THE IDENTIFICATION OF UNKNOWN POULTRY VIRUSES THROUGH ESTABLISHED METHODS

by

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DECLARATION

I, Ji-Yun Lee, hereby declare that the dissertation hereby submitted by me for the degree Magister Scientiae at the University of the Free State (U.F.S) is my own independent work and has not previously been submitted by me at another university/institution/faculty.

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The experimental work conducted and discussed in this thesis was carried out in the Department of Microbiology and Biochemistry, University of the Free State, Bloemfontein. The study was conducted during the period January 2006 to May 2008 in the laboratory of the Veterinary Biotechnology group under the supervision Prof. R.R. Bragg, of the Department of Microbial, Biochemical and Food Biotechnology.

Signature:_____________________

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Date: 23rd March 2009

List of Abbreviations

APMV	Avian Paramyxovirus
APV	Avian pneumoviruses
ARC	Agricultural Research College
ART	Avian rhinotracheitis
CAM	Chorio-allantoic membrane
CCEM	Centre for Confocal Electron Microscopy
CIA	Chicken infectious anemia
CAV	Chicken infectious anemia virus
CEF	Chicken embryo fibroblast
CEFC	Chicken embryo fibroblast cells
CPE	Cytopathic effects
EDS	Egg drop syndrome
EDSV	Egg drop syndrome virus
EM	Electron microscopy
ESEM	Environmental scanning electron microscope
F	Fusion protein
FAdV-1	Fowl adenovirus-1
FAO	Food and Agriculture Organisation
FBS	Foetal bovine serum
FESTEM	Field emission scanning transmission electron microscope
HA	Haemagglutination assay
HI	Haemagglutination inhibition
HPAI	Highly pathogenic avian influenza
HREM	Higher resolution electron microscope
HVEM	Higher voltage electron microscope
HN	Haemagglutinin neuraminidase
IB	Infectious bronchitis
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
L	Large RNA polymerase protein
LPAI	Low pathogenicity avian influenza
LT	Laryngotracheitis
LTV	Laryngotracheitis virus
MDT	Mean death time
MEM	Minimal Essential Medium
NAD	Nicotinamide adenine dinucleotide
NCL	National Control Laboratories
ND	Newcastle disease
NDV	Newcastle disease virus
NP	Nucleocapsid protein

OVI	Onderstepoort Veterinary Institute
OIE	Office Internationale des Epizooties
P	Phosphoprotein
PBFDV	Psittacine beak and feather disease virus
PBS	Phosphate Buffer Saline
RBC	Red blood cell
PCR	Polymerase chain reaction
RT-PCR	Reverse-transcriptase polymerase chain reaction
SEM	Scanning electron microscope
SHS	Swollen head syndrome
SPF	Specific pathogen free
TEM	Transmission electron microscope
TRT	Turkey rhinotreichitis
TSA	Tryptone soy agar
UFS	University of the Free State
WTO	World Trade Organization

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Chapter 1. Literature Review

1.1 Introduction

Poultry viruses are as varied and diverse as the poultry hosts that they infect. For the various species of chickens, ducks, guinea fowl, turkeys, geese, quail and other poultry species, there are numerous poultry viruses that are able to infect these poultry species.

Poultry farmers across the globe face the devastating effects that poultry viruses have as they infect commercial poultry and spread disease. The economic decline that is consequent to the loss of poultry and poultry products is also brought on by viral diseases. This emphasises the importance of research concerning poultry viruses as well as the production and implementation of vaccines against these viruses. Therefore, investigation into techniques that allows for the early detection and identification of the virus is imperative and may lead to the eventual eradication of the poultry viruses.

The first step to eliminating the effects that the poultry viruses have on poultry is the identification of the different viruses as well as an understanding of how they operate and how they can be either manipulated or stopped.

1.2. Sample Background and Aims of Study

The Onderstepoort Veterinary Institute (OVI) of Tshwane is well renowned for dealing with veterinary diseases in poultry and other agriculturally important animals. A number of virus samples, that were isolated at the OVI from poultry samples, were sent to the Veterinary Biotechnology Laboratory of the University of the Free State (UFS) as suspected Newcastle disease viruses (NDV) of poultry.

