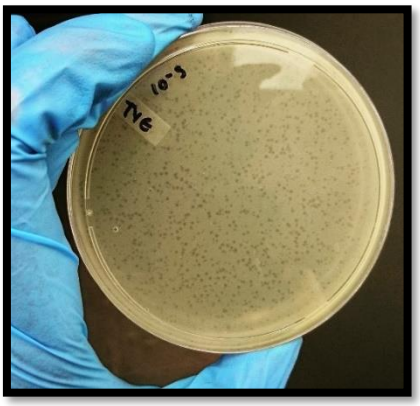

Figure 3: Titre plaques (10^{-5} dilution) of the KM13 helper-phage and TG1 TR *E. coli* host present in H-top agar on TYE plates.

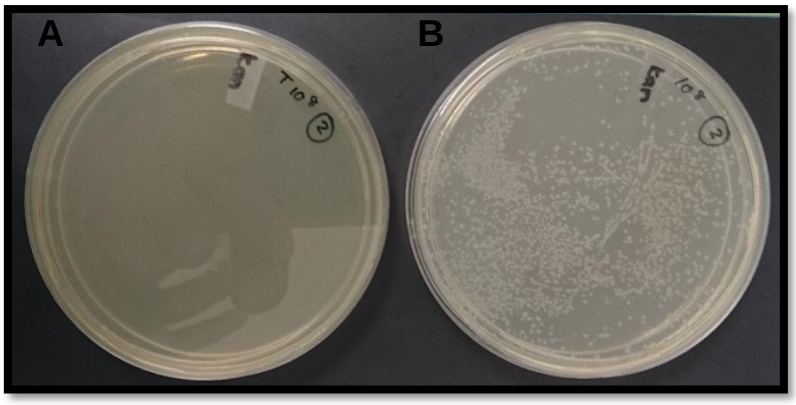

Figure 4: Tryptone yeast extract (TYE) plates supplemented with 50 µg/ml kanamycin with colonies of the trypsin treated phage (A) and non-trypsin treated phage at a dilution of 10^{-8} (B).

2.6.1.2 Selection of phage clones against IBV antigens

Phage clones containing scFvs with the ability to bind to IBV antigens were selected by the use of the dAb library by three consecutive selection rounds. The input of phage amount remained constant while the output differed during each selection round. The eluted phage amount was determined by using Equation 1. Table 1 indicate the phage clones that was put into the library (input) and the clones that were retrieved after one round of selection (output) and the percentage recovery of the output against the input (Equation A1, Appendix 1). The PFU per ml is also shown per selection round (Table A3, Appendix 1). The amount of phage clones that were put into the library stayed constant as per protocol but the amount of output increased.

The increase in phage clone output was expected as the specificity increased with each round of selection (Table 1). Initially, the phage recovery was 102% after round 1, with a phage input of 5.0×10^{12} but increased to 170% after round 2, and 180% after round 3, respectively. The PFU (per ml) (Table 1) also increased with each selection round. For round 1 only 1.6×10^{5} PFU /ml were obtained, increasing to 1.2×10^{6} after round 2, and a final of 3.0×10^{10} PFU /ml were obtained after round 3 (Table A3, Appendix A).

Table 1: The phage input, phage output, phage recovery percentage, and PFU per ml for three consecutive rounds of selection using the dAb library against the IBV antigen.

Selection	Input	Output	Percentage phage recovery (%)	PFU (per ml)
Round 1	5.0×10^{12}	5.1×10^{12}	102	1.6×10^{5}
Round 2	5.0×10^{12}	8.6×10^{12}	170	1.2×10^{6}
Round 3	5.0×10^{12}	9.0×10^{12}	180	3.0×10^{10}

2.6.1.3 Screening of selected phage clones by monoclonal sandwich ELISA

Twenty four colonies were selected from each round of selection and were screened against IBV antigens with the use of a commercialised IBV coated ELISA (BioChek, UK) to assess whether the selected phage clones showed specificity to the IBV antigens (Figure 5). Table 2 indicates number of positive monoclonal phage clones that were selected from of the dAb library at each round of selection. Individual clones were determined as positive by using the standard commercialised ELISA equation (Equation A2 and Equation A3 in Appendix A) and the percentage of positive clones in each round was determined as the proportion of positive clones to the total number of clones screened (Table 2) (Table A4 and Table A5 in Appendix A).

One positive clone was identified during round 1, two in round 2 and, nine in round 3. The percentage positive clones increased from 4.2 % after round 1 to 37.5 % after round 3. A total of 12 monoclonal clones, from all three rounds of panning, were determined to potentially bind IBV antigens (Table 2). Although only 16.7 % of the clones that were selected showed specific binding of IBV antigens, the increase of positive clones per selection round indicates progressive enrichment by the selection process.

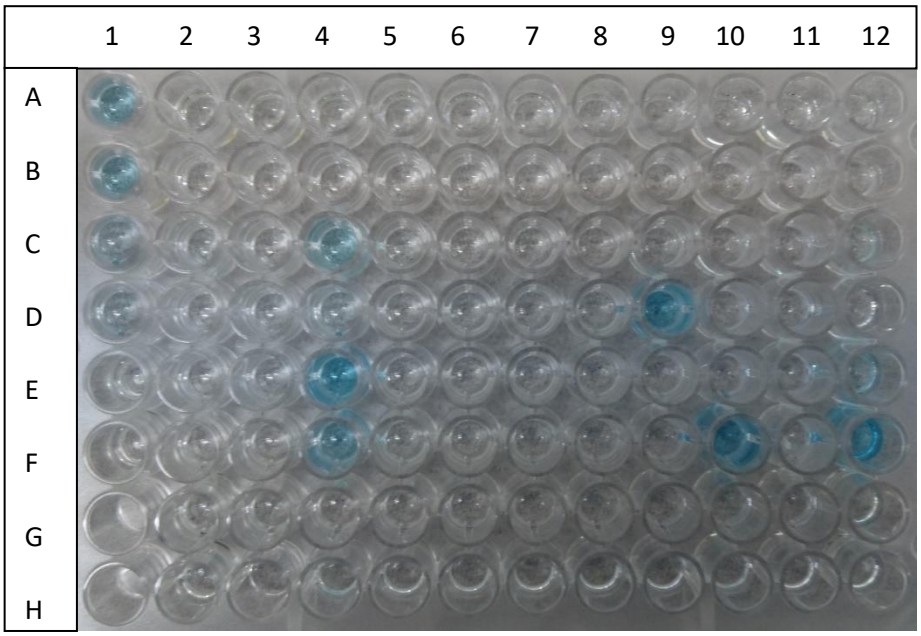

Figure 5: A monoclonal sandwich ELISA, with selected phage clones recovered from three selection rounds. All monoclonal sandwich ELISA experiments were conducted in triplicate. The ELISA plate used was the commercialised ELISA plate coated with IBV strains (M41 and H120) (BioChek, UK).

Binding of the phage clones were detected by using mouse anti-M13 bacteriophage (Bio-Rad Laboratories Inc., UK), anti-mouse HRP-conjugated antibodies (Bio-Rad Laboratories Inc., UK) and a TMB substrate (Thermo Scientific, USA). Wells A1-D1 are the positive controls and wells E1-H1 are the negative controls. A total of 24 phage clones were selected from each round of selection. (Round 1: wells A2-A12, B2-B12 and C2-C3. Round 2: wells C4-C12, D2-D12 and E2-E5. Round 3: wells E6-E12, F2-F12 and G2-G7).

Table 2: Individual specific phage clones selected from three consecutive selection rounds that showed positive binding to IBV antigens during the monoclonal sandwich ELISA.

Selection	Selected	Positive	Percentage (%) Positive phage clones
Round 1	24	1	4.2
Round 2	24	2	8.3
Round 3	24	9	37.5
Total	**72**	**12**	**16.7%**

2.6.2 Genetic characterisation of phage clones specific to IBV antigens

After the positive individual phage clones expressing scFvs that showed ability to bind IBV antigens were determined, the scFv of these clones were genetically characterised by sequencing to identify scFvs with a complete variable heavy (VH) reading frame. Plasmid DNA was extracted from selected clones and sequenced with the reverse primer provided with the dAb library (Source BioScience, Australia). The sequencing results were analysed and the completeness of the scFv reading frames as well as the amino acid sequence diversity of the scFvs were determined. A non-binding clone, scFv 15 underwent the same experiments to serve as a non-binding clone control.

2.6.2.1 Plasmid extraction

Shown in Table 3 are the quality and concentrations of extracted plasmid DNA from selected clones, measured spectrophotometry. The $A_{260/280}$ range of 1.7 to 1.9 falls within the acceptable quality range for DNA. The accepted value for pure DNA is 1.8. The $A_{260/230}$ values ranged from 1.7 to 2.4, with a range of 1.8 to 2.2 indicative of pure DNA. The values are dependent on pH and ionic strength and can therefore influence the value ranges by +/- 0.2 to 0.3. This indicates that all samples obtained in this experiment are of usable quality.

Table 3: The plasmid concentration (ng/µl), $A_{260/280}$ and $A_{260/230}$ values of extracted plasmid DNA.

Phage clone	Plasmid concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
1	95.27	1.82	1.80
2	93.33	1.83	2.13
3	124.95	1.82	2.16
4	143.55	1.84	1.88
5	149.29	1.77	1.84
6	254.17	1.85	2.33
7	409.97	1.88	2.34
8	481.77	1.89	1.82
9	496.66	1.89	1.84
10	372.36	1.82	1.76
11	588.13	1.87	1.76
12	658.23	1.85	1.78
15	140.58	1.88	2.17

2.6.2.2 DNA sequencing of scFvs

The completeness of the variable heavy (VH) scFv sequences were determined by the use of Geneious V 11.1.4 (http://www.geneious.com, Kearse *et al*., 2012). Sequence analysis was required as some scFvs with the specificity to bind to IBV antigens contained a stop codon or lacked components needed for correct expression of the scFv within a non-suppressor strain, for example the HB2151 *E*. *coli* strain. Indicated in Figure 6 are four nucleotide sequences from the 12 scFvs obtained from the positive selected clones with specificity for the IBV antigen that contained complete and unique VH sequences. From the 12 phage clones with scFvs that were determined to be positive with the monoclonal sandwich ELISA, only five scFvs had a complete VH sequence namely, scFv 7, 8, 9, 11 and 12 (Table A6 in Appendix A). From these five scFvs, only four unique VH amino acid sequences were obtained (Figure 7) that were identified as the variable heavy (VH) chain of *Homo sapiens* (Table A8 in Appendix A) by the abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017). The predicted amino acid sequence in Figure 7 shows diversity within the following regions: CDR1 (H27, H30-H33), CDR2 (H50, H53 and H54) and in the CDR3 (H94-H96, H100b, H100c, H100g, H100j and H101), while the rest of the predicted amino acid sequences shows similarity.

Figure 6: Nucleotide acid sequence from four complete variable heavy (VH) regions of the scFv from positive binding clones obtained from the monoclonal sandwich ELISA. The blue box indicates the *NcoI* restriction site and the purple box the *XhoI* restriction site.

The variation of the amino acid sequences obtained from the selected phage clones are indicated in Figure 7. The predicted amino acid sequence from scFvs 9 and 11 has no variation between the two sequences whilst scFv 7 and 8 has variations between one another and between scFv 9 and scFv 11. All the predicted amino acid sequences contained a total of 9 insertions within the CDR3 (H100a-H100i) and H82a, H82b and H82c and one insertion within the CDR2 (H52a) region (Figure A1, A2, A3 in Appendix A).

Figure 7: Predicted amino acid sequences of four variable heavy (VH) regions of scFvs that has binding ability to IBV antigens. Amino acid differences are displayed in grey and white.

The Chothia database from the abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017) was used to compare the variations of the amino acids located at each position and determine unusual residues. Table A7 in Appendix A indicates the most frequently observed amino acids at the positions within the CDRs in the variable heavy VH chain of *Homo sapiens*, based on the Chothia numbering scheme, from the predicted amino acid sequences. Table 4 indicates the unusual residues that were detected in the CDRs from the predicted amino acid sequences. The sequence from scFv 7 revealed a total of seven unusual residues present in the CDRs while the sequences obtained from scFv 8, scFv 9 and scFv 11 had a total of three unusual residues present within the CDRs. Amino acid positions H28, H52A and H101 all display unusual residues in the different predicted amino acid sequences. At positions H28

and H101 of all sequences, an arginine (R) is present. The inserted H52A position in scFv 7, scFv 9 and scFv 11 all have a methionine (M) present while scFv 8 has a leucine (L) present.

The amino acid sequence of scFv 7 has an asparagine (N) at amino acid position H27 while, the rest of the sequences have a tyrosine (Y) present, which is considered to be the most frequent amino acid at that site according to the Chothia numbering scheme on the abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017) (Table A6 in Appendix A). Furthermore, scFv 7 has a histidine (H) present at position H30, while the other sequences have asparagine (N). At position H31 a tyrosine (Y) was found to be present in scFv 7, while serine (S) was present within the other scFv sequences, which is the most frequent amino acid for this amino acid position (Table A6 in Appendix A). Furthermore, position H53 on scFv 7 has a valine (V) present while sequence 8 has a serine (S) and sequences 9 and 11 have an arginine (R) present at this site. Position H91 has an aspartate (D) present in scFv 7, while the rest of the sequences have a tyrosine (Y) present. The sequence of scFv 7 has an additional aspartate (D) present at position H92, while the rest of the scFv sequences have the most frequent amino acid for this site, namely cysteine (C) (Table A6 in Appendix A).

Table 4: Unusual residues and the most frequently observed amino acids in the CDRs that was determined by abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017) based on the Chothia numbering scheme from the predicted amino acid sequences of the four selected phage clones.

Amino acid position with unusual residue	H27	H28	H30	H31	H52A	H53	H101
Most frequent occurring amino acid	Y	S/T	S/T	S	P	S	D
Sequence 7	N	R	H	Y	M	V	R
Sequence 8	Y	R	N	S	L	S	R
Sequence 9	Y	R	N	S	M	R	R
Sequence 11	Y	R	N	S	M	R	R

C- Cysteine; D- Aspartate; H- Histidine; L- Leucine; M- Methionine; N- Asparagine; P- Proline; S- Serine; T- Threonine; R- Arginine; Y- Tyrosine

Similar amino acid as most frequent amino acid

Unusual amino acid

2.7. Discussion

The aim of this study was to find highly specific scFvs that bind IBV antigens by using phage display technology (Source BioScience, Australia) together with commercial ELISA plates coated with IBV antigens (BioChek, UK). This was plausible, due to the selection and screening methods that allowed for the retrieval of the desired antigen-specific scFvs. The aim was achieved by the selection of scFvs that may play a role in the neutralisation of IBV. In other words, this worked aimed at finding scFvs that bind to IBV and which could be used to neutralise the virus, thus preventing the binding of the virus to the host cells. Firstly, phage clones with scFvs that bind to IBV were selected by various selection rounds. Thereafter, the clones were screened for by a monoclonal sandwich ELISA to retrieve monoclonal phage clones from a polyclonal mixture. These clones were sequenced and analysed to determine sequence variations.

2.7.1 Selection and Screening

Selection was done with the dAb library that makes use of the phagemid display to ensure the most monovalent display of the scFv fused to the Gene III (Hoogenboom *et al*., 1998). The helper-phage was used to superinfect bacteria with the phage clone to ensure production of phage particles carrying the selected scFv. The helper-phage stock was cultivated and titrated for the ease of plaque selection, as seen in Figure 3 that indicated successful growth of the helper-phage stock. The helper-phage titre was determined by the use of Equation 1, the empirical formula that was provided with the dAb library kit. The equation is based on the use of absorbance values read at a wavelength of 260 nm as this detects the major coat protein of a bacteriophage, in a volume of 100 µl and a 10^{10} dilution. Alternative to measuring the phage titres by spectrophotometry, as was done during the study, the phage can be diluted and added to the host cells to get a more accurate phage titre results by plating on selective agar (Lee *et al*., 2007).

According to the dAb library (Source BioScience, Australia), the helper-phage is supposed to be trypsin sensitive. The reason for this is that trypsin is used to elute the phage clones fused to scFv that binds to the antigen. If the selected helper-phage were not trypsin sensitive, it would influence the results of the selection process by causing background infectivity from the helper-phage (Lee *et al*., 2007). A trypsin cleavage test was conducted to assess whether or not the selected helper-phage will be efficient to use in the selection process. According to the results obtained as displayed in Figure 4, no colonies were observed for the trypsin treated helper-phage while there were colonies present for the non-trypsin treated helper-phage. This indicaes that the phage is trypsin sensitive and was cut by trypsin. The results are in agreement with the dAb library that requires the trypsin-treated helper-phage to have 10^6 fold

lower PFU/ml than non-trypsin treated helper-phage, making it sufficient to use during the selection process (Lee *et al*., 2007).

The use of the dAb library proved successful for the selection of phage clones with specific scFvs that have the ability to bind to IBV antigens. Successful selection depends on whether or not the number of phage clones retrieved after each round increases (Weisser & Hall, 2009). When the number of phage clones increases, this indicates an increase in specificity and affinity to the antigen. When phage output and percentage phage recovery increased with each round of selection this indicated a progressive enrichment of specific binding phage clones and therefore the successful recovery of specific scFvs with the ability to bind to IBV antigens (Table 1). The obtained screening results of phage clones with the ability to bind to the IBV antigens that were selected after each round of selection by the monoclonal sandwich ELISA also indicated successful enrichment and recovery of antigen-specific phage clones (Table 2).

2.7.2 Plasmid extraction and transformation

The plasmid extraction was successful and the purity was assumed adequate for further use as the extracted plasmid DNA samples had an $A_{260/280}$ values ranging between 1.7 and 1.9. DNA and other nucleic acids are measured at 260 nm while, proteins are measured at 280 nm. The ratio can be influenced by change in sample acidity, wavelength accuracy of the spectrophotometer and the nucleotide mix of the sample (Leninger, 1975; Wilfinger *et al*., 1997). The latter is influenced by the composition of the sample as adenine has an absorbance of 4.5, while guanine has an absorbance of 1.15. The difference in composition will therefore influence the absorbance value and the ratio (Leninger, 1975). The $A_{260/230}$ values were also determined from the DNA extracted and ranged from 1.7 to 2.4, which indicates that the plasmid DNA that was extracted was of usable quality. The $A_{260/230}$ ratio is the ratio between the absorbance measured at 260 nm and 230 nm. As mentioned, nucleic acids are measured at 260 nm while phenol, EDTA, TriZol, and other similar substances are measured at 230 nm. Both of the mentioned ratios are used to indicate the purity of the extracted plasmid DNA sample and in this instance the extracted plasmid DNA sample can be deemed as reasonably pure based on the $A_{260/280}$ and $A_{260/230}$ values.