However, two of the virus samples that were sent to the UFS, namely strains 834/05 and D1446/95, did not show the usual profile of NDV when tested for haemagglutination

(an ability to bind red blood cells) and did not show amplification when specific primers were used in an optimized reverse-transcriptase polymerase chain reaction (RT-PCR) test with primer sets designed for the F-protein gene of NDV (Boucher, 2006). The F-protein was chosen due to it being the protein responsible for NDV to fuse with the receptor proteins on the host cells, gaining access for the virus into the cells. It is also one of the regions recognized as conserved within the NDV species sequence (Abenes *et al.*, 1986).

There is a possibility that the two samples, 834/05 and D1446/95, may be poultry viruses other than NDV. Their inability to haemagglutinate and the negative RT-PCR results suggest that this is most likely the case. There are certain poultry viruses that do not haemagglutinate. Some of these viruses that do not haemagglutinate include Avian pneumovirus (Gough, 2003), infectious bronchitis [IB] (Cavanagh & Naqi, 2003), laryngotracheitis [LT] (Guy & Bagust, 2003), infectious bursal disease (Lukert & Saif, 2003) and the Chicken infectious anemia virus [CAV] (Schat, 2003). The presence of so many, mostly common, viruses that cannot haemagglutinate indicate that it may be possible that the samples are indeed a completely different virus from NDV. Or they may possibly be unknown variations of NDV that haemagglutinate under specific circumstances or perhaps do not haemagglutinate to the point of forming a lattice visible to the naked eye.

The ability of NDV to haemagglutinate is an important factor that is used in the preliminary steps of identification of the virus. If the virus sample shows haemagglutination in a haemagglutination assay (HA), the subsequent step would be to identify the virus using a haemagglutination inhibition (HI) test. In an HA the interaction between a) red blood cells (RBCs) and b) the antigen or virus is determined. The presence or absence of haemagglutination is based on the presence or absence respectively of the haemagglutination neuraminidase (HN) protein on the assayed virus (Cobaleda *et al.*, 2002). The HN protein attaches onto the protruding sialic acid-containing proteins or receptors of the RBCs (Cobaleda *et al.*, 2002). Through the action of an individual virus attaching itself onto two different RBCs at once, an

interlinking ‘floating’ carpet/lawn of RBCs is created. This depicts the presence of the HN protein on a virus. In the absence of the HN protein there is nothing that interlinks the RBCs and they would drop to the bottom of the container, pulled by gravity. In this way the absence of the HN protein on the virus would be represented by the absence of this lawn of RBCs.

Where the HA only indicates whether or not the virus is present and can agglutinate erythrocytes, the application of an HI test assists in identifying the virus by using serum containing antibodies that are specific for a certain virus. If the virus reacts with the antibodies of the serum in the HI test, it can be concluded that the virus sample contains that specific virus. In this way it would be possible to identify the nature of the virus according to the antibodies that it had tested positive against.

However, due to the unexpected results obtained with the two above-mentioned virus strains; they were regarded as “unknown” viruses for this project. The main aim of this project was thus to identify these “unknown” viruses.

The aim of this study will firstly be to determine whether or not the viruses are NDV, and if it is determined that they are not NDV, to further determine which viruses they are, using mainly classical methods and simple molecular methods.

1.3. Disease in poultry

Disease in poultry occurs when the normal body functions are damaged and the scale of damage determines the severity of the disease (Bermudez, 2003). Disease may result from malnutrition, physical damage caused by stress, infection from pathogenic agents or due to a toxic substance which has been consumed. However, where disease has been caused by infectious or parasitic micro-organisms, it becomes more complicated and is based on the characteristics of the host, agent, and environmental conditions in the breeding enclosure of farms. While nutritional deficiencies may be reversible with the correct amount of nutrients, most of the conditions of disease from

pathogenic agents are usually not so (Bermudez, 2003).

Disease can be either directly or indirectly transmitted. Direct routes of transmission can be either through the vertical (congenital) route or the horizontal transmission route (Mc Guire & Scheideler, 2005). Vertical transmission refers to the transmission of pathogens from the hen to the progeny in the eggs. In this case the embryonated eggs that have been infected develop into infected chicks and unless the chick dies it becomes a carrier infecting more progeny that is via horizontal transmission as well as vertically through the eggshell surface (Chandra *et al.,* 2001). The alternate route is the horizontal route of transmission which is through aerosol droplets from infected carrier birds to healthy birds. These droplets allow for the pathogens to attach onto dust particles and to be moved long distances by wind. Newcastle disease (ND) and infectious laryngotracheitis both spread using this route (Chandra *et al.,* 2001). Indirect transmission of most diseases occurs through attendants, visitors, wild birds, crates, chick boxes, equipment, contaminated feed, water and litter (Figure 1.1).

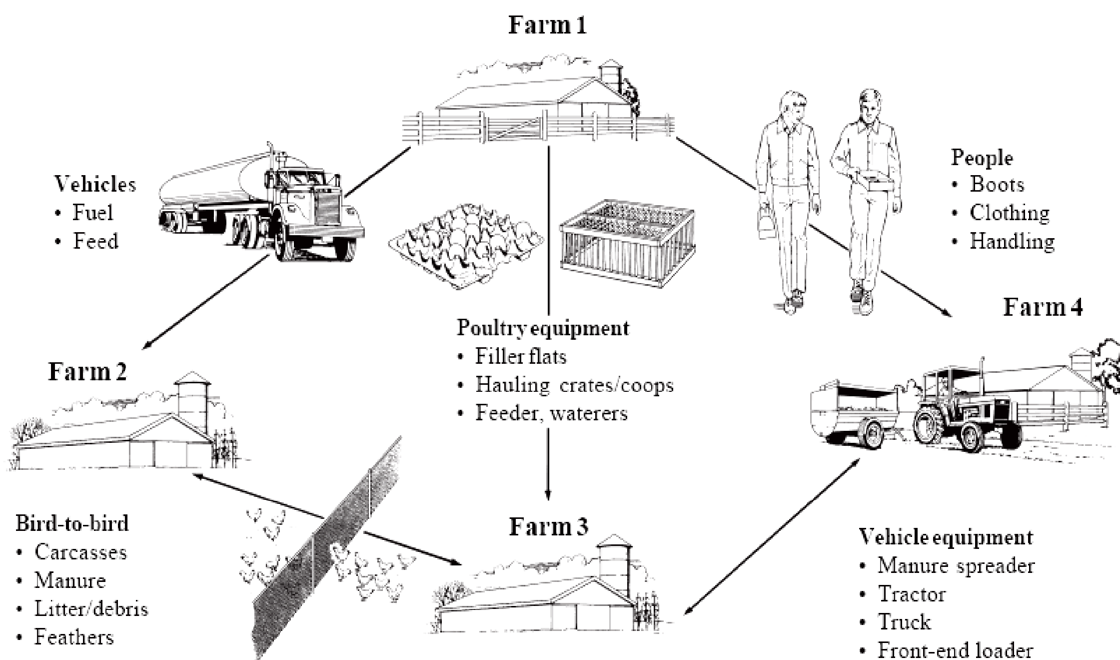

Figure 1.1. How poultry disease spreads. The different routes that affect the spread of poultry disease. The interlinking points of potential infection and carrier routes of disease as portrayed by the producing and delivering of various farms showing the ease with which viruses and disease can be spread with ease. (Mc Guire & Scheideler, 2005)

Other sources of infections are biological agents. Wild birds can assume the role of reservoirs of bird flu and *Pasteurella* sp. Rodents have been found to harbour salmonellosis and pasteurellosis and carrying these over to poultry via contamination of the feed (Chandra *et al.,* 2001). Insects like mosquitoes, *Argus* ticks and beetles have been associated with the spreading of poxvirus, spirochatosis and Marek's disease respectively (Chandra *et al.,* 2001).