2.7.3 Genetic analysis of sequences

According to Azzazy & Highsmith (2002) the number of panning rounds varies from three to five and states that the more selection rounds are done, the more specific phage clones can be selected. However, Almdni (2013) states that the increase of selection rounds do not necessarily increase the specificity or the quality of the of the phage clones obtained as it is known that phage clones with fragments with an incomplete framework is smaller in size and

can grow at a faster pace, allowing the opportunity to dominate the output of each selection round. Along with the incomplete variable heavy (VH) frameworks, it is known that the amino acid sequence and amino acid placements and substitutions have influences on the affinity and binding ability of an antibody (Betts & Russell, 2007). Considering this, the positive phage clones selected from this work were genetically characterised to determine the completeness of the scFv sequences, and the difference in diversity between the amino acid positions was observed. Diversity was introduced into the human domain (dAb) library at CDR1, CDR2 and CDR3 with residues H27-33; H35; H50; H52-H54; H94; H95-100; H101; H102 (Lee *et al*., 2007).

All the selected phage clones with scFvs were able to bind the IBV antigens during selection and screening using the monoclonal sandwich ELISA, even though the sequencing results determined only four of the fragments to have unique and complete sequences (Figure 6). The rest of the sequences had deletions, amber stop codons within the CDRs and were partial sequences (Table A6, Appendix A). An explanation for this is due to the scFv phage clones that infected the suppressor TG1 TR *E. coli* strain. The TG1 TR *E. coli* strain allows the expression of the scFvs by suppressing the TGA amber stop codons that occurs within the CDRs during the selection and screening process (Lee *et al*., 2007). For soluble expression of the scFvs a non-suppressor HB2151 *E. coli* strain that do not suppress the TGA amber stop codon is used and it is therefore necessary to determine if the scFvs that are able to bind IBV antigens does not contain additional stop codons in the CDRs. According to Almdni (2013), the expression of antibody fragments can also be toxic to a bacterial cell allowing selective pressure to cause deletions and partial sequences can arise. Bacterial expressing fragments with partial sequences grow at a faster pace, dominating the selection outcome (Lee *et al*., 2007).

According to Lin *et al*. (2015) the CDR3 has the most diversity in terms of sequence and length. The CDR3 of the dAb library was meant to be as short as possible, while still containing adequate binding properties (Lee *et al*, 2007). Although all six CDRs within a scFv play a role of binding to an antigen, the heavy chain in a larger sense, the CDR3 is known to possess the most binding activity (Lin *et al*., 2015). The amino acid sequence within the CDR3 can therefore influence interaction and binding affinity of a scFv with an antigen (Betts & Russell, 2007). Variations within the specified sites of the CDRs were expected and the five sequences showed diversity within these sites while the conserved regions remained similar. Analysing the different amino acid sequences using the abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017) based on the Chothia numbering scheme of the human VH framework, the CDRs were identified along with the detection of unusual

residues that is present within the sequence. Unusual residues are defined as an occurrence at a specific site that is less than 1% as calculated with abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017).

Variations in the nucleotide sequences were observed within the entire sequence but variations of amino acid sequences were only observed within the CDRs, especially within the CDR3. The same variation was observed by Lin *et al.* (2015) where a high degree of sequence diversity in the CDRs between different clones, indicates the importance of CDRs in antigen recognition. Based on the Chothia numbering scheme, the CDR1 showed unusual residues at H27 (Table 4). The most frequent observed amino acid is tyrosine (Y). According to Lin *et al*. (2015) tyrosine (Y), arginine (R) and serine (S) play roles in antibody-antigen binding as these amino acids have high binding energy and serine aids by providing conformational flexibility that leads to more optimal binding contacts. A presence of asparagine (N) residue instead of a tyrosine can influence the protein structure as tyrosine (Y) is preferably phosphorylated, partially hydrophobic and tends to be buried within the hydrophobic core, while asparagine (N) is hydrophilic and is therefore commonly found on the surface of a protein (Betts & Russell, 2007). Differences in the amino acid sequence may lead to a difference in the protein structure which may have an influence on the antibody-antigen binding (Betts & Russell, 2007; Lin *et al.*, 2011). Position H28 showed the presence of arginine (R) within all the obtained sequences, while the most frequent occurring amino acid at this position is serine (S) or threonine (T), which are polar amino acids (Table 4). According to Tiller and co-workers (2017) the presence of arginine (R) within antibody germ-lines are much lower than that of tyrosine (Y) and serine(S) but has an important influence on binding ability. The interactive nature of arginine (R) has also been found to contribute to non-specific binding. The presence of arginine (R) may therefore influence the binding ability and specificity of an antibody fragment (Tiller *et al*., 2017).

Serine (S) and threonine (T) are also the most frequent occurring amino acids at position H30 within the CDR1 but a histidine (H) is present in sequence 7 and asparagine (N) is present in the other sequences. Both histidine (H) and asparagine (N) are polar amino acids that play a role in protein active binding sites, which are able to substitute serine. But as mentioned, serine plays a role in the binding of antibodies with antigens (Betts & Russell, 2007). A tyrosine (Y) is present at position H31, while the most frequent occurring residue is serine (S). Tyrosine (Y) plays a role in the phosphorylation of intracellular proteins, this role is also carried by serine but the substitution of serine with tyrosine is very rare. This is due to the highly specific nature of the protein kinases that is responsible for phosphorylation. The substitution can therefore influence the role of the amino acid (Hanks *et al.,* 1988).

An insertion occurred at site H52A in the CDR2 of all the sequences obtained and an unusual amino acid was identified to be present within all of the sequences (Table 4). The sequence obtained from scFv 8 showed a leucine (L) to be present at position H52A and the other sequences showed the presence of methionine (M). Taking into account that the most frequent occurring amino acid at this position is proline (P), a substitution of the amino acid to leucine (L) can influence the structure, as proline (P) forms part of the protein backbone (Betts & Russell, 2007). However, the presence of methionine (M) is not believed to have a big influence on the protein binding as both methionine (M) and proline (P) are non-reactive amino acids but may have an influence on the structure (Betts & Russell, 2007).

The arginine (R) that is present at the amino acid position H53 in sequence 9 and 11 is not considered to have a major influence on structure but may have an influence on binding ability as the most frequent amino acid is serine (S) and arginine (R) present plays a role in the binding activity (Lin *et al*., 2015). The obtained sequence from scFv 7 has a valine (V) present and the change from a polar amino acid to a non-polar amino acid may have an influence on the structure and function (Betts & Russell, 2007).

Amino acid variations have a vast range of influence on the protein structure and function and the variation within the CDR3 has the most prominent effect on binding activity and the affinity to an antigen. A study conducted by Lin and co-workers (2011) aimed to identify the role of specific residues in the CDR3 in antigen binding. The amino acids in the CDR3 were replaced with alanine (A) and it was found that substitution at site H99-H101 with alanine (A) may influence the binding of the antigen and may therefore play an important role in antigen recognition and the affinity of an antibody. Nine insertions were identified in all the sequences at residues H100a-H100k. Taking into account that the CDR3 plays the most prominent role in determining antibody specificity and affinity the insertions can play a role in the antigen recognition and the affinity of an antibody. The amino acid insertions therefore have the potential ability to influence the affinity between an antibody and an antigen (Lin *et al*., 2011).

In conclusion the objective of this chapter, namely to obtain various scFvs with binding activity towards IBV, was successfully achieved by the use of the dAb library. Specific scFvs were successfully selected and screened for by use of a monoclonal sandwich ELISA. A total of 12 positive binding phage clones were identified followed by sequence analysis using Geneious V 11.1.4 (http://www.geneious.com, Kearse *et al*., 2012) and abYsis software (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017). A total of four sequences had unique and correct sequences and from analysis of the sequencing data, it became apparent that the selected phage clones had amino acid variations within the CDRs that are predominantly responsible for antigen binding.

Chapter 3

Expression and purification of single chain variable fragments

Introduction

Expression of recombinant proteins differs depending on the type of protein as well as the expression system used. *E. coli* expression systems are most commonly used as a baseline for small-scale expression before other systems such as yeast can be implemented (Leong & Chen, 2008). The reason is due to *E. coli* being the most cost-effective and efficient system to date (Leong & Chen, 2008; Sivashanmugam *et al.*, 2009). *Escherichia coli* has been used in the expression of recombinant antibody fragments, and according to Jurado and colleagues (2002) utilising an *E. coli* expression system to produce antibody fragments can contribute to proteomics and therapeutics. Since *E. coli* is considered the most commonly used expression system for the production of recombinant antibodies, all advantages and disadvantages should be taken into account when determining the expression success. Disadvantages of using an *E. coli*-based expression system includes the lack of post-translational modifications and retention of the amino terminal methionine, which affects stability and immunogenicity of the fragments, and codon bias, is a reoccurring problem. These problems can be reduced by the use of phage display libraries as phage selection is based on both high affinity antigen binders as well as adequate antibody fragment expression (Verma *et al.*, 1998).

3.1. Expression of single chain variable fragments

Antibodies are used for research, diagnostic, and therapeutic applications. Regardless of the type of recombinant antibody, all fields of application require the production of purified proteins with ease (Miller *et al.*, 2005). As with any other recombinant protein, single chain variable fragment (scFv) expression is dependent on the efficiency of transcription, translation and correct folding (Leong & Chen, 2008). A recombinant antibody that is expressed in *E. coli* can be localised to the cytoplasm or periplasm. The periplasm is the preferred locality as protein folding is more precise due to the more oxidising environment, resulting in the correct formation of disulphide bonds. This is advantageous as protein folding is considered to be the yield-limiting step. Protein folding is influenced by the amino acid composition of the selected antibody or antibody fragment (Schaefer & Plückthun, 2010).

According to Weisser and Hall (2009) high concentrations of reasonably pure scFv fragments can easily be obtained by directing expression to the periplasmic space. In addition, Schaefer and Plückthun (2010) stated that the periplasmic expression had become the standard for preparing functional antibody fragments fast and conveniently. The reason is the ease of

protein extraction and success of purification attributable to the small contamination spectrum. Therefore, periplasmic localisation allows large scale expression to be efficient and economically feasible due to rapid screening and ease of purification (Leong & Chen, 2008). During this method of expression, proteins have been reported to leak through the outer membrane and can be purified from the media. This is dependent on the bacterial strain used as well as the induction conditions and sequence of the protein. An example of where this can be observed is in the case of IPTG (Isopropyl β-D-1 thiogalactopyranoside) induction with the use of the *E. coli* HB2151 bacterial strain (Lee *et al*., 2007).

One specific factor influencing the expression efficiency and solubility of scFvs in *E.coli* is the presence of rare codons (Rosano & Ceccarelli, 2009). Sequence variety plays an important role in the regulation of protein expression and therefore on the translation efficiency. The relationship between the codons are highly influenced by codon bias especially in the case of rare codons (Boel *et al.*, 2016). The reason why this is problematic in the expression of scFv in *E. coli* is due to the difference in codon usage of the mRNA coding for the scFv and that of *E. coli*. During translation if the ribosome encounters a rare codon, it pauses and may even detach from the mRNA, decreasing the yield of expression (Rosano & Ceccarelli, 2009). Possible solutions to this phenomenon have been researched and applied by including recombinant strains that replaces the rare codons, but this introduces a whole additional range of problems including the misfolding and insolubility of the protein as shown by Rosano and Ceccarelli (2009).

3.2. Aims and objectives

This chapter focusses on the direct expression of highly specific single chain variable fragments (scFv). The fragments were obtained from selected phage clones using a human domain (dAb) phage display library (Source BioScience, Australia). Expression was conducted using the non-suppressor HB2151 *E. coli* strain followed by the characterisation of the expressed scFvs. The characterisation consisted of determining the size of the scFvs on a SDS-PAGE gel, determining the concentration of the scFvs with the Bicinchoninic Acid (BCA) assay (Thermo Scientific, USA) and determining whether the expressed fragment contained the ability to bind to the IBV antigen by means of a direct competitive ELISA.

3.3. Materials and methods

Twelve specific phage clones with scFvs that possessed the ability to efficiently bind to IBV antigens were selected out of a large number of phage clones from the human domain (dAb) library (Source BioScience, Australia) and screened by a monoclonal ELISA (Chapter 2, section 2.6.1.3). From these 12 phage clones, only four showed unique and variable heavy

(VH) sequences (Chapter 2, section 2.6.2.2) and these were chosen for protein expression experiments. An additional phage clone with a scFv (scFv 15), possessing a complete VH sequence but without efficient binding ability to the IBV antigen, was selected for the expression as a control scFv. The plasmid DNA of the selected phage clones were extracted (Chapter 2, section 2.6.2.1) and used to transform the HB2151 *E. coli* strain. This strain was provided by the dAb library (Source BioScience, Australia) and is a non-suppressor strain. After transformations were conducted, the expression of the scFvs followed by means of isopropyl β-D-1 thiogalactopyranoside (IPTG) induction. Five selected scFvs were expressed in the HB2151 *E. coli* strain by IPTG induction, followed by the precipitation of scFvs from the supernatant by the addition of a saturated ammonium sulphate solution, followed by dialysis to remove the salt. After precipitation, the scFvs were purified by immunoprecipitation. The size and purity of the scFvs were characterised by SDS-PAGE and the concentrations were determined by a BCA (Bicinchoninic Acid) assay. Thereafter, the binding efficiency of the purified scFv samples were analysed by means of a direct competitive ELISA.

3.3.1. Transformations

3.3.1.1. Preparation of competent HB2151 *E. coli* cells

After streaking out TG1 TR *E. coli* on M9 minimal plates [1.3 % w/v disodium phosphate (Na_2HPO_4-$7H_20$), 0.3 % w/v monopotassium phosphate (KH_2PO_4), 0.05 % w/v sodium chloride (NaCl), 0.1 % w/v ammonium chloride (NH_4Cl), 0.4 % v/v glucose, one mg/ml VitB1, 2.0 mM Magnesium sulphate ($MgSO_4$-$7H_2O$), 0.1 mM calcium chloride ($CaCl_2$) and 1.5 % w/v agar] and HB2151 *E. coli* strain on tryptone yeast extract (TYE) plates [1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.8 % (w/v) NaCl and 1.5 % (w/v) agar], the plates were incubated for 24 hours at 37 °C. A single colony was then inoculated into 5 ml 2x tryptone yeast (TY) medium [1.6% (w/v) tryptone, 1 % (w/v) yeast extract and 5 % (w/v) NaCl] and incubated for 24 hours at 37 °C and shaking at 250 rpm. The overnight culture was then diluted 1:100 into 50 ml 2x TY media. The media was incubated at 37 °C at 250 rpm until an OD_{600} of 0.4 was reached. A total of 40 ml culture was centrifuged at 3200 x *g* and the supernatant was discarded. The pellet was resuspended into 20 ml 50 mM ice-cold calcium chloride ($CaCl_2$). The cells were placed on ice for 20 minutes and centrifuged again at 3200 x *g*. The supernatant was discarded and the pellets resuspended in 2 ml ice cold 50 mM $CaCl_2$, aliquoted and stored at -20 °C.

3.3.1.2. Transformation of the HB2151 *E. coli* cells

The extracted plasmid DNA from the selected clones obtained in Chapter 2, section 2.6.2.1 (Table 3) was thawed on ice with competent *E. coli* HB2151 cells. A total of 50 µl of competent

cells were transferred to ice cold 1.5 ml microcentrifuge tubes. Two microliters of the extracted plasmid DNA was added to the microcentrifuge tube and mixed gently by pipetting. The microcentrifuge tubes were then incubated on ice for 15 minutes, followed by incubation at 42 °C for 45 seconds. The microcentrifuge tubes were then cooled on ice for two minutes where after a total of 900 µl room temperature super optimal medium (SOC) [2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 8.56 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate and 20 mM glucose] was added to the microcentrifuge tubes. The tubes were then incubated for 45 minutes at 37 °C with shaking at 250 rpm. After incubation, 10 µl of the mixture was spread on a TYE plate containing 100 µg/ml ampicillin and incubated at 37 °C for 12 hours.

3.3.2. Expression of the selected scFvs

3.3.2.1. Expression of scFvs with IPTG induction

A single colony from each selected phage clone was selected from the transformation plates and inoculated into 5 ml 2x TY media, incubated overnight at 37 °C, with shaking at 250 rpm. The overnight culture was then diluted 1:100 into fresh 150 ml 2x TY medium (supplemented with 1% (w/v) glucose and 100 µg/ml ampicillin) and incubated at 37 °C with shaking at 250 rpm until an OD_{600} of 0.9 was reached. The culture was induced with a final concentration of 1 mM IPTG, and incubated for 24 hours at 30 °C with shaking at 250 rpm. Thereafter, the culture was centrifuged for 10 minutes at 5000 x *g* at 4 °C. The supernatant was used for precipitation by salting out using ammonium sulphate [$(NH_4)_2SO_4$].

3.3.2.2. Ammonium sulphate precipitation

Ammonium sulphate [$(NH_4)_2SO_4$] was gradually added to 150 ml of supernatant to a final concentration of 561 g/L and saturation of 80% at 25 °C. The mixture was incubated at 4 °C for 24 hours which allowed precipitation to occur. After 24 hours, the mixture was centrifuged at 4000 x *g* for 10 minutes and the pellet was resuspended in 3.5 ml nuclease-free water. The ammonium sulphate was removed from the sample by dialysis in PBS, pH of 7.4. The resuspended pellet was placed in a SnakeSkin® Dialysis Tube (Thermo Scientific, USA) and placed in one litre of PBS (pH 7.4) at 4 °C. This was repeated six times with the buffer changed every one hour. Thereafter, the dialysis tube was placed in three litres of PBS (pH 7.4) for 24 hours. The sample was then collected and concentrated using an Amicon®Ultra- 4 10K Centrifugal Filter Device with a molecular weight cut-off value of 10 kDa (Merck Millipore, IRL). The sample was placed into the filter device and centrifuged at 4000 x *g* for 17 minutes. The concentrated sample was collected in a 1.5 ml microcentrifuge tube and stored at -20 °C, prior

to purification by immunoprecipitation. Prior to purification expression of the scFvs were confirmed by the use of a 12 % SDS-PAGE gel.