Disease resulting from infectious agents depends on the number, types and virulence of the pathogen in conjunction with the route of entry to the host body and the defence status and abilities of the host (Bermudez, 2003). The host's defence status is based greatly on previous exposures to disease including nutritional deficiencies, environmental stresses and the use of drugs or environmental changes used to eradicate previous disease encounters.

Finally, contaminated poultry vaccines may contain specific pathogens and transmit the diseases caused by the respective agents via vaccination. This has been reported when a fowlpox vaccine, contaminated with Reticuloendotheliosis virus had caused an outbreak of lymphomas in commercial broiler breeder chickens (Fadly *et al.,* 1996). Egg drop syndrome (EDS) was a new disease during the time when it was prevalent in 1976 (Zsák & Kisary, 1981). The disease was known by its characteristic ability to decrease egg production in laying hen flocks hence making it economically important in the degree of its seriousness that resulted in thin-shelled or shell-less eggs by seemingly healthy birds. The disease was caused by an Adenovirus which was probably also introduced into the hosts through a contaminated vaccine (McFerran & McConnell Adair, 2003b).

1.4. Common poultry viruses and their economic impact

Globally, poultry are used as an animal protein source. In addition they have various advantages adapting to most geographical areas and conditions, are relatively inexpensive, have rapid generation time, a high rate of productivity and generally do not need large areas of land in which to be kept (Marangon & Busani, 2006).

All these advantages consequently make poultry highly valuable and profitable. Throughout the poultry industry there are a multitude of diseases which cost the industry exorbitantly in terms of losses. It is important to remember that some poultry diseases are bacterial in nature like the *Salmonella* infections, which include the more common Fowl Typhoid, which is caused by *Salmonella* Gallinarum. Viral diseases of poultry include some of the better known poultry viruses, such as Fowl Pox, ND, Infectious Bronchitis, Avian Influenza (AI) and Marek's Disease among others. Movement of people and equipment contribute the highest risk of spreading the disease during an outbreak, which is generally due to the traffic of personnel and vehicles (feed and chicken trucks, egg collectors, advisers, helpers, veterinarians etc.) moving from one flock to another (McFerran & McNulty, 1993).

As the main objective of this project was to identify two samples of viruses, which were submitted to this laboratory as ND, but could not be confirmed as NDV, it is advisable to briefly review all of the viruses which have been associated with diseases in poultry.

Some of the most infectious agents of poultry are viral. The most important viral pathogen for poultry is Avian paramyxovirus 1 (APMV-1) with NDV classed as the representative APMV-1. The other main viral disease of poultry is Avian Influenza virus. An idea of what kind of impact viral diseases of poultry have on the poultry market is shown by the outbreaks of viruses such as the highly pathogenic avian influenza (HPAI) in ostriches in South Africa during August 2004, which led to a loss in income for the South African ostrich producers. Although South Africa may not have a great impact on poultry markets worldwide, it is the leader in ostrich and ostrich product production. The

South African ostrich industry is said to provide 90% of ostrich products in the world and exports the majority of its meat to Europe (CEI, 2004).

AI has been causing havoc within the poultry industry worldwide from as early as 1878 when it was first referred to as “fowl plague” in an outbreak in Italy (Jacob *et al.,* 2003). There were outbreaks of AI in 1924 and 1929 in the United States and were successfully eradicated both times (Jacob *et al.*, 2003). Thereafter, HPAI broke out in the North-eastern United States during 1983 which took more than 2 years to eradicate, over 70 million dollars at the time and 17 million birds. The AI virus has been detected serologically and characterized as nonpathogenic to chickens through virus isolation. However, the effects of the outbreaks have been devastating on the poultry industry. The syndromes associated with AI infection may range from asymptomatic infection to respiratory disease and drop in egg production and even mortality of up to 100% (Swayne & Halvorson, 2003). The HPAI is the responsible strain for the 100% mortality rate that had occurred while other milder forms of AI are less severe than HPAI. The greatest losses from HPAI are experienced on large commercial farms keeping live poultry. These losses have included depopulation and disposal costs, high morbidity and mortality losses, quarantine and surveillance costs and expenses paid for the elimination of birds (Lukert & Saif, 2003).