3.3.3. Purification by Immunoprecipitation

Immunoprecipitation (IP) was conducted according to the protocol supplied with the Pierce Anti-c-Myc Agarose (Thermo Scientific, USA). Briefly, IP was done in microcentrifuge tubes. A total of 100 µl Pierce Anti-c-Myc Agarose slurry with 0.63 % resin was added to a microcentrifuge tube and centrifuged at 12 000 x *g* for 10 seconds. The supernatant was discarded and 50 µl of PBS (pH 7.4) was added to the resin followed by centrifugation at 12 000 x *g* for 10 seconds. A volume of 200 µl of the expressed scFv sample (lysate) was added to the resin pellet and incubated for 24 hours at 4 °C on a rocker (Benchmark Scientific Inc., USA). This step allowed the Myc-c tag, which is fused to the expressed scFvs, to bind to the resin that is coupled with anti-Myc-c antibodies. The pellet was washed three times with 500 µl PBS (pH 7.4) supplemented with 0.05 % Tween®20 (Sigma, USA) by inverting the microcentrifuge tube to remove the unbound scFvs and then centrifuged at 12000 x *g* for 10 seconds at 4 °C. The supernatant was discarded, and the elution of the Myc-c tagged scFvs was conducted.

The elution was done according to the gentle elution protocol provided by the manufacturer (Thermo Scientific, USA). First, the Pierce Myc-c peptide (Thermo Scientific, USA) was prepared in distilled H_2O to a final concentration of 0.5 mg/ml. One bed volume was added to the resin and incubated at 37 °C for 15 minutes to compete with the bound Myc-c tags fused to the scFvs. The supernatant was collected after centrifugation at 12000 x *g* for 10 seconds at 4 °C. This step was repeated three times. The resin was reused for all samples and therefore the bound Pierce Myc-c peptide required removal after each purification by the addition of 3 M potassium cyanide (KSCN). The remaining Pierce Myc-c peptide within the purified sample was removed by dialysis in PBS (pH 7.4) (as previously described in section 3.3.2.2) and the purified scFvs concentration determined with the BCA protein assay. Dialysis was needed as the presence of the Pierce Myc-c peptide would have influenced the BCA protein assay by being detected as a protein, resulting in the detection of higher concentrations. The purified samples were then aliquoted and stored at -20°C.

3.3.4. Bicinchoninic Acid (BCA) Protein Assay

A Pierce™ BCA Protein Assay kit (Thermo Scientific, USA) was used to determine the concentration of expressed and purified scFv samples. A total of nine standards with a BCA working range of 20 µg/ml to 2000 µg/ml were prepared. The microplate procedure of the Pierce™ BCA Protein Assay kit (Thermo Scientific, USA) was followed based on a working

reagent to sample ratio of 1:8. A total of 25 µl standards and 25 µl scFv samples were added into a flat bottom 96-well plate (Sigma, USA), followed by the addition of 200 µl of the working reagent. The 96-well plate was covered and placed on a rocker (Benchmark Scientific Inc., USA) for 30 seconds and then incubated for 30 minutes at 37 °C, without agitation. Thereafter, the 96-well plate was cooled to room temperature and the absorbance was read at a wavelength of 560 nm with an iEMS micro-well plate reader (Labsystems, Finland). The absorbance values of the standard samples were used to construct a standard curve from which the scFv concentrations were determined (Thermo Scientific, USA).

3.3.5. Visualisation by SDS-PAGE

A sodium dodecyl sulphate polyacrylamide gel was prepared with a stacking gel [4.2 % acrylamide/bis-acrylamide 37.5 to 1 ratio; 0.25 M Tris (pH 6.8); 0.1 % (w/v) sodium dodecyl sulphate (SDS); 0.05 % (w/v) ammonium persulfate [$(NH_4)_2S_2O_8$]; and 0.004 % tetramethylethylenediamine (TEMED)] and a 12 % resolving gel [12 % acrylamide/bis-acrylamide 37.5:10.375 M Tris-HCl (pH 6.8),0.1 % (w/v) sodium dodecyl sulphate (SDS); 0.85 % (w/v) ammonium persulphate [$(NH_4)_2S_2O_8$]; and 0.002 % tetramethylethylenediamine (TEMED)]. Samples containing the purified scFvs were prepared by adding 5 µl of sample buffer [10 % (w/v) sodium dodecyl sulphate (SDS); 10 mM β-mercaptoethanol; 20% (v/v) glycerol; 0.2 % Tris-HCl (pH 6.8) and 0.05 % (w/v) bromophenol blue] to 14 µl of purified scFv sample that was obtained in section 3.3.3. The sample was then boiled at 95 °C for five minutes. The prepared purified scFv samples and a Bio-Rad Precision Plus Protein™ standard (Bio-Rad laboratories, USA) were loaded onto prepared SDS-PAGE gels and electrophoresis was performed at 120 V for 65 min.

3.3.6. Direct competitive ELISA

To determine the binding efficiency of the purified scFvs, a direct competitive ELISA was performed by modifying a commercialised ELISA coated with IBV antigens (BioChek, UK). The purified scFvs that was obtained in section 3.3.3 were used as the “primary antibody” while, the horseradish peroxide (HRP)-conjugated IgG IBV antibodies, that were provided in the kit (BioChek, UK) as a positive control, were used as a secondary competitive antibody. Both primary and secondary antibodies were incubated at the same time and temperature. A total of 50 µl of the positive and negative controls, provided by the kit, were added to the commercialised ELISA plate coated with IBV antigens (BioChek, UK) and 50 µl of the purified scFv samples. All controls and samples were added to the plate in triplicate. After the controls and samples were added, the plate was incubated for 30 minutes at 28 °C. The wells were washed four times with the provided wash buffer. Thereafter, a substrate solution, provided by the commercialised ELISA kit (BioChek, UK), was added to the wells and the ELISA plate

was incubated for 15 minutes at 28 °C. After the incubation time, the provided stop solution was added and the absorbance values of each well was read at a wavelength of 405 nm with the BioTek EL x 800 ELISA plate reader (BioTek, USA).

3.3.7. Statistical analysis

Statistical analysis was conducted using the two-tail T-test with a p value ≤ 0.05, using Microsoft Office Excel 2010 (V 14.0) (Microsoft Corp., Redmond, WA).

3.4. Results

The scFvs were expressed within the culture supernatant and precipitated by 80% ammonium sulphate precipitation followed by dialysis. The scFvs were then concentrated and purified by the use of a Pierce anti-Myc-c agarose kit (Thermo Scientific, USA). After the scFvs were expressed and purified, the size and purity were characterised by SDS-PAGE (Figure 8) and the concentrations were determined by a BCA assay (Figure 9). The binding ability of the purified scFvs were determined by a direct competitive ELISA (Figure 10).

3.4.1. Transformations

Table 5 shows the concentrations and quality of the extracted plasmid DNA from selected clones with an $A_{260/280}$ range of 1.8 to 1.9, $A_{260/230}$ values range from 1.8 to 2.2 and a concentration ranging between 140.58 ng/µl and 658.23 ng/µl. The $A_{260/280}$ and $A_{260/230}$ ratio indicates that the extracted plasmid DNA from all clones were assumed to be of usable quality. Transformations were performed and the transformation efficiency was found to range from 24.77 to 63.69 transformants/ng plasmid DNA. The transformation efficiency was calculated based on the concentration of the plasmid, plasmid added to competent cells and the colony forming units (CFU) per plate (Table A9 in Appendix A).

Table 5: The plasmid concentration (ng/µl), $A_{260/280}$ and $A_{260/230}$ values of extracted plasmid DNA and the transformation efficiency of the five selected phage clones with the binding ability (7, 8, 9, 11 and 12) and one selected phage clone without the IBV binding ability (15) into the HB2151 *E. coli* strain.

Phage clone	Plasmid concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$	Transformation efficiency (transformants/ng plasmid DNA)
7	409.97	1.88	2.34	28.48
8	481.77	1.89	1.82	34.56
9	496.66	1.89	1.84	31.26
11	588.13	1.87	1.76	27.44
12	658.23	1.85	1.78	24.77
15	140.58	1.88	2.17	63.69

3.4.2. Expression and purification of scFvs

Figure 8 illustrates that four selected scFvs with binding ability to IBV antigens (lane 2 to 5) and the scFv without binding ability (lane 6) were expressed and purified. The expected size of a scFv is estimated to be 27 kDa and the bands illustrated in Figure 8 are an approximate size of 27 kDa. The purification method using the Pierce Anti-c-Myc Agarose kit (Thermo Scientific, USA) was very specific, as no other bands are visible on the gel.

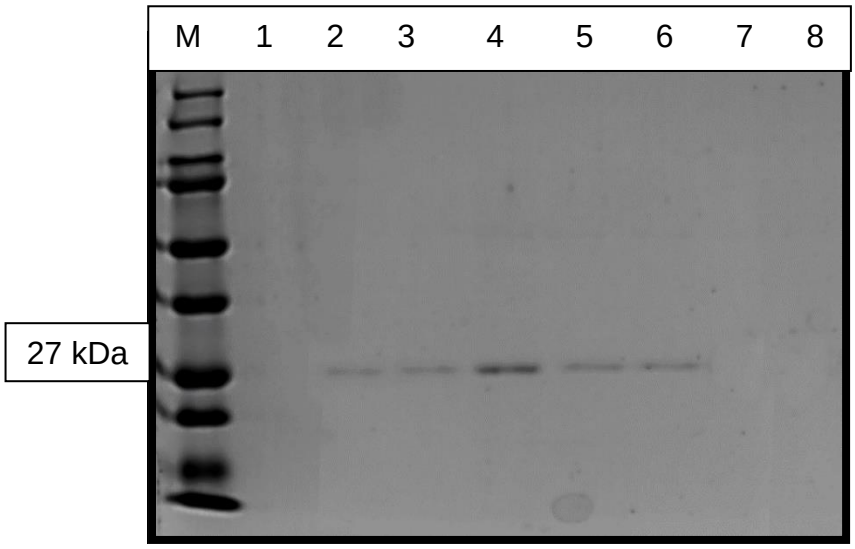

Figure 8: A 12 % SDS-PAGE gel showing the scFvs obtained from expression and purification with the Pierce Anti-c-Myc Agarose kit (Thermo Scientific, USA). The first lane after the Bio-Rad Precision Plus Protein™ Standard (Bio-Rad Laboratories, USA) marker is open, followed by scFv 7 in lane 2, scFv 8 in lane 3, scFv 9 in lane 4, scFv 11 in lane 5, and a non-binding scFv 15 in lane 6. Lane 7 and 8 are negative controls of non-expressing HB2151 *E. coli* strains. All expressed and purified scFvs are within the correct size range of approximately 27 kDa.

3.4.3. Bicinchoninic Acid (BCA) Protein Assay

After the successful expression and purification, the concentrations of the purified scFvs were determined using a Pierce™ BCA Protein Assay kit (Thermo Scientific, USA). Figure 9 illustrates the standard curve of the BCA assay ranging from 0 µg/ml to 2000 µg/ml with the determined concentrations of the five purified scFvs indicated on the standard curve. More precise concentrations are presented in Table 6. The scFvs had a concentration range between 204.37 µg/ml and 257.77 µg/ml.

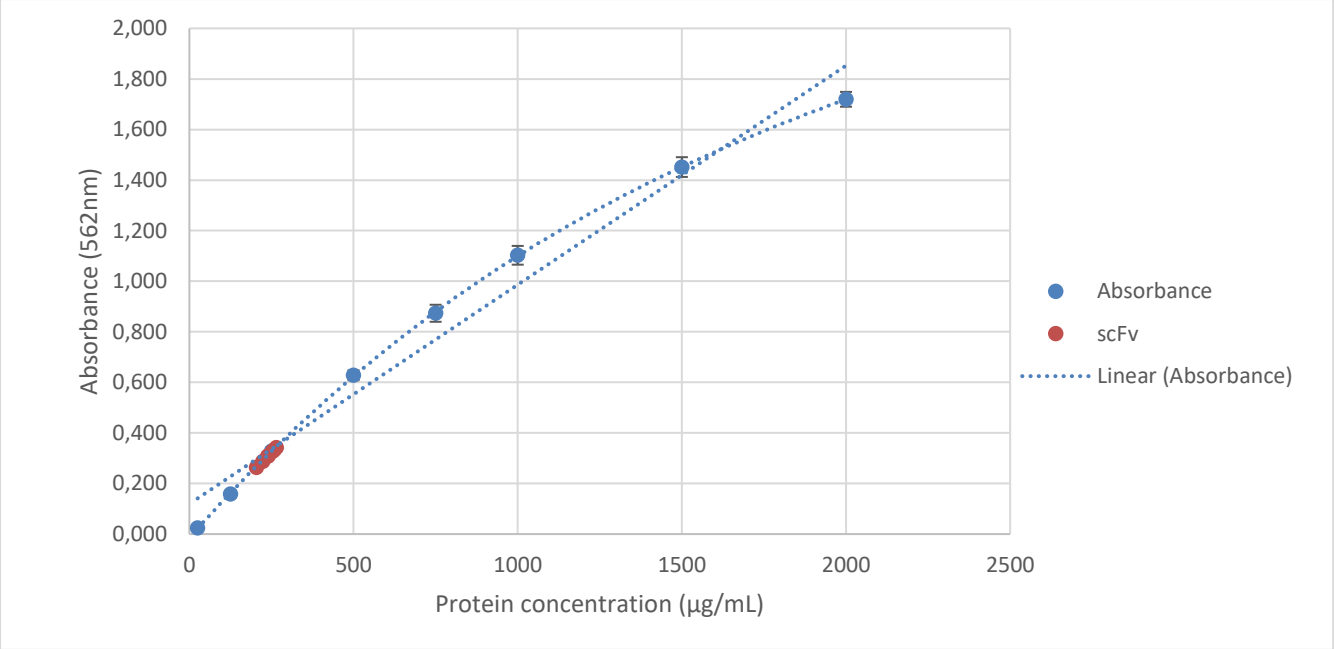

Figure 9: Standard curve of the BCA assay ranging from 0 µg/ml to 2000 µg/ml with the concentrations of the five purified scFvs indicated in red. Error bars are included along with the equation of the curve (y = 0.0009x + 0.1194) and the R^2 value that is equal to 0.9744.

Table 6: Concentrations of the six expressed and purified scFvs calculated by the y = 0.0009x + 0.1194 equation that was determined by the standard curve of the BCA Assay Pierce™ BCA Protein Assay kit (Thermo Scientific, USA).

scFv	Absorbance value at a wavelength of 562 nm	Concentration (µg/ml)
scFv 7	0.332	257.77
scFv 8	0.263	204.38
scFv 9	0.288	223.62
scFv 11	0.309	239.87
scFv 15	0.342	265.07

3.3.4. Direct Competitive ELISA

A direct competitive ELISA was performed to determine if the expressed and purified scFvs (that was obtained in section 3.3.3) retained the ability to bind to the IBV antigens coated on a commercialised ELISA plate (BioChek, UK). The concentrations of the scFvs were not standardised, as it was not needed for the direct competitive ELISA that determined the binding ability of the scFvs to the IBV antigens at optimal concentrations. Table 7 indicates the average absorbance values that was obtained from the direct competitive ELISA that was

measured at a wavelength of 405 nm (Table 9A in Appendix A indicates the absorbance values of three replicates, average and standard deviation). The absorbance values of the scFvs were compared to the positive control, which was provided by the manufacturer of the commercialised ELISA plate that was coated with IBV antigens (BioChek, UK). The comparison is illustrated in Figure 10. The negative control had an absorbance value of 0.51. The red line indicates absorbance value (0.207) of the positive control and the absorbance values of the scFvs (7, 8, 9 and 11) with binding ability all showed lower absorbance values that may indicate binding of the scFvs. The scFv without binding ability showed a higher absorbance value to that of the positive control. These results were expected based on the principle of a direct competitive ELISA. However, statistical analysis of this data by a two-tailed T-Test with a *p* value of 0.05, using Microsoft Office Excel 2010 (V 14.0) (Microsoft Corp., Redmond, WA) indicated that the difference of absorbance value of the scFvs and the positive control is not of significant value (Table 8) as the *p* values of all scFv absorbance value compared to the positive absorbance value was larger than 0.05. The null hypothesis stated that there is no significant difference in the absorbance values between that of the scFvs and the positive control. The null hypothesis is accepted, as the *p* value is larger than 0.05, indicating that there was no significant difference in the measured absorbance values between the scFvs and the positive control.

Table 7: The average absorbance values from the five scFvs, positive and negative controls during direct competitive ELISA that was measured at a wavelength of 405 nm.

Sample	Absorbance (405nm)
Negative control	0.051
Positive control	0.207
ScFv 7	0.195
ScFv 8	0.205
ScFv 9	0.186
ScFv 11	0.193
ScFv 15	0.233

Table 8: The mean, variance, observations, degree of freedom and *p* value from the two-tailed T-test with a *p* value of 0.05, using Microsoft Office excel 2010 (V 14.0) (Microsoft Corp., Redmond, WA) of comparison of observed absorbance values between the purified scFv and the positive control that was the conjugated IgG anti-chicken IBV antibody (BioChek, UK).

	Positive control	scFv 7	scFv 8	scFv 9	scFv 11	scFv 15
Mean	0.216	0.195	0.205	0.186	0.193	0.233
Variance	0.00024375	0.001225	0.001521	0.000784	0.001521	0.000225
Observations	3					
Degree of freedom	2					
two-tail *p* value		**0.298113**	**0.587902**	**0.121125**	**0.312897**	**0.123874**

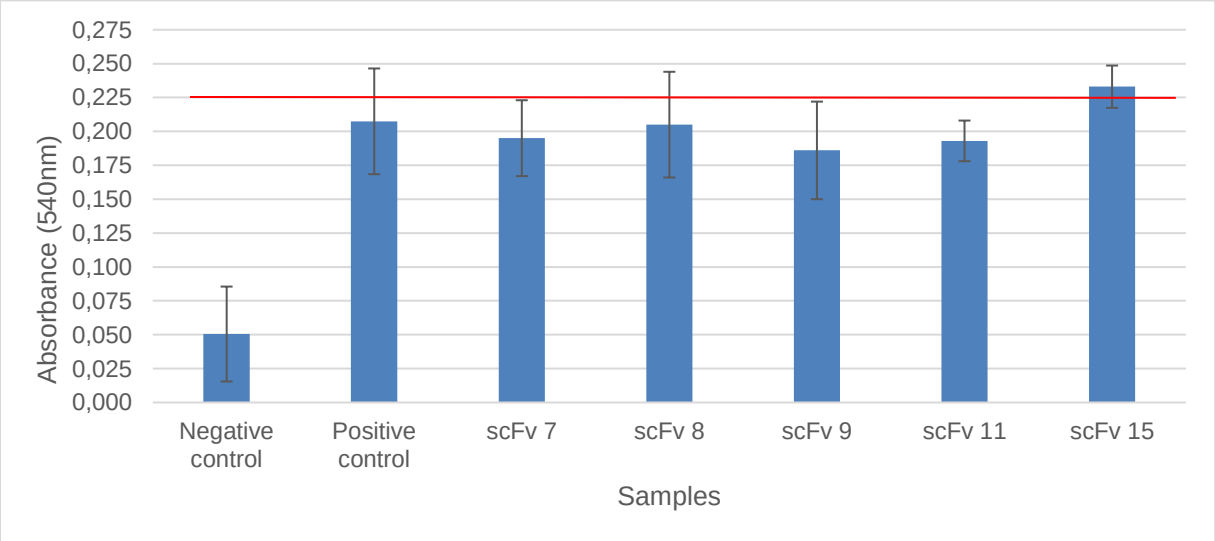

Figure 10: The average absorbance values from the purified scFvs, positive and negative controls from a direct competitive ELISA. The red line indicates the average absorbance value of the positive control (0.207). All scFvs with binding ability have lower absorbance values and does not exceed the indicated red line.

3.5. Discussion

An ideal expression system will produce a large quantity or reasonable quality, be easy to genetically modify, and should express functional fragments in terms of affinity (Floss *et al*., 2007). The expression of recombinant antibodies is dependent on a range of factors including whether the recombinant antibody is a full-length antibody or an antibody fragment, expression

vector, and the expression system used (Leong & Chen, 2008). According to Jurado *et al*. (2002) the expression of antibody fragments in an *E. coli* expression system are more efficient than the expression of full-length antibodies. This is due to *E. coli* that possess mechanisms that aid in promoting the correct folding of antibody fragments and therefore contribute to high levels of expression (Jurado *et al*., 2002). A study by Miller *et al*. (2005) stated that single chain variable fragments (scFvs) have a higher expression level within an *E. coli* expression system when compared to yeast expression systems. Other studies conducted on scFv expression in *E. coli* expression systems determined that it is plausible to increase the quantity of scFvs by the expression of scFvs within the cytoplasmic space while obtaining soluble scFvs (Miller *et al*., 2005; Guglielmi & Martineau, 2009). According to Dimitrova and co-authors (2009) the production of scFvs is dependent on three steps namely, transformation, expression and purification. The chapter focussed on producing scFvs by the transformation of the HB2151 *E. coli* strain, the expression of scFvs and purification the expressed scFvs. Characterisation of the expressed scFvs were also conducted based on the purity, size and binding ability.

The first step of producing scFvs was the transformation of extracted plasmid DNA into the HB2151 *E. coli* host cell. Transformation is dependent on the purity of DNA used during the transformation process. The quality of plasmid DNA that was extracted from selected phage clones in Chapter 2 (section 2.5.6.2) were of acceptable quality as it fell within the accepted range for pure DNA. The transformation efficiency indicated that successful transformation occurred as single colonies were obtained from the competent HB2151 *E. coli* cells that were transformed with the extracted plasmid DNA from the selected phage clones. This is in accordance with other studies conducted on the transformation of plasmid DNA that was extracted from phage clones all showed successful levels of transformation (Persic *et al*., 1997; Shi *et al*., 2003; Almdni, 2013; Khan *et al*., 2017).

The expression of the scFvs resulted in bands with an approximate size of 27 kDa as visualised on the SDS-PAGE gel. The estimated size of a scFv is in the range of 27 kDa (Weisser & Hall, 2009; Wang *et al*., 2013; Lin *et al*., 2015). The purification of scFvs is usually conducted by affinity purification and in this instance a Pierce anti-c-Myc Agarose purification kit (Thermo Science, USA) was used because of a myc-c tag that was fused onto the scFvs gene sequence by the manufacturer. This method of purification is considered to be a highly specific immunopurification method (Laroche-Traineau *et al*., 2000). The anti-c-myc antibody is coupled with a high affinity resin, which recognises the c-myc epitope tag at the C-terminus of the scFv (Thermo Scientific, USA). The fact that there were no additional bands visible on the SDS-PAGE gel, after the purification, indicate that the purification was highly specific to

the scFv of interest. Determining the concentration of the expressed and purified scFvs were conducted by a Pierce™ BCA Protein Assay kit (Thermo Scientific, USA). The Pierce Anti-c-Myc Agarose (Thermo Scientific, USA) kit recommends dialysis of the purified scFvs after purification to remove the c-myc peptide that was used during elution, as the presence of the peptide within the sample may influence the concentration determination. The determined concentrations of the expressed scFvs are all within the working range of the Pierce™BCA Protein Assay kit (Thermo Scientific, USA) that was used. According to Wiechelmann *et al.* (1988) protein concentrations measured by a BCA assay is based on functional groups in reference to a common protein, for example bovine serum albumin (BSA). The colour formation is influenced by the presence of a macromolecule, the number of peptide bonds, and by the presence of certain amino acids (Thermo Scientific. Undated *b*).

The determined concentrations for all expressed scFvs and the presence of bands on the SDS-PAGE gel with an approximate size of 27 kDa, indicates that expression occurred within the *E. coli* expression system. Factors that can influence the expression of scFvs are the variation of the variable regions within the scFvs, structural and conformational alterations caused by synonymous codons, expression system, expression vector, presence of rare codons, and the three intramolecular disulphide bonds in the structure of scFvs (Rippmann *et al*., 1998; Ye *et al*., 2008; Schaefer & Plückthun, 2010; Malpiedi *et al*., 2013).

After expression and the determination of the expressed scFvs concentrations, a direct competitive ELISA was performed to determine whether the expressed and purified scFvs retained the ability to bind IBV antigens. Binding of the expressed and purified scFvs to IBV antigens were expected. Lower absorbance values of the scFvs were observed at a wavelength of 405 nm in comparison to the positive control. Statistical analysis of the measured absorbance values indicated that the difference was not significant as the *p* values for all purified scFvs were all higher than 0.05. However, it cannot be said that insufficient binding of the purified scFvs occurred due to the lack of knowledge needed on the quantity of the scFvs that were added and needed to create sufficient reaction to take place. The main reason for this occurance is based on the fact that the secondary competitive antibody is a divalent IgG antibody whereas the scFvs are monoclonal antigen binders. This therefore causes a difference in valency between the two types. The divalent antibodies has a stronger antigen binding energy arriving from it's enhanced avidity and therefore it makes it impossible for the monoclonal scFvs to displace the divalent antibody during the experiment.

The commercialised IBV antigen coated kit (BioChek, undated) used was optimised for the purpose of diagnostics. Modification to the protocol could therefore have influenced the calculations and the results. A solution is a manual set-up of an ELISA by the coating of an

ELISA plate with the appropriate concentration of IBV antigens and optimising the ELISA protocol as conducted by Lin and co-workers in 2015. The binding did not differ statistically significantly to the positive control, although binding was observed as this was seen by the absorbance values that are lower than the positive but higher than the negative control and this is what was expected. The ideal would have been to have seen a significant decrease in absorbance compared to the positive control. As mentioned experimental optimisation in terms of scFvs concentrations added should be considered. Other studies that were conducted on the binding of scFvs to the IBV antigen showed to have binding ability (Lin *et al*., 2015). Therefore, the expressed and purified scFvs indicate a potential binding ability after alteration of the experimental procedure.

In conclusion, the selected scFvs were expressed, purified and characterised successfully. The expression system produced reasonable quality and quantity scFvs. The quantification of the expressed scFvs cannot be stated to be as required, as it is unclear whether sufficient quantities were produced. However, it should be noted that pure fragments were produced. If sufficient quantities were produced a significant drop in absorbance compared to positive control would have been apparent throughout the experiment. Never the less, the purification was highly specific and the characterisation in terms of size and purity was successful. The ease of production indicates the possible use of recombinant antibody fragments as a therapeutic treatment against Infectious bronchitis virus.

Chapter 4

In ovo virus neutralisation

Introduction

Vaccination acts to stimulate the immune system to produce antibodies against viral antigens. Antibodies are an effective way for the immune system to provide protection against a virus. The binding of antibodies to a virus can potentially lead to virus neutralisation, preventing further infection (Magnus, 2013). Virus neutralisation assays measure the number of antibodies (titre) that is required to prevent a virus from entering cells, by preventing attachment to host cells. The results are expressed as the reciprocal of the highest dilution at which infection with the virus is prevented. This is also referred to as the end-point virus titre (Truelove *et al*., 2016). Virus neutralisation assays are time consuming, expensive to perform, requires a live virus and has been shown to result in poor reproducibility between laboratories (Truelove *et al*., 2016). However, virus neutralisation assays are more sensitive in comparison to other assays as it represents the most realistic reaction of the virus with the presented antigens (Truelove *et al*., 2016).

4.1. Virus titre determination

Specific pathogen-free (SPF) embryonated eggs are used for the cultivation and propagation of different types of viruses, including infectious bronchitis virus (IBV), which is an epitheliotropic virus (Kwon *et al*., 1993; Kint *et al*., 2015). Only SPF eggs are used for cultivation, as non-SPF eggs could possibly possesses antibodies against IBV that may interfere with the replication of IBV (Kint *et al*., 2015). The process requires inoculation of the 9-11 day old SPF eggs with virus at different dilutions and monitoring of observed mortalities (Truelove *et al*., 2016; OIE, 2013). The virus can be inoculated by several methods, the simplest being the allantoic route followed by the amniotic, yolk sac or chorioallantoic membrane routes (Kint *et al*., 2015). The inoculated eggs are monitored for a specific time period, depending on the virus type (OIE, 2013). Infectious bronchitis virus causes morphological changes within infected embryonated eggs and may lead to embryonic death within four to seven days (Kint *et al*., 2015). Morphological changes may include the curling of embryos, wry neck, deformed feet compressed over the head, retardation of growth, thickened amniotic membrane and less amniotic fluid (National Research Council, 1959). Upon monitoring of the eggs, the virus end-point titre can be determined by means of the Reed and Meunch or the Spearman-Karber method (Reed and Meunch, 1938; Spearman, 1908). The virus end-point titre is defined as the reciprocal of the dilution at which 50 % of the inoculated

embryos die (ELD_{50}) while the Embryo-Infectious-Dose-50 % (EID_{50}) is the amount of virus that will infect 50 % of eggs (Kint *et al*., 2015).

4.2. Virus neutralisation assay

A virus neutralisation (VN) assay is used to detect antibodies that have the ability to neutralise virus infectivity (Hitchner, 1973). Neutralising antibodies are highly specific and target important antigens of the viral surface that play a role in the process of cell absorption (Villareal, 2010). Virus neutralisation assays can be carried out in SPF embryonated eggs, cell cultures or in tracheal ring cultures (De wit, 2000; Bande, 2016). To perform these assays, the virus amount should be standardised (Hitchner, 1973). Further, two- or ten-fold dilutions are needed of a standardised serum, which is then mixed with a fixed amount of infective virus antigen. The reaction between the antigen and antibody is allowed for a standard time and temperature, depending on the virus type (OIE, 2013). The virus-serum mixture is then assayed for virus infectivity using end-point titration methods to determine the highest dilution of sera that possesses detectable neutralising antibodies (Hitchner, 1973). Comparison of VN assays are challenging due to the difficulty in standardisation between different assays, a VN assay should therefore be decided on by sensitivity, reproducibility and titre detection (Wooley *et al.*, 1976; De wit, 2000). Virus neutralisation assays can be altered to determine if recombinant antibody fragments are efficient in neutralising virus particles (Guo *et al.*, 2010). A study conducted in 1984 by Mockett and co-workers on the Massachusetts M41 strain of IBV, determined that the virus was neutralised by the majority of monoclonal antibodies that bound to the S1 glycoprotein and by some which bound the anti-membrane protein, indicating monoclonal antibodies can neutralise the virus.

4.3. *In ovo* virus neutralisation assays

According to OIE (2013) all IBV sera should be inactivated by heating for 30 minutes at 56 °C. Inactivation is needed due to non-specific, heat-labile inhibitors present in the serum that are active against IBV (Truelove *et al*., 2016). *In ovo* virus neutralisation assays require the mixture of both the virus and the potentially neutralising serum. Virus particles are mixed with serum and incubated at 37 °C or room temperature for 30 to 60 minutes. To determine the neutralising ability of antibodies two methods can be implemented. The first method, called the alpha method, requires a constant serum concentration and varying dilutions of the virus. The results are expressed as a neutralisation index (NI) that represents the log_{10} difference in virus titres (OIE, 2013). The second method, called the beta method is more commonly used within embryonated eggs. The principle of the beta method relies on two-or four-fold serial dilutions of the serum, with a constant concentration of the virus, usually at the EID_{50} (OIE,

2013). Both of these methods are usually performed along-side the virus strain end-point virus titration as a control (OIE, 2013).

4.4. Aim and objectives

The aim of this chapter was to determine if the four expressed and purified scFvs (obtained as described Chapter 2 and 3), which indicated binding activity to the IBV antigen, were able to neutralise the IBV H120 strain by conducting an *in ovo* virus neutralisation assay. This assay was conducted with SPF eggs and the H120 IBV vaccine strain (AviPro, Germany). Two different experiments were conducted to reach the aim. First, the ELD_{50} of the H120 IBV vaccine strain (AviPro, Germany) was determined. Second, the alpha virus neutralisation assay with the H120 IBV vaccine strain (AviPro, Germany) and the four scFvs.

4.5. Materials and methods

4.5.1. IBV H120 strain virus end-point titre

The end-point titre determination was performed in duplicate with each batch divided into two groups (Group A and Group B) of five eggs per dilution. The groups were inoculated eight hours apart. It should be noted that this process requires the manipulation of embryonated eggs under sterile conditions and therefore sterile techniques were applied in all aspects including disinfecting equipment and surfaces with 70% ethanol.

Specific pathogen-free (SPF) eggs were obtained from the Agriculture Research Council, Glen, Free State, South Africa. The eggs were incubated in an egg incubator with set temperature and humidity controls (Surehatch, South Africa) for 9 days at a temperature of 37 °C and a humidity of 60 %. After 9 days of incubation, the eggs were candled to determine fertility, by placing the eggs in a darkroom and holding a light against the egg to observe blood vessels and movement. Eggs were deemed fertile if blood vessels leading to an embryo was visible within the egg yolk. The eggs were then returned to the incubator within a time-period of 30 minutes to minimise stress to the embryos.

A freeze-dried IBV H120 vaccine strain was obtained from Avipro (Lohmann Animal Health Gmbh, Germany) with a minimal EID_{50} of $10^{3.0}$ per dose. The vial was stored at 4 °C until needed and resuspended according to the guidelines supplied by the manufacturer: The freeze-dried virus was resuspended in one millilitre PBS (pH 7.4) and a ten-fold serial dilution was prepared (10^{0} - 10^{-5}). The virus was incubated at 56 °C for 30 minutes to inactivate non-specific heat-labile inhibitors active against IBV. Ten, 9-day-old, SPF embryonated eggs were inoculated with 0.1 ml virus per dilution via the allantoic inoculation route, as described by Gelb (1989) as follows; the eggs are placed in a darkroom and positioned with the air sac

facing upwards. The eggs are candled and a marker is used to indicate the air sac position. The eggs are disinfected and placed in a Biosafety level-2 laminar-flow cabinet where an egg puncher is used to make a small hole above the air sac. The virus dilutions are to be injected at a 45° angle into the eggs, followed by the closure of the hole with wood glue (National Adhesive Distributers, South Africa). The eggs were incubated and observed every eight hours over a time-period of six-days, as indicated in literature (Kint *et al.,* 2015). Six SPF eggs served as controls, of which three eggs were injected with PBS that served as experimental controls and three eggs were not inoculated. Embryos that showed mortality after 24-hour post-infection were removed, and deaths were ascribed to shock. The allantoic fluid was extracted from all embryos and streaked for bacterial contamination. The results of the mortality of the embryos, which could be attributed to the effects of the virus, were used to calculate the virus end-point titre by means of the Spearman-Karber method as given by Equation 2 (Spearman, 1908).

$$\text{Log}_{10}\ 50\%\ \text{end point dilution} = -\left(x^{\circ} - {}^{d}/_{2} + d\ \Sigma\ {}^{ri}/_{ni}\right) \quad \text{.....Equation 2}$$

x° = $\log_{10}$ of the reciprocal of the highest dilution (lowest concentration) at which all eggs are positive;

d = $\log_{10}$ of the dilution factor;

ni = number of animals* used in each individual dilution (after omitting accidental deaths);

ri = number of positive animals (out of ni) -Summation is started at dilution x°.

*animals referring to embryonated SPF eggs

4.6. Confirmation of the presence of IBV

4.6.1 Allantoic fluid extraction

Eggs were candled in a darkroom and embryo mortalities were identified by the absence of blood vessels leading to the embryo within the egg yolk and the absence of embryo movement. Allantoic fluid was extracted from eggs that showed embryonic death. The eggs were disinfected with 70% ethanol and the glue that was placed to cover the hole was removed with tweezers. A 2 ml syringe (avacare™, South Africa) and hypodermic needle (Medispo, Saudi Arabia) was used to extract the allantoic fluid. The fluid was placed in a 2 ml microcentrifuge tube and 10 µl was streaked onto trypticase soy agar (TSA) plates to determine the absence of bacterial contamination. The extracted allantoic fluid was stored at -20 °C until further use.