Although NDV will be reviewed in detail later; the economic impact it has will be described in this section. ND is a noted major cause of economic losses in the poultry market. Due to this it is of high priority that NDV are identified according to their virulence in order to create vaccines. Reduction of the risk can be achieved by strict application of biosecurity measures at even the smallest of small-scale farms. However, free-living birds and other wildlife, the poultry markets, poultry shows, fighting cocks (which are very difficult to trace and are usually be illegal), are also noteworthy factors causing or spreading ND (McFerran & McNulty, 1993). These factors make it very difficult for the quarantining and, more importantly, control of outbreaks. In addition, even the control measures of NDV vaccination and biosecurity, amongst other means, are cost ineffective and sustain continuing losses to the poultry industry in developed

countries with reputable poultry industries (Leslie, 2000) further accentuating the global economic impact of ND.

According to serological tests, the paramyxoviruses affecting avian species are classified into nine different serovars which are APMV-1 to APMV-9. The serovars are known for having different effects in various poultry. For example, APMV-2 is known to cause disease in turkeys and passerines (songbirds), APMV-3 infects turkeys, APMV-6 causes disease in ducks and APMV-7 infects pigeons and doves (Alexander, 2003b). While the other APMVs may cause infection and disease NDV is still the most prevalent APMV. There are over 250 species of birds that are reportedly vulnerable to NDV and it is most probable that there may be several other susceptible existing species that have not yet been identified as such (Alexander, 1997). Due to the severe nature of the disease and the associated consequences, ND is included as an Office Internationale des Epizooties (OIE) list A disease and most countries, including all European Union countries, implement constitutional control (Aldous & Alexander, 2001). Newcastle disease is also an existing control disease in South Africa and has been designated as being present as assessed by the National Department of South Africa (Status of South Africa 2005). The other paramyxoviruses, APMV 2-9, cause major respiratory disease and decreased egg production in laying hens. Avian paramyxoviruses have the greatest economic impact as they may increase the infectivity of existing bacterial and virus infections (Alexander[2], 2003).

Avian pneumovirus (APVs) is another of the viruses which are important in the poultry industry. The APVs lead to turkey rhinotreichitis (TRT), swollen head syndrome (SHS) and avian rhinotracheitis (ART) (Gough, 2003). Avian pneumovirus was first detected and described in South Africa during the late 1970s (Buys *et al.,* 1980) and thereafter was reported in Europe, the United Kingdom and France. The clinical signs following APV infection are generally upper respiratory tract infection and chickens with swollen heads. APVs cause grave economic and animal welfare problems. This group of poultry viruses is still the most important respiratory diseases in turkeys other than AI (Gough, 2003). It has affected economic problems resulting from the major losses of Minnesota

turkeys in America ever since the first outbreaks in 1997.

Another virus causing major problems in the poultry market is Infectious bronchitis virus (IBV).This virus is the sole agent responsible for IB which is an acute, highly contagious respiratory disease of chickens. A lot of the symptoms of infectious bronchitis are of respiratory tract difficulties including tracheal rales, coughing and sneezing (Cavanagh & Naqi, 2003). Infectious bronchitis is a highly contagious viral respiratory disease affecting chickens. It causes poor weight gain, low feed efficiency, airsacculitis in mixed infections with other viruses or bacteria, declining egg production and egg quality. These effects of IB inevitably affect the economy of poultry markets. In addition, due to the multiple serovars of the IBV, the cost of vaccine production has increased radically adding onto the losses already suffered from inefficient production (Cavanagh & Naqi, 2003).

The Laryngotracheitis virus (LTV) transmits another respiratory tract infection that is known as infectious laryngotracheitis (LT). This virus is found in chickens and is responsible for severe production losses that are due to mortality or decreased egg production (Guy & Bagust, 2003). This disease is one of several viral respiratory tract infections of chickens and has been estimated to incur multimillion dollar losses annually in the American poultry industry alone (Guy & Bagust, 2003). Losses from LTV including mortality and decreased egg production may be similarly experienced in other countries.