4.6.2 Total RNA extraction

Total RNA was extracted from the allantoic fluid using the TRIzol method (Chomczynski & Sacchi, 1987). A total of 0.2 ml allantoic fluid was added to one millilitre TRIzol reagent to

solubilise nucleic acids and denature the proteins, followed by the addition of chloroform to separate the proteins from the RNA and to resolve DNA. After phase separation occurred, 0.5 ml isopropanol was added per one millilitre TRIzol reagent used to precipitate the RNA. A washing step with 70 % ethanol and 0.1 % (v/v) diethyl pyrocarbonate (DEPC)-treated water followed the precipitation step. The water was treated with DEPC to inactivate any RNase enzymes within the water that might damage the extracted RNA. The quality and quantity of the extracted RNA was determined using the Nanodrop ND-1000 UV-spectrophotometer (Nanodrop Inc., USA).

4.6.3 cDNA synthesis

The extracted RNA was converted to cDNA using the high capacity RNA-to-cDNA kit (Applied Bioscience, USA) as outlined by the manufacturers protocol. The kit provided a 20X Enzyme mix that contains the Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase that synthesises complementary DNA strands, RNase inhibitor proteins, and a 2x RT Buffer Mix (which includes dNTPs, random octamers, and oligo dT-16). Reagents were placed on ice to thaw and a reaction volume of 20 µl was prepared by adding 10 µl 2x RT buffer, one microliter 20x RT enzyme mix, two microliter total RNA (up to 9 µl) and nuclease-free water. The reaction was incubated at 37 °C for 60 minutes, followed by the inactivation of enzymes by heating the reaction to 95 °C for five minutes in a thermal cycler (Applied Biosystems, Singapore). The cDNA was stored at -20 °C and the quality and quantity were determined using the Nanodrop ND-1000 UV-spectrophotometer (Nanodrop Inc., USA).

4.6.4 Amplification of the S1 gene

Amplification of the S1 gene serves as indication for the presence of IBV. A PCR was performed on synthesised cDNA to verify the presence of IBV within the extracted allantoic fluid. This was done by the amplification of the S1 gene conserved region with the following primer pair (Meir *et al*., 2010): The forward primer S1NEWOLIGO 5'-TGAAAACTGAACAAAAGAC-3' and the reverse primer Degenerate 3 5' -CCATAAGTAACATAAGGRCRA-3' (Integrated DNA Technologies, South Africa). A final reaction volume of 25 µl was prepared by the addition of 1X ThermoPol®buffer (New England BioLabs, USA), 200 µM dNTP's (New England BioLabs, USA), 0.2 µM of each primer (Integrated DNA Technologies, South Africa), 1.25 units/ 50 µl Thermopol Taq polymerase (New England BioLabs, USA) and a final amount of one nanogram of template cDNA. The reaction mixture was placed in a 2720 Thermal Cycler (Applied Biosystems, Singapore) with the following conditions as described by Meir and co-workers (2010), 35 cycles of denaturation at 94 °C for 80 s, annealing at 50 °C for 60 s and elongation at 74 °C for 3 min was performed. Followed by a final elongation at 74 °C for 5 minutes and a 4 °C hold.

Thereafter, a total of 3 µl PCR product was loaded onto a 1 % (w/v) agarose with 0.001 % (v/v) ethidium bromide as stain and electrophoresis was conducted at 8 V/cm for 45 minutes. The remaining PCR products were used for sequencing PCR using the BigDye® Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with both the forward and reverse primers. Two different reactions were performed, one for each primer. For each PCR product; a mixture with an end volume of 10 µl containing 20 ng amplified S1 PCR product, 3.2 pmol/µl primer (Integrated DNA Technologies, South Africa), 5x BigDye Sequencing Buffer (Applied Biosystems, USA), 0.6x Ready Reaction Premix (Applied Biosystems, USA) and nuclease-free water.

Thermal cycling conditions were as follows; the sample was incubated at 96 °C for one minute, followed by 25 cycles of an initial denaturation step at 96 °C for 10 seconds, annealing at 50 °C for five seconds and elongation at 60 °C for four minutes (Applied Biosystems, USA). Upon completion of the sequencing PCR, the PCR products were cleaned using the EDTA/ethanol PCR product cleaning method. The 10 µL sequencing PCR product was adjusted to 20 µL by the addition of ultrapure H_2O. A total 7.35 mM ethylenediaminetetraacetic acid (EDTA) and absolute ethanol was added to a final volume of 70.5% (v/v), followed by centrifugation at 20 000 x *g* for five minutes at 4 °C to remove the unincorporated di-deoxynucleotidetriphosphates (ddNTPs). The supernatant was aspirated and 60 µL ice cold 70 % ethanol was added to the pellet. Centrifugation was repeated, the supernatant was aspirated, and the pellet was dried using a miVac vacuum chamber (SP Scientific, UK). The pellet was stored in a darkroom at 4 °C until Sanger sequencing was performed at the Department of Biochemical, Microbiological and Food Biotechnology, University of the Free State.

After sequencing, contigs were assembled using Geneious V 11.1.4 (http://www.geneious.com, Kearse *et al*., 2012). The contigs were analysed and compared to known sequences in the Nucleotide collection of the GenBank Database using a nucleotide BLAST analysis tool (http://www.ncbi.nlm.nih.gov.Blast.cgi). Blast was performed to determine if the PCR amplicons were indeed the IBV S1 gene, indicating the presence of IBV in the extracted allantoic fluid.

4.7. Virus neutralisation assays

4.7.1 Preparing scFv and IBV H120 strain sample

The alpha method of virus neutralisation was used during this experiment. A series dilution of the IBV H120 strain was made in PBS (pH 7.4) from 10^{-1} to 10^{-4}, this range was selected as the EID_{50} was determined to be $10^{4.6}$/ml in section 4.2.1. The purified scFvs were standardised

to ensure the end-point titre results obtained can be compared using the alpha virus neutralisation assay method (OIE, 2013). The concentration of the purified scFvs was standardised at 200 µg/ml, as this was the lowest limiting concentration obtained from the BCA assay conducted in Chapter 3 (Section 3.4.3, Table 8). The concentrations were standardised by diluting scFvs in a PBS solution (pH 7.4) to a final concentration of 200 µg/ml. Each standardised purified scFv was mixed with 100 µl IBV H120 strain of each dilution. The mixture from henceforth will be referred to as the scFv-viral mixture. The scFv-viral mixture was incubated for 30 minutes at 56 °C to ensure that all heat-liable proteins were inactivated.

4.7.2 scFv-viral mixture end-point titre

A total of 20 eggs were used for each of the scFv-viral mixture containing the scFvs that were expressed and purified in section 3.4.2 of Chapter 3. A total of five eggs were used for each scFv at each dilution (10^{-1} to 10^{-4}). In addition to the experimental controls receiving PBS, one scFv (scFv 15) without binding ability was expressed and purified with the aim of being inoculated along with the scFvs that had shown potential binding ability to the IBV antigen (section 3.4.2 in Chapter 3). The eggs were placed in a temperature-controlled humidified egg incubator (Surehatch, South Africa) at a temperature of 37 °C with a humidity of 60 % and tilted every hour to ensure the virus is distributed throughout the allantoic fluid of the egg. The eggs were monitored every eight hours for six consecutive days. Deaths after the first 24 hours were ascribed to shock. The end-point titre was determined using the Spearman-Karber method (Spearman, 1908) with the use of Equation 2 as described in section 4.2.1. A comparison was done between the $\log_{10}$ values of the end-point virus titres between the scFv-viral mixtures and the IBV H120 and statistical analysis was conducted by the two-tail T-test with a *p* value of 0.05, using Microsoft Office Excel 2010 (V 14.0) (Microsoft Corp., Redmond, WA).

4.8. Results

4.8.1. Determining IBV H120 strain titre

The average end-point titre for the IBV H120 vaccine strain in Table 11 calculated by Equation 2 indicates that the determined end-point titre is $10^{4.6}$/ml. Figure 11 illustrates that no bacterial growth was present within the extracted allantoic fluid that was plated out on TSA plates. Total RNA was extracted from allantoic fluid and synthesised to cDNA. This was followed by amplification of the S1 gene, and sequencing to confirm the presence of IBV within inoculated SPF embryonated eggs (Figure 9) as the sequence data showed that all S1 amplified PCR products to be the IBV spike glycoprotein gene Table 13.

4.8.1.1. IBV H120 strain end-point titre

Using the Spearman-Karber end-point method (Equation 2), the end-point virus titre for the first replicate of H120 in SPF eggs was determined to be $10^{4.4}$ ELD_{50}/ml (Table 11). The values for Equation 2 was obtained from Table 9. The x° value represents the log_{10} of the reciprocal of the highest dilution (lowest concentration) at which all animals/eggs are positive; meaning the highest concentration where all embryonated eggs died from IBV infection. In this instance it was at 10^{-1} meaning that x° = 1. The value of d is also 1 as log_{10} of a 10-fold dilution is equal to one. The *ni* value is the number of animals/eggs used for each individual dilution which was 10. The *ri* value is 39 representing the sum of all embryonated eggs that died from IBV infection, including the x° dilution. Equation 2 and Table 10 were used to determine the end-point titre to be $10^{4.7}$ ELD_{50}/ml for the second replicate of H120 in SPF eggs. The average end-point titre of the two replicates was calculated to be $10^{4.6}$ ELD_{50}/ml (Table 11). All eggs that died from both replicates were tested for bacterial contamination. No bacterial growth was observed from the extracted allantoic fluid, thus eliminating bacterial contamination as a cause of death (Figure 11).

Table 9: Results of observed mortalities of SPF embryonated eggs after inoculation with a dilution series of IBV H120 vaccine strain from Avipro (Lohmann Animal Health Gmbh, Germany) for replicate 1.

Log_{10} Dilution	**Embryonated Eggs**	
	Died	Inoculated (n)
0	10	10
-1	10	10
-2	9	10
-3	8	10
-4	5	10
-5	7	10

Table 10: Results observed mortalities of SPF embryonated eggs after inoculation with a dilution series of IBV H120 vaccine strain from Avipro (Lohmann Animal Health Gmbh, Germany) for replicate 2.

Log_{10} Dilution	Embryonated Eggs	
	Died	Inoculated (n)
0	10	10
-1	10	10
-2	10	10
-3	9	10
-4	7	10
-5	6	10

Table 11: The calculated 50% end-point dilution and log_{10} value of the end-point virus titre (ELD_{50}/ml) obtained from all two replicates by the Spearman-Karber method (1908).

Sample inoculated into embryonated eggs	50% End-point dilution	Log_{10} value of virus titre (ELD_{50}/ml)
IBV H120 (1)	$10^{-4,4}$	4,4
IBV H120 (2)	$10^{-4,7}$	4,7
Average	$10^{-4,6}$	4,6

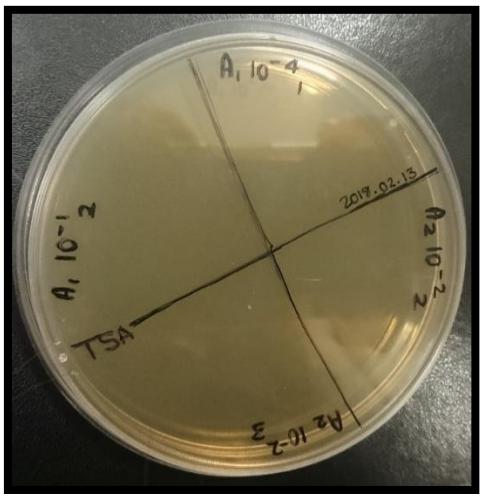

Figure 11: Extracted allantoic fluid plated on TSA media plates. Results observed after 24 hour incubation at 37 °C. A_1 embryonated egg from the first inoculated batch A_2 embryonated egg from the second inoculated batch. The dilution and number of egg from a total of 10 is also indicated.

4.8.1.2. Confirmation of the IBV presence

We confirmed that the extracted allantoic fluid from selected eggs did not die as a result of a bacterial infection as indicated by the lack of bacterial growth on the TSA plates (Figure 11). After the allantoic fluid extraction, total RNA was extracted, and the RNA concentration and absorbance ratios obtained from spectrophotometry are given in Table 12. Thereafter, cDNA was synthesised, and the cDNA concentration and absorbance ratios are given in Table 13. Using the synthesised cDNA, the S1 gene of IBV was amplified followed by sequencing to determine the presence of IBV. Table 14 represents the sequence results obtained, indicating that IBV was present within the embryonated eggs upon confirmation using the nucleotide BLAST analysis tool (http://www.ncbi.nlm.nih.gov.Blast.cgi)

Both the RNA and cDNA values were found to be within the acceptable range in terms of purity and concentration and were used to confirm IBV presence by the amplification of the S1 gene. The sequences obtained from the S1 amplified PCR products (Table 14) indicated that the S1 gene of IBV was present in the allantoic fluid from all the eggs that died (Figure 12).

Table 12: The obtained RNA concentrations (ng/µL), A260/280 and A260/230 values of extracted total RNA from allantoic fluid during IBV H120 strain titre determination.

Sample	ng/µL	260/280	260/230
Negative control	600.02	1.74	1.32
Positive control	2886.08	1.93	1.34
10^{0}	1770.92	1.85	1.77
10^{-1}	1978.52	1.93	1.59
10^{-2}	2887.74	1.95	1.58
10^{-3}	3938.75	2.00	1.65
10^{-4}	1222.52	2.03	1.92
10^{-5}	1443.12	1.84	1.74
10^{-4}S*	1805.09	1.82	1.51
10^{-5}S*	950.76	1.93	1.59

* RNA was extracted from allantoic fluid that was extracted from embryonated eggs that did not die during experiment.

Table 13: The cDNA concentrations (ng/µL), A260/280 and A260/230 values during IBV H120 strain titre determination.

Sample	ng/µL	260/280	260/230
Negative control	537.90	1.79	1.30
Positive control	555.63	1.75	1.21
10^{0}	1594.95	1.74	1.24
10^{-1}	1460.99	1.78	1.94
10^{-2}	526.28	1.79	1.71
10^{-3}	530.72	1.79	1.65
10^{-4}	539.99	1.77	1.47
10^{-5}	538.01	1.79	2.35
10^{-4}S*	536.96	1.78	1.40
10^{-5}S*	518.37	1.77	1.56

* cDNA was obtained from the allantoic fluid that was extracted from embryonated eggs that did not die during experimentation.

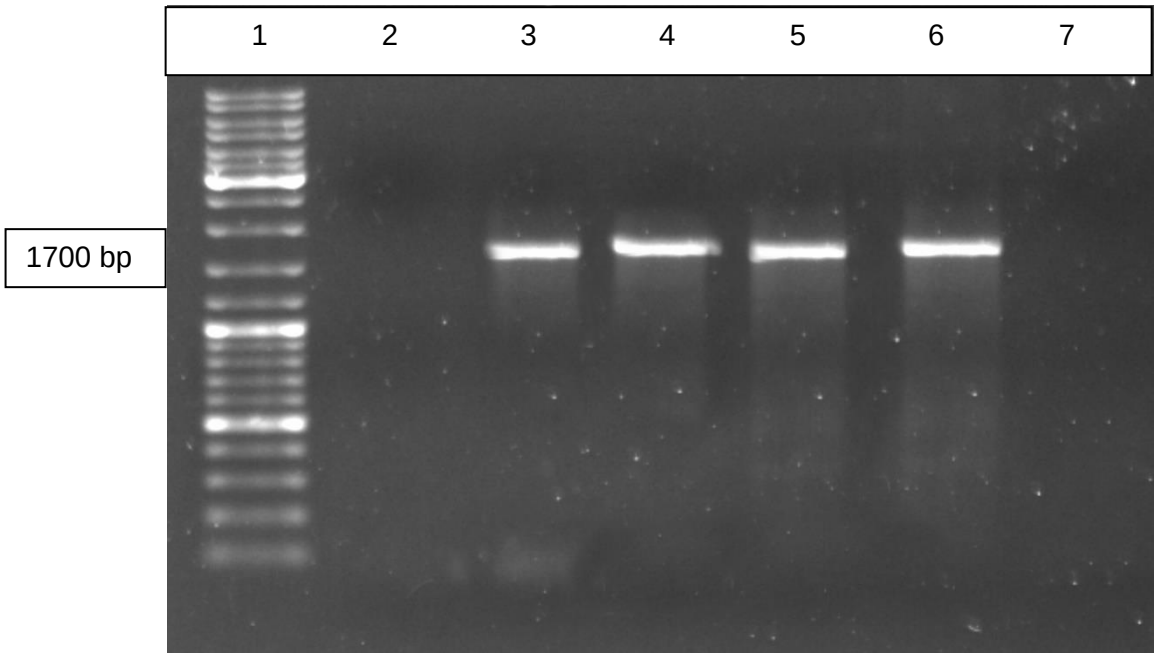

Figure 12: A 1 % agarose gel of the S1 gene (1700 bp) amplified from extracted allantoic fluid from selected embryonated eggs upon inoculation with IBV H120, during the end-point titre determination. MM indicates the O'GeneRuler™ DNA Ladder Mix molecular weight marker (Thermofisher Scientific). Lane 2 no-template control; lanes 3-5 the S1 gene obtained from three selected embryonated eggs (10^{-1}, 10^{-3} and 10^{-4}), and lane 6 the positive IBV control obtained from Avipro (Lohmann Animal Health Gmbh, Germany).

Table 14: Results obtained from the nucleotide BLAST analysis tool (http://www.ncbi.nlm.nih.gov .Blast.cgi) for all dilutions sequenced that tested positive during the S1 PCR for IBV detection after inoculation with the H120 virus strain.

Sample	Description	Query cover	E value	Identity	Accession
Positive	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
10^0	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
10^{-1}	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
10^{-2}	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
10^{-3}	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
10^{-4}	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1

4.8.2. Virus neutralisation assays

The end-point virus titres of the IBV H120 strain that was mixed with the purified scFvs (referred to as scFv-viral mixtures) was determined by the Spearman-Karber method (Equation 1) using the results obtained in Table 15 and Table 16. The results of the average 50 % end-point dilution and $\log_{10}$ end-point virus titre values are presented in Table 17. The

log_{10} values of end-point virus titres (neutralising index) of the IBV H120 strain and the log_{10} values of end-point virus titres (neutralising index) of the scFv-viral mixtures were compared and a log difference between the neutralising index were observed (Figure 13). The statistical analysis (Table 18) indicated that the neutralising index of scFv-viral mixture 9 (that was a mixture of scFv 9 and the IBV H120 strain) had a significant difference in comparison to the IBV H120 strain.