One of the viruses that elicit a highly contagious infection in young chickens is the Infectious bursal disease virus (IBDV). This virus primarily targets lymphoid tissue and results in extreme kidney damage in birds that are infected. The disease is found to be highly immunosuppressive at a young age and it is of high priority that birds are protected from the point of hatching through maternal antibodies (Lukert & Saif, 2003).

Chicken infectious anemia (CIA) is caused by infection with CAV. This virus is a member of the *Circoviridae* family which is also the family of viruses that include the Beak and feather disease virus (BFDV) that affects psittacine species (parrots). However, CAV was reclassified in 1999 to be under the new genus, Gyrovirus of the *Circoviridae* and remains the only existing member of this genus. The CAV is diagnosed by aplastic anemia, lymphoid atrophy and a suppressed immune system (Schat, 2003).

A very common group of viruses affecting poultry are the adenoviruses. They mostly infect healthy birds without detection and succeed in revealing their opportunistic pathogenicity during bouts of infection from other sources and negatively affecting the host's health. An adenovirus that is a primary pathogen is the egg drop syndrome virus (EDSV). There have been reports of up to 10% drop in egg production due to adenovirus of the *Aviadenovirus* genus (subgroup 1 Avian adenovirus) (McFerran & McConnell Adair, 2003a). These results are usually not repeatable in laboratory cases and may suggest that the environment of the infected host may, along with the virus, concurrently affect the host negatively in terms of egg production.

Pox is a fairly common viral disease of commercial poultry targeting mostly chickens and turkeys and is referred to as fowlpox (Bolte *et al.,* 1999). The reason for fowlpox's economic importance is its relation to the drop in egg production and mortality in poultry. Pox has been found to be a slow-spreading disease that characteristically has either a cutaneous or diptheritic form of infection and subsequent disease. Nodular proliferation in the form of skin lesions on non-feathered parts of the body are representative of the cutaneous form while the diptheritic form results in the proliferation of lesions in the mucous membranes of the upper respiratory tract (Tripathy & Reed, 2003). Although there have been concerns of whether or not avian pox should be of public health significance it does not cause productive infection in mammalian species.

Rotaviruses are a major cause of enteritis and diarrhoea in a wide range of mammalian species including humans (Tzipori, 1985). Avian rotavirus infections were initially reported in 1977 when viral particles that were morphologically identical to rotaviruses were found in the watery droppings of poults that had intestinal infections and resulting increased mortalities (McNulty, 2003).

1.5. Newcastle disease virus

The order *Mononegavirales* consists of three virus families namely *Rhabdoviridae, Filoviridae* and *Paramyxoviridae*. The NDV (Figure 1.2) is a serovar of the genus *Avulavirus* which belongs to the subfamily *Paramyxovirinae*, family *Paramyxoviridae* (Alexander, 1997) and is known to have a non-segmented single-stranded RNA (ss-RNA) genome of negative sense. The viral genome codes for six proteins which are the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), HN, the large RNA polymerase protein (L) and two non-structural proteins W and V (Rojs *et al.*, 2002).

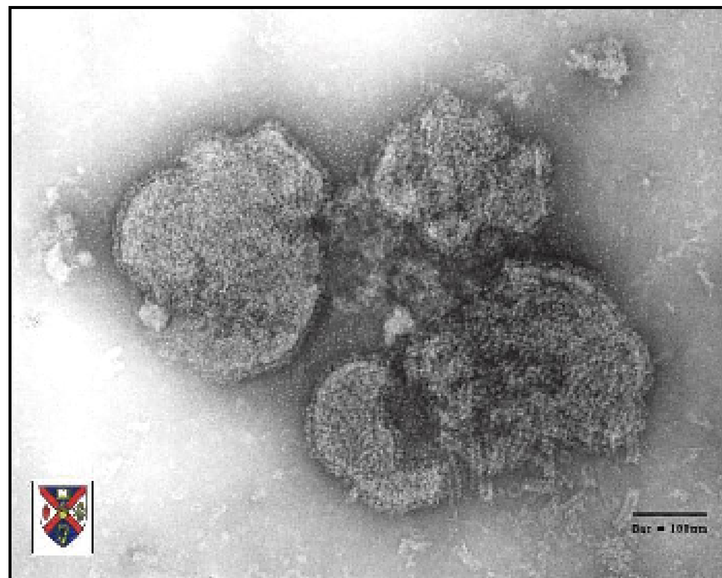

Figure 1.2. A reference electron micrograph of a known *Newcastle disease virus*. Taken by Prof. S. McNulty of The Queen's University of Belfast. Bar insert measuring 100 nm. (ICTVdb [ICTV database] http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm)

Four pathotypes of NDV are recognised, namely viscerotropic velogenic, neurotropic velogenic, mesogenic and lentogenic strains (Rojs *et al.,* 2002). The first two pathotypes result in a high mortality of up to 100% while mesogenic strains show moderate mortality due mainly to respiratory disease. Lentogenic isolates cause low virulence and induce mild respiratory or enteric infections (Wise *et al.*, 2004). Although these different serovars are known, they are not always defined and identified as distinctly as described.

More often than not, there is a combination of the attributes of the different serovars. Some of the clinical signs expected with the infections of ND viruses can vary from asymptomatic enteric infections to systemic infections with 100% mortality. These can be briefly described as the viscerotropic velogenic strains displaying highly virulent disease in which haemorrhagic lesions are characteristically present in the intestinal tract (Beard & Hanson, 1984). Poultry infected with neurotopic velogenic strains experience high mortality following respiratory and nervous signs. Mesogenic strains cause respiratory and nervous signs and usually result in low mortality while lentogenic strains direct a mild or inapparent infection of the respiratory tract. In some cases the signs show an asymptomatic enteric nature of virus where there is an unnoticeable intestinal infection.

Typical clinical signs are a state of prostration and depression in the birds, ruffled feathers and greenish white diarrhoea (Alexander *et al.*, 2004) and in birds surviving the disease, often the head is turned to one side, a condition known as torticollis. Along with this there may be paralysis of the legs, wings or other neurological signs. Some common characteristics of the disease include a rapid spread, death within 2 to 3 days, a mortality rate of over 50 percent in unexposed populations, and an incubation period of 3 to 6 days or, on rare occasions, 2 to 15 days (Alexander *et al.*, 2004). Several of the preceding signs and lesions can be caused by other diseases highlighting the need for a definitive diagnosis of ND where both virus isolation and laboratory characterisation are necessary. Clinical signs of disease may be exacerbated in milder strains of NDV when infections of other organisms coincide. Thus, using clinical

symptoms to diagnose chickens is not a reliable diagnostic identification of NDV. ND is able to affect a wide range of birds, seemingly all types of birds, and of all ages. In addition, it may also be present in humans and other mammals in the form of a mild conjunctivitis (Butcher *et al.*, 1999).

Currently NDV virulence is assessable on *in vivo* testing. This may include a combination of inoculation in embryonated eggs to determine the mean death time (MDT) of an embryo, the intracerebral pathogenicity index in 1-day-old chickens or the intravenous pathogenicity index in 6-week-old chickens (Wise *et al.*, 2004). NDV is known to grow in embryonated SPF chicken eggs as well as in various cell culture systems. Most NDV strains grow well in embryonated chicken eggs inoculated into the yolk sac, allantoic cavity and amnion or on the ectodermal layer of the chorio-allantoic membrane (CAM) (McFerran & McNulty, 1993).

The virulence of NDV isolates may also be identified according to the sequence of their F protein cleavage sites using certain cellular proteases. In the case of lentogenic NDV isolates there are fewer basic amino acids in the F protein cleavage site as compared to those of mesogenic or velogenic strains. Thus, the isolation of virus in embryonated chicken eggs, the ability of the virus to agglutinate RBCs as well as HI assays with NDV-specific antibodies are used as a standard operating procedures for identification of NDV (Wise *et al.*, 2004).