4.8.2.1. ScFv-viral mixture end-point titre

The scFv and H120 mixture that was inoculated into five embryonated eggs per dilution with a dilution range of 10^{-1} to 10^{-4} and this was repeated in duplicate experiments (Table 15 and Table 16). The end-point titres for all scFv-viral mixtures from both replicates are listed in Table 11A in Appendix A. The average end-point titre obtained from all the scFv and H120 mixtures ranged between $10^{-3.1}$ and $10^{-4.1}$ while the end-point dilution of the average IBV H120 strain was determined to be $10^{-4.6}$ (Table 16).

Table 15: Number of embryonated eggs that was injected at each dilution and number of mortalities observed during the first replicate of the end-point virus titre from scFv-viral mixtures within embryonated eggs during replicate 1.

Log_{10} dilution	N	ScFv-viral mixture 7*	ScFv-viral mixture 8*	ScFv-viral mixture 9*	ScFv-viral mixture 11*	ScFv-viral mixture 15*
10^{-1}	5	5	5	5	5	5
10^{-2}	5	5	5	4	5	5
10^{-3}	5	4	3	3	3	4
10^{-4}	5	2	1	2	2	4

N= Number of eggs inoculated per dilution for each scFv-viral mixture
* = Number of embryonated egg mortalities for scFv-viral mixture

Table 16: Number of embryonated eggs that was injected at each dilution and number of mortalities observed during the second replicate of the end-point virus titre from scFv-viral mixtures within embryonated eggs during replicate 2.

Log_{10} dilution	N	ScFv-viral mixture 7*	ScFv-viral mixture 8*	ScFv-viral mixture 9*	ScFv-viral mixture 11*	ScFv-viral mixture 15*
10^{-1}	5	5	5	5	5	5
10^{-2}	5	5	5	4	5	5
10^{-3}	5	3	3	2	3	4
10^{-4}	5	3	2	2	3	4

N= Number of eggs inoculated per dilution for each scFv-viral mixture
* = Number of embryonated egg mortalities for scFv-viral mixture

Table 17: The average 50 % end-point dilution and the log_{10} values of the end-point titres (neutralising index) obtained for the all the scFv and H120 mixtures determined by the Spearman-Karber method (Spearman, 1908).

Sample inoculated into embryonated eggs	50% End-Point dilution (ELD_{50}/ml)	Virus Neutralisation Index (NI)-Log_{10} value
IBV H120 strain	$10^{4.6}$	4.6
ScFv-viral mixture 15	$10^{4.1}$	4.1
ScFv-viral mixture 7	$10^{3.7}$	3.7
ScFv-viral mixture 8	$10^{3.4}$	3.4
ScFv-viral mixture 9	$10^{3.2}$	3.2
ScFv-viral mixture 11	$10^{3.6}$	3.6

4.8.2.2. Comparison between the IBV H120 strain and scFv-viral mixture end-point titres

As seen in Table 17 and 18, there is a difference in the end-point dilution and the end-point virus titre between the IBV H120 strain alone and with the scFv-viral mixtures. Figure 13 illustrates that there is a log difference between the log_{10} value of the end-point titres from the IBV H120 strain (4.6) and scFv-viral mixture 8 (3.4), scFv-viral mixture 9 (3.2) and scFv-viral mixture 11 (3.6). The log_{10} value of the end-point titre of the scFv-viral mixture 7 is 3.7. The results indicate that the scFvs that were mixed with the IBV H120 strain all had lower log_{10} end-point titre values as the IBV H120 strain and scFv-viral mixture 15 that contained a scFv that did not show any indication of binding ability to the IBV antigens in section 2.5.5. in Chapter 2 and section 3.3.4. in Chapter 3.

Regardless of the observed lower log_{10} end-point titre value, statistical analysis of the comparison between the IBV H120 log_{10} end point titre value and the log_{10} end point titre values from the scFv-viral mixtures indicated that only scFv-viral mixture 9 has a *p* value lower than 0.05 (0.02357) (Table 18). This indicated that the observed difference was only significant for scFv-viral mixture containing the scFv 9. The other scFv-viral mixtures all showed a *p* value higher than 0.05. The null hypothesis stated that there is no significant difference between the log_{10} end-point titre values between that of the scFv-viral mixtures and the IBV H120 strain. The null hypothesis was thus accepted, indicating that there was no significant difference in the log_{10} values of the end-point titre values except for scFv-viral mixture 9.

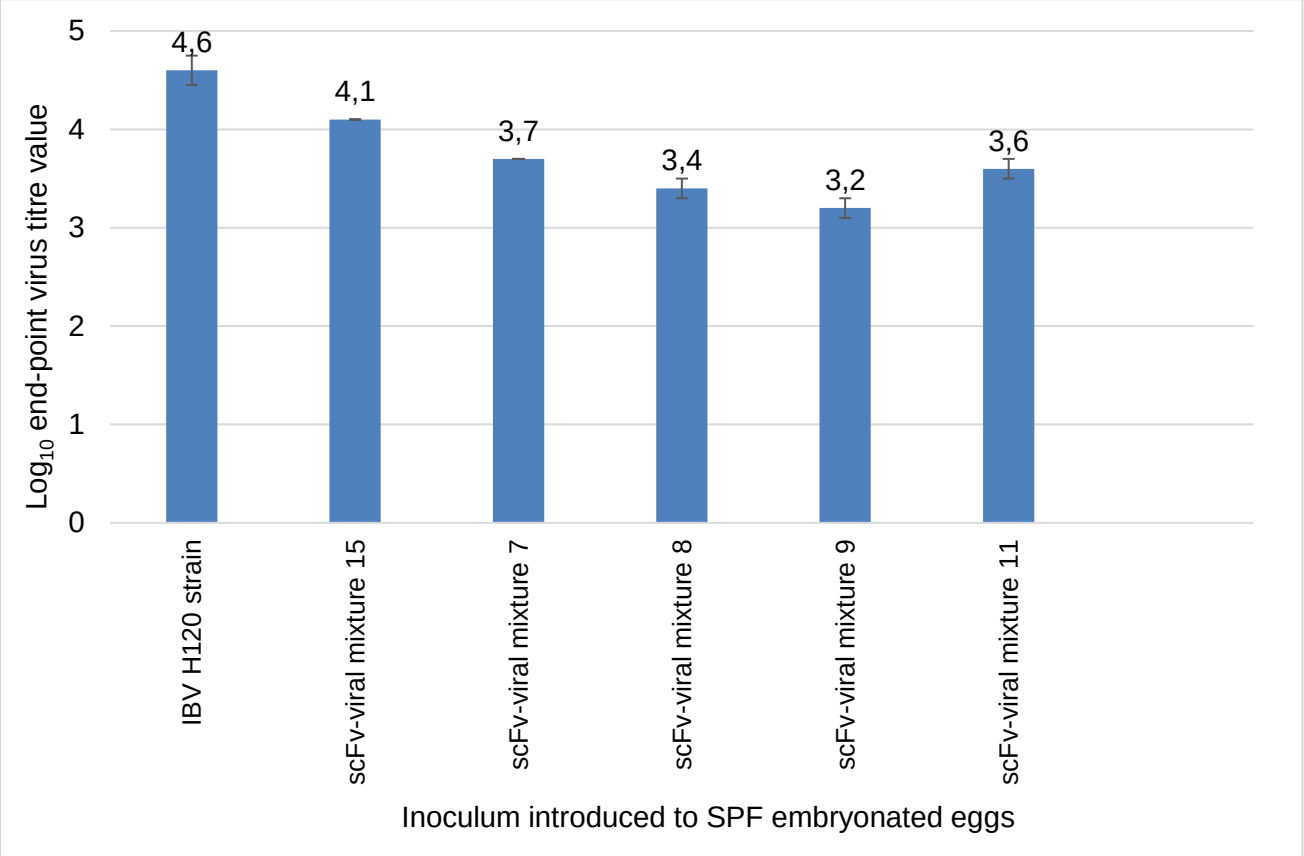

Figure 13: The log_{10} values of the end-point virus titre from all scFv-viral mixtures and IBV H120 strain that were calculated using the Spearman-Karber method (Spearman, 1908).

4.8.2.3. Statistical analysis

Table 18: The mean, variance, observations, degree of freedom and *p* value from the two-tailed T-test with a *p* value of 0.05, using Microsoft Office excel 2010 (V 14.0) (Microsoft Corp., Redmond, WA) of the comparison of the neutralising index of scFv-viral mixtures and the IBV H120 strain.

	Positive control	ScFv-viral mixture 7	ScFv-viral mixture 8	ScFv-viral mixture 9	ScFv-viral mixture 11	ScFv-viral mixture 15
Mean	4.55	3.7	3.4	3.2	3.6	4.1
Variance	0.045	0	0.02	0.02	0.02	0
Observations	2	2	2	2	2	2
Degree of freedom	1	1	1	1	1	1
two-tail *p* value		**0.1112**	**0.13628**	**0.02357**	**0.16382**	**0.204833**

4.8.2.4. Confirmation of the presence of IBV

The RNA that was extracted from allantoic fluid by the TRIzol method showed high concentrations of RNA with fair $A_{260/280}$ but low $A_{260/230}$ values. The $A_{260/280}$ values ranged between 1.74 and 2.05 and the $A_{260/230}$ values between 1.24 and 1.92 (Table 19). The concentration of the extracted RNA was adequate for cDNA synthesis as 2 ng of RNA was required by the high capacity RNA-to-cDNA kit (Applied Bioscience, USA). The cDNA concentrations in Table 20 showed adequate concentrations of cDNA with concentrations ranging between 1239.44 ng/µL and 2098.21 ng/µL. The $A_{260/280}$ and $A_{260/230}$ values indicated that the cDNA was of adequate quality to be used for amplification of the S1 gene through PCR to confirm the presence of IBV. The negative control, extracted allantoic fluid from the negative control during the virus neutralisation assay, that was used for RNA extraction and cDNA synthesis was not positive for the S1 gene, indicating no IBV was present. All other samples tested positive for the S1 gene indicating the presence of IBV within the embryonated eggs that died during the experiment. The nucleotide BLAST analysis tool (http://www.ncbi.nlm.nih.gov.Blast.cgi) provided the description and all samples were identified with 99% confidence to be an Infectious bronchitis virus isolate spike glycoprotein gene (KX107816.1). The query coverage was 100% and an E value of 0 was obtained for all samples.

Table 19: The RNA concentrations (ng/µL), $A_{260/280}$ and $A_{260/230}$ values of extracted RNA from the allantoic fluid obtained from selected embryonated eggs that was inoculated with the scFv-viral mixtures.

Sample	RNA concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
Negative control	617.22	1.74	1.32
Positive control	3886.08	1.92	1.24
ScFv-viral mixture 7	1770.92	1.96	1.58
ScFv-viral mixture 8	1978.52	2.01	1.65
ScFv-viral mixture 9	2887.74	2.05	1.92
ScFv-viral mixture 11	744.80	1.74	1.26
ScFv-viral mixture 15	378.52	1.85	1.77
ScFv-viral mixture 9S*	1222.52	1.78	1.44
ScFv-viral mixture 11S*	950.76	1.64	1.32

* RNA was extracted from allantoic fluid that was extracted from embryonated eggs that did not die during experiment.

Table 20: The cDNA concentrations (ng/µL), $A_{260/280}$ and $A_{260/230}$ values that was obtained from the synthesised cDNA from the extracted RNA of embryonated eggs inoculated with scFv-viral mixture.

Sample	cDNA concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
Negative control	1511.16	1.71	0.67
Positive control	1539.45	1.72	0.67
ScFv-viral mixture 7	1522.27	1.73	0.68
ScFv-viral mixture 8	1545.29	1.72	0.70
ScFv-viral mixture 9	1765.00	1.69	0.76
ScFv-viral mixture 11	1329.67	1.75	0.86
ScFv-viral mixture 15	1239.44	1.77	1.58
ScFv-viral mixture 9S*	1372.11	1.74	1.01
ScFv-viral mixture 11S*	1319.89	1.74	0.77

* cDNA was obtained from the RNA that was extracted from allantoic fluid retrieved from embryonated eggs that did not die during experiment.

Table 21: Results obtained from the NCBI nucleotide-nucleotide BLAST database from sequenced positive S1 PCR products for IBV detection after inoculation with scFvs and H120 mixture virus titre.

Sample	Description	Query cover	E value	Identity	Accession
Positive	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
7	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
8	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
9	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
11	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1
15	Infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene, partial cds	100%	0	99%	KX107816.1

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4.9. Discussion

This focus of this chapter was to determine if expressed and purified scFvs have a neutralising effect on the IBV H120 strain. This was achieved by determining the end-point virus titre of

the H120 IBV strain which was shown to be $10^{4.6}$/ml. Following this, an *in ovo* virus neutralisation assay was conducted by means of the alpha method (Kint *et al*., 2015).

According to the manufacturer of the Avipro IBV H120 vaccine strain (Lohmann Animal Health Gmbh, Germany) the vaccine is expected to have a minimum end-point titre of $10^{3.0}$/ml per dose. The end-point titre of the IBV H120 strain was determined in this study using 9-day old SPF embryonated eggs and is in accordance to the specifications of the manufacturer. To determine the end-point virus titre, different methods can be used, including the use of SPF embryonated eggs, tracheal rings and chorionic allantoic membranes (Cavanagh, 2003; Yan *et al*., 2017). The use of embryonated eggs are considered to be time consuming, expensive and this method has a low level of reproducibility between laboratories (Truelove *et al*., 2016). However, this method provides the best model to observe reproducibility of a virus within an *in vivo* environment (Truelove *et al*., 2016). Steps were taken to overcome the mentioned disadvantages. Problems with reproducibility were overcome by repeating the IBV H120 strain end-point titre determination and by obtaining the SPF embryonated eggs from the same supplier namely the ARC (Glen, Free State, South Africa). In addition to the repetition, the total number of eggs (n= 10) used during the experiments were divided within two different groups (Group A and Group B), which were inoculated eight hours apart. All experiments were performed in a BSL-2 laboratory and the live IBV H120 vaccine strain was obtained from a vaccine manufacturer Avipro (Lohmann Animal Health Gmbh, Germany).

After the end-point titre was determined, the absence of bacterial contamination within the allantoic fluid confirmed that the observed mortality of the embryonated eggs were not due to bacterial contamination and that the deaths are due to the IBV H120 strain infection. The presence of the IBV H120 strain should therefore be detected within the allantoic fluid. The extracted RNA of all eggs had adequate concentrations of RNA that was extracted, but low A_{260}/A_{230} ratios were obtained. Low A_{260}/A_{230} ratios indicate contamination of the RNA sample with phenols and other substances (Kim *et al*., 2014). This occurred due to the TRIzol-RNA extraction technique that was used as the TRIzol cause guanidine to co-precipitate with RNA resulting in low A_{260}/A_{230} ratios (Wang & Stegemann, 2010). Although this can influence the cDNA synthesis (Wang & Stegemann, 2010), it was not the case as the cDNA values obtained was also considered to be of usable quality for the amplification of the S1 gene. All extracted allantoic fluid from the inoculated eggs showed amplification of the S1 gene of IBV and were identified to be similar to infectious bronchitis virus isolate CK/CH/JS/LYG1506-2 spike glycoprotein gene (KX107816.1) by the GenBank nucleotide BLAST analysis tool (http://www.ncbi.nlm.nih.gov.Blast.cgi). According to Yan and co-workers (2011) who submitted the KX107816.1 sequence, the spike glycoprotein originated from a vaccine IBV

strain, supporting the results obtained as the IBV H120 strain was also obtained from a vaccine (Avipro, Germany). Other studies, including the study conducted by Handberg et al. (1999) concluded that the detection of the S1 gene from allantoic fluid can be successfully performed to indicate the presence of IBV (Handberg *et al*., 1999; Acevedo *et al.*, 2013; Jahantigh *et al.*, 2013; OIE, 2013; Lin & Chen, 2017). The S1 gene was amplified from the allantoic fluid of all embryonated eggs that died during the experiment indicating that the mortality was due to the infection of IBV, while the negative control eggs were devoid of the S1 gene, as expected.

After the end-point virus titre of the IBV H120 strain was determined, the virus neutralisation assays were conducted using the alpha method (Kint *et al*., 2015). The alpha method determines whether neutralising antibodies are present within a serum and it has been used in previous studies with various types of viruses, including coronaviruses (OIE, 2013; Kint *et al*., 2015). Although the beta method is more frequently used for VN assays in embryonated eggs, the alpha method was more suited in this case as this method requires a standard concentration of the sera, or purified scFvs, to be added to a ten-fold dilution range (OIE, 2013).

Comparing the neutralising index of the scFv-viral mixtures to the IBV H120 strain, a log reduction difference was seen, indicating the neutralising effect of the scFvs that was added to the scFv-viral mixtures. This is plausible as seen by the results demonstrated in Chapter 2 and Chapter 3 where it was concluded that the selected scFvs displayed binding ability to the IBV antigen. The binding of scFvs may be responsible for the blocking of the H120 that prevents the binding to the host epithelial cells, which then influence the end-point virus titre and the $\log_{10}$ value (Wang *et al*., 2009). A study conducted by Lin *et al.* (2015) conducted a similar study that concluded that expressed scFvs, resulted in a partial neutralising effect against the IBV H120 strain via the infection of Vero cells.

The difference in log values were observed between the H120 and the scFv-viral mixture of scFvs with H120 mixture, indicate a partial neutralising effect from the scFvs against the IBV H120 strain that could be seen using embryonated eggs. According to Rockland Inc. (undated) the most commonly used concentration of antibody used during a virus neutralisation assay is 5 mg/ml. However, this is not necessarily the norm and just an indication, as the amount needed to neutralise different viruses differs. In this case, an end concentration of 0.2 mg/ml of the purified scFvs were used in the VN assay. It is possible that an increased scFv concentration will contribute to a higher virus neutralisation effect (Magnus, 2013). To definitely state that a scFv has a neutralising effect on a virus, a most basic question with a highly complicated answer should first be considered. The question being, how many scFvs are needed to bind to one virus particle to lead to neutralisation? After answering this question

then only the neutralising ability of the individual scFvs can be determined with certainty. The question seems straight forward, but according to Magnus (2013) no one has yet been able to answer that question but further investigation on kinetic neutralisation models could be useful to determine the answer.

As differences in the $\log_{10}$ end-point titre values were observed for all scFv-viral mixtures in comparison to the IBV H120 strain, statistical analysis of the data showed that only of scFv-viral mixture 9 had a significantly different value with a *p* value less than 0.05. The difference in the $\log_{10}$ end-point titre values between the scFvs in the sample may be due to the genetic differences such as the amino acid composition. These genetic differences are based on the nucleotide as well as the amino acid sequence (section 2.6.2, Chapter 2). The scFvs were selected from a human domain antibody library that had variations introduced into the CDR 3 regions. According to Hoet and co-authors (1999) and Lin and co-workers (2015) the CDR 3 region plays an important role in antibody-antigen binding and antigenic specificity. It can therefore be concluded that diversity within the amino acid sequence of the scFvs can have an effect on the scFv folding and therefore influence the binding ability. Although genetic differences may play a role in the $\log_{10}$ end-point titre values between the scFvs other influence can be due to valency differences between the scFvs and natural antibody fragments.

Comparisons of the amino acid composition of the scFvs, especially the CDR 3 (section 2.6.2, Chapter 2) may explain the differences of $\log_{10}$ end-point titre values seen between the scFvs in the scFv-viral mixtures. The amount of rare amino acid residues and substitutions within the scFvs should also be taken into account as this can influence antigen specificity and binding ability (Lin *et al*., 2011). Results obtained by a study conducted by Kant and co-workers (1992) illustrated that the differences in the efficiency of neutralising antibodies were influenced by the substitution of proline with glutamine; and tyrosine with histidine. The substitution of residues within the CDR may therefore influence the binding ability of the expressed scFv to the IBV H120 strain antigen.

In conclusion, a log difference of the $\log_{10}$ end-point titre value of the scFv-viral mixtures were observed in comparison to the IBV H120 strain in embryonated eggs. This indicates a partial neutralising ability by the scFvs. Further investigation is however needed in terms of the concentrations of scFvs that will have a complete neutralisation effect as well as conducting virus neutralisation assays in other *in vivo* systems and furthering the investigation of kinetic neutralisation models of scFvs.

Chapter 5

Discussion and Conclusions

Infectious bronchitis virus (IBV) is distributed worldwide and is the etiological agent of infectious bronchitis (IB) in poultry. The first isolation of IB was in 1931 where it was defined as an acute, highly infectious, respiratory disease of chickens (Beach & Schalm, 1936). Infectious bronchitis is transmitted by direct and indirect methods between chickens of all ages (Ignjatovic & Sapats, 2000; OIE, 2013). Avian IBV has been isolated in broilers, layers and breeder chickens (Butcher *et al*., 2015). The main effects of this virus on chickens include respiratory tract infections, egg laying problems and renal damage (Pattison *et al.*, 2008). The economic impact of IBV on the poultry industry is seen on production losses due to delayed maturity, decline of egg quantity and quality, poor weight gain, downgrading of carcasses, and mortality (Ignjatovic & Sapats, 2000; Pattison *et al.*, 2008). Inadequate vaccination and antigenic variants play roles in the reoccurrence of IBV within the poultry industry (Ignjatovic & Sapats, 2000). Therefore, the main issue regarding IBV is the reported variance amongst IBV strains that occur worldwide (Bakhshesh *et al.*, 2016). The variance is not only due to mutations that are introduced during replication but also due to recombination events that occur among strains (Bande *et al.*, 2015). The occurrence of variations among IBV strains may affect viral characteristics, thus lowering the vaccination efficacy, resulting in inadequate protection in the birds (CEVA, 2005; Kint *et al.*, 2015). Variation in the spike glycoprotein (S1 subunit) of IBV, is comparable to the variation reported in the HIV-1 envelope glycoprotein that hampers effective vaccine development (Khan *et al.*, 2017).

A study conducted by Khan and co-workers (2017) on the HIV-1 envelope glycoprotein investigated the neutralising ability of antibody fragments with the utilisation of phage display technology. Phage display technology contributes to therapies by delivering improved efficacy, higher safety, and potentially lower production costs (Hougaard *et al.*, 2017). The study conducted by Khan and co-workers (2017) engineered single chain variable fragments (scFvs) that proved to possess neutralising abilities. Neutralising abilities can be defined as the decrease in infectivity, preventing the attachment of a virus to a host cell by binding of antibodies or antibody fragments to the surface proteins of the virus (Klasse, 2014). The principle has been applied to a vast number of viruses and can therefore be applied to IBV (Kwon *et al*., 1993; Kint *et al*., 2015). The aim of the present study was to detect, identify and express scFvs that display viral binding ability, and thus neutralisation capability against the most frequent occurring IBV strains namely, H120 and M41. According to the results obtained, a partial neutralising effect from the expressed scFvs were observed against the IBV H120 antigen.

Objective 1 was to screen and identify scFvs using a commercial ELISA kit coated with IBV antigens and a human domain (dAb) library (Source BioScience, Australia). The results obtained in Chapter 2 shows that the selection of scFvs were successful. The commercial ELISA plate (BioCheck, UK) was coated with the most frequently identified IBV strains, namely the M41 and H120 strain. The commercial ELISA plate (BioCheck, UK) was easy to incorporate into the protocol provided by the human domain antibody library (Source BioScience, Australia). A large number of phage clones were found to contain antigen specific scFvs that were able to bind IBV antigens during screening. Specific clones were selected from the screening process in order to further select, more stringently, by a monoclonal sandwich ELISA. The selection was successful as the specificity of the phage clones with the scFvs increased with each panning round. A total of 12 individual phage clones containing antigen specific scFvs were identified. The 12 selected phage clones with antigen specific scFvs were sequenced, and sequence analysis indicated a total of five scFvs with a complete sequence and correct open reading frames. However, two of the five sequences were identical, resulting in four unique and complete sequences. The results from Chapter 2 are in alignment with a number of recent studies that were successful in selecting antibody fragments with phage display (Ahmad *et al.*, 2012; Almdni, 2013; Awad *et al*., 2015; Hougaard *et al.,*2017). The detection and sequencing of four scFvs, which showed binding to the M41 and H120 strain of IBV in this section facilitated the expression of scFvs (Chapter 3).

The second objective of this project was to successfully express the selected scFvs. The expression of five selected scFv (four binding and one non-binding) was successfully performed with the use of the *E. coli* expression system supplied by the manufacturer of the human dAb phage display kit (Source BioScience, Australia). These expressed scFvs were purified and the obtained results are discussed in Chapter 3. The four expressed scFvs were purified by means of immunoprecipitation with the Pierce Anti-c-Myc Agarose kit (Thermo Scientific, USA) and the concentrations were determined using the Pierce™ BCA Protein Assay kit (Thermo Scientific, USA). The concentrations of the expressed scFvs ranged between 204.38 µg/mL and 265.07 µg/mL falling within the range of other studies (Almdni, 2013). The concentrations that were determined differ due to the difference in nucleotide and amino acid sequence between the four scFvs (Chapter 2, Section 2.2.2). Expression is dependent on the scFv nucleotide sequences as it can influence the transcription and translation as well as the folding of the scFvs. The binding affinity of the expressed and purified scFvs against IBV antigens were tested by means of a direct competitive ELISA. Although, a decrease in the absorbance values that were measured at a wavelength of 405 nm was observed, the results showed to be statistically insignificant for any scFvs when compared to

the positive control. This is due to the monoclonal scFvs that were not able to out compete with the divalent antibodies used.

The amount of the scFvs that can possibly bind to an antigen differs from the amount of complete antibodies as the binding position and size differs between antibody fragments and complete antibodies. Single chain variable fragments are smaller in size when compared to complete antibodies and the binding position of the scFvs to antigens has not yet been intensly studied and remains to be unknown. The antibodies used in the experiment were also IgG divalent antibodies with a much higher avidity than that of the monoclonal scFvs which made it nearly impossible for the scFvs to outcompete the dicalent antibodies. Taking these facts into account the amount of scFvs that a possibly bind to the IBV antigen in comparison to the amount of complete antibodies would definitely differ (Almdni, 2013). In addition, it should be noted that a commercial ELISA plates were used and these plates are optimised for diagnostic application and the detection of complete antibodies in a serum.

The third objective was to determine if the selected, expressed, and purified scFvs have the ability to neutralise the Avipro H120 IBV virus strain (Lohmann Animal Health Gmbh, Germany) *in ovo*. The results obtained from Chapter 4 indicate that partial virus neutralisation was obtained. The data from the neutralising assay showed a one log difference of the end-point titre of the sample mixtures in comparison to the end-point titre of the IBV H120 strain, which indicates a partial neutralising effect of the scFvs. However, complete neutralising was not obtained. The optimal concentrations for the scFvs, mixed with the IBV H120 strain were however not determined, as the purpose was to see whether any neutralising effect can be observed.

Similar results regarding the increase of specificity of scFvs during selection and panning has been reported in other studies (Almdni, 2013; Lin *et al*., 2015). These studies also utilised scFvs obtained from a human domain antibody library but the selection method and the method of neutralisation detection of the scFvs differed. It should be taken into account that there are different selection methods and the success of both panning and selection is dependent on various factors such as; the phage type, display type, antibody fragment, type of library and the surface to which antigens are bound to during selection (Hougaard *et al.,* 2017). According to Hougaard and co-workers (2017) the use of the dAb library has been highly successful and the utilisation of scFvs are more efficient than other fragments. The use of an immune library, such as the human domain library, also has the advantage of selecting a great range of phage clones with high affinity against the specific antigen due to the greater range of introduced variety (Ahmad, 2012).

The display types of a phage display library has been reviewed in Chapter 1 and the most advantageous display type is one that utilises the dAb library, namely the phagemid display (Lee *et al.*, 2007; Ahmad, 2012). The fact that the fragments have the same binding ability and binding functionality of full length antibodies, ease of permeation into tissues, and manipulation of fragments, highlights the advantage of this system (Malpiedi *et al.*, 2013). However, the linker chain between the two functional units in a scFv determines the folding and binding characteristics. According to Klein and co-authors (2014), the linkage between functional units of fragments can have an influence on epitope access and can therefore affect the antigen binding ability and potentially the neutralising effect. The most commonly used linker is the $(GGGGS)_n$; as is the case in the current study. Studies conducted by Evers and co-workers (2006) found that linkers provide limited domain separation due to the unstructured nature. More rigid linkers, for example an all helical linker $A(EAAAK)_nA$ is reported to provide more efficient separation thus promoting adequate binding of recombinant scFvs (Klein *et al.*, 2014).

Another aspect regarding the selection of scFvs is the selection method, which was performed with a monoclonal sandwich ELISA in this study. According to Hougaard and co-workers (2017) the binding of the antigen directly to a plastic surface during selection is the most commonly used method. However, the presentation of antigens can be increased with the immobilization of biotinylated antigens to streptavidin via a linker that will result in better selection results (Hougaard *et al*., 2017).

The use of a mixture of IBV antigens (M41 and H120) coating the commercial ELISA kit provided a bigger chance of selection but the desired aim was to select one specific antigen, thus it would have been more beneficial to have used a plate only coated with the specific antigen to ensure higher specificity. In addition by doing this, the time it takes to move from selection to expression is speeded up (Hougaard *et al*., 2017). Selecting small plaques rather than large plaques will also increase the selection of specific phage clones and eliminate some of the growth bias that is found within phage libraries as it was stated by Hougaard and co-workers (2017). The reason why growth bias occurs is due to growth of incomplete phage clones being much quicker to reproduce than the complete phage clones, therefor resulting in larger clones. Selecting small plaques will therefore increase the chance of obtaining complete phage clones (Lee *et al*., 2007).

To obtain more specific selection results it may be advisable to increase the level of binding during the selection process by limiting the amount of phages added during the selection process. This will increase the level of specificity as it was observed during a study conducted by Almdni (2013). It should, however, be noted that increasing the number of selection rounds will not necessarily increase the quality of the scFvs obtained. After selection occurs, the

selected antibody fragments have to be analysed for the correct and complete sequences (Almdni, 2013). According to Hougaard and co-workers (2017) the presence of amber codons within phage display libraries are also a prominent issue as it was also observed by Almdni (2013) and this contributes to low number of clones with a correct open reading frame. The presence of the stop codons influences the reading frames and therefore the successful downstream expression experiments. The stop codons occur due to utilising the suppressor strain during selection. The stop codon is located between the terminal Myc-c tag and the pIII protein. The non-suppressor strain does not suppress the amber stop codon to ensure the expression of the scFv without the pIII protein (Lee *et al*., 2007). Lee and co-workers (2007) recommended a mutation of the amber stop codons into glutamate to ensure high levels of expression.

The use of a well-optimised *E. coli* expression system creates an advantage as it has been modified to provide high yield of soluble proteins (Rosano & Ceccarelli, 2009). However, the expression of scFv in *E. coli* is problematic due to codon bias and amber stop codons (Lee *et al.*, 2007). Other issues that should be taken into account is the quantity of expressed scFvs and solubility of fragments. A study conducted by Wang and co-authors (2013) proposed a few solutions to overcome expression woes with scFvs by the co-expression of chaperones. The use of expression systems other than *E. coli* and various vectors, should also be considered. For example, scFvs have successfully been expressed within multiple plant-based expression systems as well as insect cells (Ahmad, 2012).

The purification of expressed scFvs was achieved using affinity chromatography with a Myc-c tag ligand, resulting in low quantities of pure scFvs. Other studies that utilised this method also reported on low amounts of purified fragments obtained after one round of purification (Malpiedi, 2013). The purification process proved to be expensive and time consuming while not delivering large quantities of purified peptide fragments. Improving the expression and the purification would therefore be advisable for future studies. A study conducted by Malpiedi (2013) has proven that affinity chromatography provided the purest scFvs but the smallest amount of it, while other methods such as precipitation, liquid-liquid extraction and cryogels produced a larger volume of scFvs but not as pure. The question of which method to use will therefore be based on what the downstream application of the expressed scFvs will be.

The expression of scFvs were sufficient as the end goal of the scFvs were to detect whether or not a neutralising effect can be detected against the IBV H120 antigen. The results obtained resulted in one statistical significant difference of the virus neutralisation log, indicating a neutralising effect against the IBV H120 antigen. Possible explanations to why only one statistical significant results was obtained may be due to the presence of IBV M41 antigen

binding the scFvs that are present. As the level of cross-reactivity between these two strains have not been determined the scFvs may have higher binding ability to the IBV M41 antigen as the scFvs were originally screened against both the IBV M41 and H120 antigens. Further, the binding position of the selected scFvs have not been determined, leading to the possibility of non-neutralisation of the virus to occur even if binding does occur. The main reason can however be linked to the difference in valency between the scFvs and natural antibodies.

Almdni (2013) showed scFvs to have partial neutralisation against toxin A by means of ELISA and cytopathogenic effect, while Lin and co-workers (2007) illustrated partial neutralisation by the means of real-time PCR experiments based on the mRNA expression levels of the S1 protein. No study has yet proven complete neutralisation by scFvs even though the method of virus neutralisation differs between the different studies. This should be taken into account when referring to the results obtained during the current study. A higher concentration of scFvs might have had an increased neutralising effect as recommended by Lin and co-authors (2016). Thus, a checkerboard assay by means of SPF eggs would be beneficial to detect the maximum effective concentrations in terms of the neutralising ability *in vitro* and the neutralising ability should be tested within other *in vivo* systems to provide more insight on the neutralising effects (Lin *et al*., 2016). It would also be beneficial to determine the structural protein of the IBV antigen that the expressed scFv binds to, using western blots. Infectious bronchitis virus have four structural proteins namely the spike (S) glycoprotein, nucleocapsid (N) phosphoprotein, membrane (M) glycoprotein and the envelope (E) protein (Al-Beltagi *et al*., 2014). A study conducted by Lin *et al.* (2015) proved a potential neutralising scFv that binds to the S1 protein. According to Bakhshesh (2016) scFvs may also bind to the nucleocapsid phosphoprotein and membrane glycoprotein but the neutralising effects have not yet been fully studied. In addition to determining the structural protein where binding of the scFv takes place, other *in vitro* experiments such as real-time PCR and mRNA expression of structural proteins will also be beneficial. The correlation of *in vitro* and *in ovo* experiments provides a broad overview of the purified and selected scFvs binding abilities and neutralising effect (Ignjatovic & Galli, 1995; Bakhshesh, 2016).

Khan and co-workers (2017) stated that neutralising antibodies with definite epitope specificities are more effective in providing a neutralising effect. Therefore, determining the epitope region of specific scFvs will contribute to the understanding of the neutralising ability (Khan *et al*., 2017). According to Joseph and de Brevern (2014) a detailed understanding of the function of a protein can be achieved by studying the three-dimensional structure. Therefore, determination of the predicted structure of both the scFv and the antigen will be beneficial as the binding activity of scFvs are influenced by the structure and the structure is

influenced by the amino acid sequence. This information will then contribute to the therapeutic success of scFvs and product development by ensuring the production of more antigen-binding specific scFvs (Bertonati & Punta, undated).

In conclusion, the selection process showed a progressive enrichment of specific clones from the dAb library against IBV antigens. The scFv proteins of the selected phage clones were successfully expressed, purified and characterised by determining binding abilities. A partial neutralising effect from the scFvs were observed during the *in ovo* neutralising assay against the IBV H120 antigen.

Future work on the current study should entail deeper investigation into the expression of scFvs in terms of the expression system and vectors as well as the purification methods used. This should be combined with downstream applications of the scFvs. Neutralising abilities of the scFvs should be determined within other *in vivo* systems such as e.g. live SPF chickens to determine whether the neutralising ability will occur within chickens and to what extent this occurs. Cross-neutralising assays should be conducted between strains as well as a checkerboard neutralising assay to determine the optimum concentration of scFvs that would neutralise the virus effectively. If the results obtained from future work prove to be sufficient, large-scale production can be attempted and routes of administration can be considered. As mentioned, this study serves as a pilot study to look at rendering potential therapeutic fragments with neutralising abilities against IBV. The results obtained prove that scFvs expressed using a phage display library can render possible candidates for treatment of IBV and perhaps at a later stage, expand to search for treatment candidates against other viral infections.

This project does not pose an alternative method to control IBV but should rather be viewed as an additional treatment option that can be implemented in the case of vaccination breaks. This study provides educational value to increase the knowledge on virus neutralisation regarding concentrations and methods, phage display technology, and expression of peptide fragments with the use of human domain antibody library. In addition to the educational aspect, the study provides insight to a possible application and implementation within the poultry industry. Furthermore, knowledge obtained during the completion of the study can potentially be applied as a possible treatment option for other diseases.

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Appendix A

Equation A1: Calculation to determine the percentage of phage recovery after each round of selection.

$$\text{Phage recovery percentage} = \frac{\text{Phage output}}{\text{Phage input}} \times 100$$

Equation A2: Calculation of monoclonal ELISA S/P ratio provided by commercialised IBV coated ELISA kit (BioChek, UK).

$$S/P\ \text{ratio} = \frac{\text{Mean of test sample-Mean of negative control}}{\text{Mean of positive control-Mean of negative control}}$$

Equation A3: Calculation to determine antibody titre indicating positive phage clones in the monoclonal ELISA from a polyclonal antibody mixture provided by commercialised IBV coated ELISA kit (BioChek, UK).

$$\text{Log}_{10}\text{Titre} = 1.0 \times \text{Log}_{10}(S/P) + 3.62$$

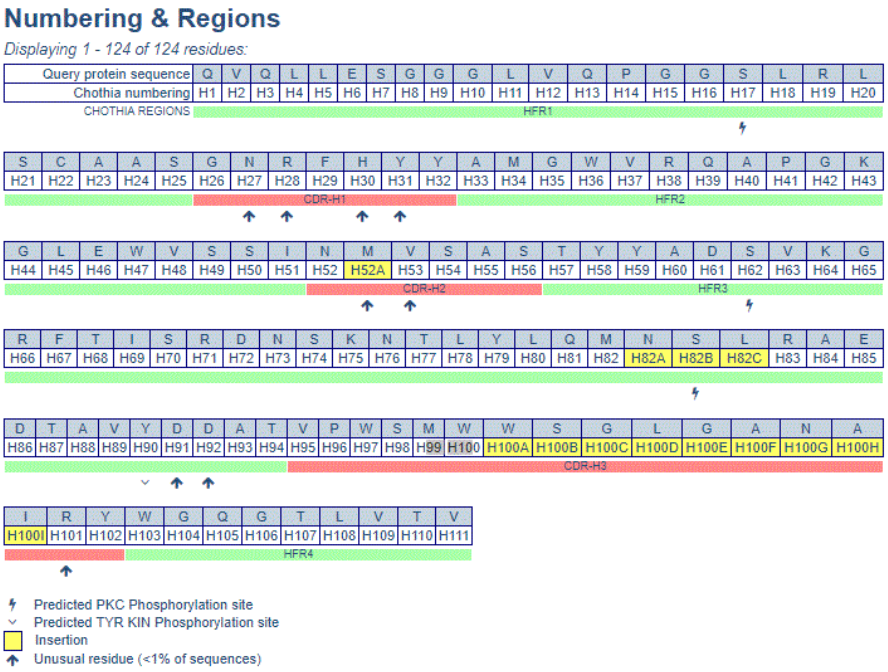

Figure A1: The amino acid numbering of the predicted sequence obtained from scFv 7 based on the chothia numbering scheme in the abYsis system (V 3.1) (http://www.abysis.org/. Swindells *et al*., 2017).

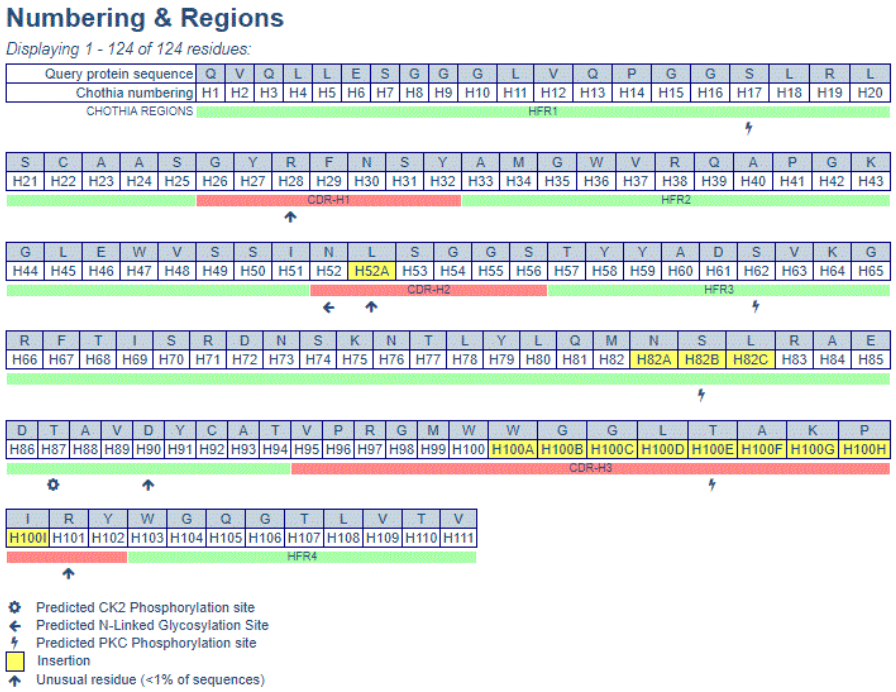

Figure A2: The amino acid numbering of the predicted sequence obtained from scFv 8 based on the chothia numbering scheme in the abYsis system (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017).

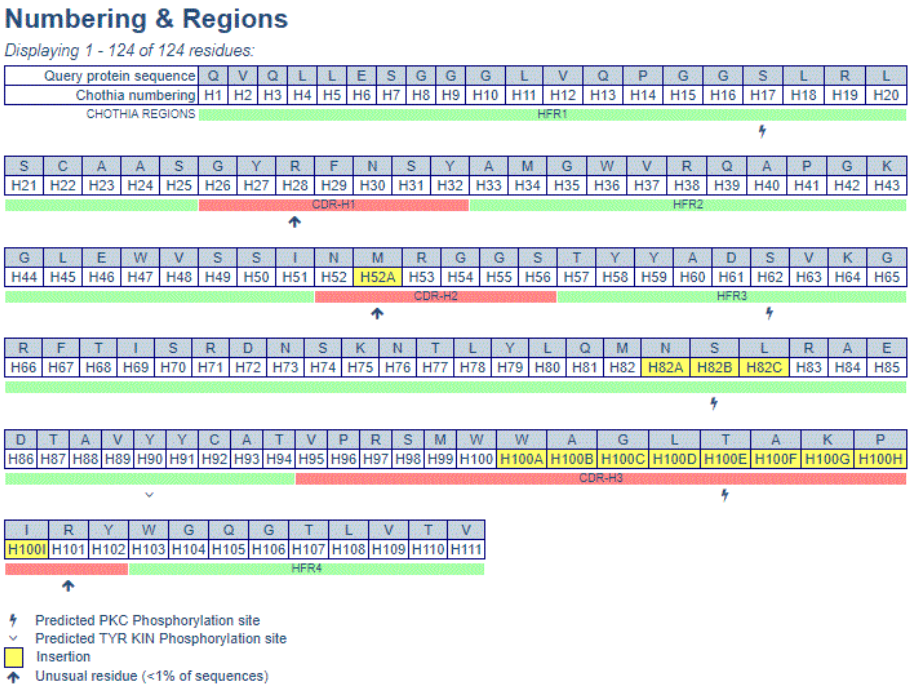

Figure A3: The amino acid numbering of the predicted sequence obtained from scFv 9 and scFv 11 based on the chothia numbering scheme in the abYsis system (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017).

Table A1: Calculated phage forming units (PFU/mL) after polyethylenglycol (PEG) precipitation of selection rounds.

Selection	A_{260} Value	$A_{260}*100*22.14*10^{10}$
Round 1	0.23	5.1×10^{12}
Round 2	0.39	8.6×10^{12}
Round 3	0.41	9.0×10^{12}

Table A2: Colony forming units (CFU/mL) of the KM13 helper-phage during trypsin cleavage test.

Dilution	Trypsin treated phages (CFU/mL)	Non-trypsin treated phages (CFU/mL)
10^{-9}	0	TNTC
10^{-8}	0	TNTC
10^{-7}	0	TNTC
10^{-6}	0	TNTC
10^{-5}	0	TNTC
10^{-4}	0	TNTC
10^{-3}	0	TNTC
10^{-2}	0	TNTC

TNTC - Too numerous to count

Table A3: Colony forming units (CFU/mL) of the KM13 helper-phage during three selection rounds.

Round 1												
Dilution range	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**	**10^{-11}**	**10^{-12}**	
Repeat 1	TNTC	131	14	1	0	0	0	0	0	0	0	
Repeat 2	TNTC	174	6	0	0	0	0	0	0	0	0	
Repeat 3	TNTC	188	4	1	0	0	0	0	0	0	0	
Average	TNTC	164	8	1	0	0	0	0	0	0	0	**1.6×10^5**
Round 2												
Repeat 1	TNTC	309	127	9	0	0	0	0	0	0	0	
Repeat 2	TNTC	302	117	13	0	0	0	0	0	0	0	
Repeat 3	TNTC	317	122	14	0	0	0	0	0	0	0	
Average	TNTC	309	122	12	0	0	0	0	0	0	0	**1.2×10^6**
Round 3												

Repeat 1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	125	34	15	7	0	
Repeat 2	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	122	27	11	8	0	
Repeat 3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	134	30	14	4	0	
Average	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	127	30	13	6	0	**3.0×10^{10}**

Table A4: The absorbance values at a wavelength of 405 nm from the selected clones in the monoclonal sandwich ELISA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.051	0.049	0.049	0.049	0.047	0.049	0.046	0.047	0.047	0.048	0.048	0.05
B	0.052	0.049	0.049	0.047	0.049	0.047	0.048	0.048	0.047	0.047	0.047	0.046
C	0.051	0.048	0.047	0.048	0.046	0.047	0.046	0.046	0.049	0.049	0.047	0.047
D	0.051	0.049	0.049	0.049	0.046	0.051	0.048	0.047	0.05	0.048	0.049	0.049
E	0.049	0.047	0.048	0.048	0.047	0.047	0.050	0.051	0.049	0.048	0.047	0.047
F	0.049	0.048	0.05	0.047	0.05	0.047	0.046	0.049	0.049	0.054	0.049	0.053
G	0.050	0.045	0.047	0.052	0.047	0.050	0.052	-	-	-	-	-
H	0.046	-	-	-	-	-	-	-	-	-	-	-

Table A5: The S/P ratio, Log10 titre value, and titre value from 12 selected clones determined from the absorbance values at a wavelength of 405 nm and average from the monoclonal sandwich ELISA.

Specific phage clone	S/P ratio	Log10 titre	Titre
1	0.44	3.28	1908.46
2	0.44	3.28	1908.46
3	0.89	3.57	3715.35
4	0.44	3.28	1908.46
5	0.89	3.57	3715.35
6	0.44	3.28	1908.46
7	0.44	3.28	1908.46
8	2.22	3.97	9332.54
9	1.78	3.87	7413.10
10	1.33	3.74	5495.41
11	0.44	3.28	1908.46
12	1.33	3.74	5495.41

Table A6: Predicted amino acid sequence of 12 full variable heavy chain of the scFv

Selected phage clone	Predicted amino acid sequence
1	QVQLLESGGGLVQPGGSLRLSCAASGFSVND*FMGWVRQAPGKGLEWVSTIAANNGSTYYADS VKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGEGWSAAGEDTMRSWGQGTLVTV
2	QVQLLESGGGLVQPGGSLRLSCAASGYRFNS*AMGWVRQAPGKGLEWVSSINMRGGSTYYAD SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATVPRSMWWAGLTAKPIRYWGQGTLVTV
3	APQALHFMLPARMLCGIVSG*QFHTGNSYDHDYAKLNSQYMSWVRQAPGKGLEWVSAITTPDG STYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGQDGNQEDIRFWGQGTLVTV
4	QVQLLESGGGLVQPGGSLRLSCAASGYRFNS*AMGWVRQAPGKGLERVSSINMRGGSTYYAD SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATVPRSMWWAGLTAKPIRYWGQGTLVTV
5	APQALHFMLPARMLCGIVSG*QFHTGNSYDHDYAKLNSQYMSWVRQAPGKGLEWVSAITTPDG STYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGQDGNQEDIRFWGQGTLVTV
6	QVQLLEYGRGLEQPGGSVRLSGSASGYRFTS*DMVWLRQIPGKGQEWVSSINMGAGSTCSADS AKGRFSISRGNSKNPLNSQKNSLRPEDTAVY*GATVPRSMWWAGLTPKPIRYWGQGTLVTV
7	QVQLLESGGGLVQRGGFMGLSCAASSNRFHSYAKGWVHQAPGKGQDSGSSINMVSASTYDTD SVKYGVSINGDISMYTDNFKKNSYRQKNSAVYDDATVPWSMWWSGLGANAIRYWGQGTLVTV
8	QVQLLESGGGLVQRGGSLRLSCAASGYRFNSYDMGWVSQAPGKGPEWVSSINLSGGSTCYAD SLKGRFTISRDNSKNKMYKKMNSLRTDDTAVDYCATVSRGMWWGGLTAKPIRYWGQGTLVTV
9	QVQLLESGGGLVQPGGSLRLSCAASGYRFNSYAMGWVRQAPGKGLEWVSSINMRGGSTYYAD SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATVPRSMWWAGLTAKPIRYWGQGTLVTV
10	GTGERTRELPGGNAWYLYSPVGFSHLRQECRFGYGLSGAGSREGSPVGIKHYGSRR*HGLCST CYVPVHPLLWRIPELPPLTKEQPLSAGQRGRAVSQ*SKKWKSAQYANEVYQVLGSGNPGHR
11	QVQLLESGGGLVQPGGSLRLSCAASGYRFNSYAMGWVRQAPGKGLEWVSSINMRGGSTYYAD SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATVPRSMWWAGLTAKPIRYWGQGTLVTV
12	QVQLLESGGGLVQPGGSLRLSCAASGYRFNSYAMGWVRQAPGKGLEWVSSINMRGGSTYYAD SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCATVPRSMWWAGLTAKPIRYWGQGTLVTV
15	VSSGGGGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAP KLLIYRASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSSNTPYTFGQGTKVEIKR

from the positive binding clones obtained from the monoclonal ELISA.

*amber stop codon

Table A7: The amino acid position in the CDRs from the four selected sequences with detected unusual residues, most occurring residues and percentage of occurrence frequency within the variable heavy (VH) chain from *Homo sapiens* based on the Chothia numbering scheme in the abYsis system (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017).

Amino acid position in CDR with detected unusual residue	Residue	Frequency of occurrence (%)
H27	Y	48
H28	S/T	55
H30	S/T	59
H31	S	47
H52A	P	27
H53	S	26
H90	Y	99
H91	Y	89
H92	C	100

C – Cysteine; D – Aspartate; S – Serine; P – Proline; T – Threonine; Y – Tyrosine

Table A8: Blast results from the four selected sequences by the abYsis system (V 3.1) (http://www.abysis.org/, Swindells *et al*., 2017).

Phage clone	Accession	Organism	Description	Score	E-bit Value
7	AEX29749.1	*Homo sapiens*	immunoglobulin G heavy chain variable region	112	5e-26
8	AQM56128.1	*Homo sapiens*	immunoglobulin G heavy chain variable region	112	5e-26
9	AAM75838.1	*Homo sapiens*	immunoglobulin G heavy chain variable region	112	5e-26
11	AAM75838.1	*Homo sapiens*	immunoglobulin G heavy chain variable region	112	5e-26

Table A9: The calculations to determine the transformation efficiency of the HB2151 *E. coli* strain with the extracted plasmid DNA that was obtained in Chapter 2 as described in section 2.5.6.2).

		#1	#2	#3	#4	#5
Clone	**ng/µL**	**Average colonies**	**Plasmid DNA amount used (ng/uL)**	**Plasmid DNA fraction on plate**	**Plasmid DNA on plate (ug)**	**Transformation efficiency (transformants/ug plasmid DNA)**
1	95.27	215.5	238.18	0.01	2.50	86.18
2	93.3	199	233.25	0.01	2.45	81.26
3	124.95	262	312.38	0.01	3.28	79.89
4	143.55	297.5	358.88	0.01	3.77	78.96
5	149.29	306	373.23	0.01	3.92	78.09
6	254.17	297.5	635.43	0.01	6.67	44.60
7	409.97	306.5	1024.93	0.01	10.76	28.48
8	481.77	437	1204.43	0.01	12.64	34.56
9	496.66	407.5	1241.65	0.01	13.04	31.26
10	372.36	291.5	930.90	0.01	9.77	29.83
11	588.13	423.5	1470.33	0.01	15.44	27.44
12	658.23	428	1645.58	0.01	17.28	24.77
15	140.58	235	351.450	0.10	3.69	63.69

Table A10: The absorbance values and average absorbance values measured at a wavelength of 405nm and standard deviations from the scFvs, positive and negative controls during replicate direct competitive ELISA.

	Positive control	**Negative control**	**scFv 7**	**scFv 8**	**scFv 9**	**scFv 11**	**scFv 15**
Repetition 1	0.2335	0.050	0.230	0.244	0.214	0.232	0.248
Repetition 2	0.211	0.052	0.160	0.166	0.158	0.154	0.218
Repetition 3	0.204	0.050	0.195	0.205	0.186	0.193	0.233
Average	0.216	0.0505	0.195	0.205	0.186	0.193	0.233
Standard deviation	0.0156	0.0009	0.035	0.039	0.028	0.039	0.015

Table A11: The Log10 end-point titre calculations of the two replicates of embryonated eggs injected with sample mixtures.

Sample mixture inoculated into embyonated eggs	Log 10 end-point titre for replicate 1	Log 10 end-point titre for replicate 2	Average Log 10 end-point titre
IBV H120 vaccine strain	4,4	4,7	4.6
Sample mixture 15	4,1	4,1	4.1
Sample mixture 7	3,7	3,7	3.7
Sample mixture 8	3,5	3,3	3.4
Sample mixture 9	3,1	3,3	3.2
Sample mixture 11	3,7	3,5	3.6