Ideally, virus isolation and identification by the above methods should be followed up with RT-PCR using ND specific primer sets and results viewed using agarose gel electrophoresis. If Real-time RT-PCR is used instead of conventional RT-PCR it saves the additional agarose gel electrophoresis step. Real-time PCR is able to detect and quantify minute amounts of specific nucleic acid sequences (Valasek & Repa, 2005). This is one of the reasons why it is frequently used as a method for molecular investigation. Furthermore, it is rapid and accurate in assessing the changes in gene expression that may be due to physiology, and in this way may compare the changes in physiology with molecular events (Valasek & Repa, 2005). Thus, using real-time PCR it

is possible to gain an understanding of the potential changes in levels and functions of protein. Real-time PCR is also used to measure viral or bacterial loads as well as to assess cancer status (Valasek & Repa, 2005).

1.6. Prevention mechanisms against disease

It is the main aim of the poultry industry to primarily guard against diseases, mentioned previously. Disease can be best prevented by the incorporation of good management practices, nutrition, sanitation, disinfection, vaccination (where available), biosecurity measures, breeding for disease resistance, early and accurate diagnosis and detection, and effective treatment (Chandra *et al.*, 2001). Prevention being dictated as being better than the cure is mainly achieved by using vaccines as in the case of diseases such as ND and IB. Vaccines are an important factor of poultry disease prevention and control worldwide. Their use in poultry production is usually aimed at avoiding or minimising the emergence of clinical disease at farm level and as a result increasing the production of poultry (Marangon & Busani, 2006). Nevertheless, even with the administration of vaccines, there are still outbreaks which result in infection and disease, and in several areas globally the disease remains epizootic. Even though vaccines are established and used worldwide, there are countries in Asia, Africa and the Americas where ND is prevalent (Alexander *et al.*, 2004).

Though vaccines may give protection to poultry at large, there is an undeniable aspect of the pathogen, whether viral or not, to change according to the environmental or physiological pressures that surround it. This is naturally a type of adaptation for the pathogen as it strives towards ensuring its continued existence. Thus, the use of vaccines to assist in the battles against pathogens may result in the alterations found in the existing population dynamics (Day *et al.*, 2008). This perceived adaptation is the consequence of widespread vaccination. The more frequently and extensively the vaccines are employed the more the environment forces the pathogen to seek out a viable course to its propagation. The obvious option would be that the pathogen either adapts to another viable host within or without its current host group. Alternatively, said

pathogen may endeavour to elude the protection that the vaccines offer (Day *et al.*, 2008). This is possible when the pathogen becomes accustomed to losing a characteristic that was either there to begin with or gaining another attribute which it did not previously own.

An example of pathogenic evasion is described in the results of Bragg (2004) concerning *Avibacterium paragallinarum* (previously *Haemophilus paragallinarum*). In this convincing research, nicotinamide adenine dinucleotide (NAD)-independent variants of *A. paragallinarum* were found to evade the immune system. At the time there was a clear distinction of the serovar distribution between the NAD-dependent isolates and the NAD-independent isolates with special regards to the decline of infection caused by serovar A NAD-dependent isolate through the use of vaccines. However, as serovar A NAD-independent isolates were recorded as the cause of previous incidents, the possibility of protective evasion by the NAD-independent isolates was considered. An approach to determining whether or not there was indeed evasion of the immune system was performed and the results according to Bragg (2004) show that there are no great differences in the disease profiles of the chickens that were vaccinated and those that weren't. This compounds the immune system evading ability of the NAD-independent isolates. The study concluded by advising a new direction of protective vaccines and control methods for the prevention of infectious coryza (IC) that would take into account the evasive measures of the NAD-independent isolates.

The countries of Oceania, in contrast, have continued to remain relatively free of ND with the exception of severe outbreaks in Australia during 1998 – 2000 (Kirkland, 2000; Westbury, 2001). This does not necessarily speak against the affectivity of the vaccine, but the outcome of such outbreaks leads to the need for treatments to be available for the respective diseases as well. However, in order to achieve either a vaccine or a treatment for any disease, it is important to know the causative agent of the specific disease. The disease may be considered present with respect to signs and lesions, which are highly suggestive, especially for village chickens.

When studying viruses which can cause diseases in poultry, laboratory based procedures which can be employed in a study of the serological characteristics, the ultrastructure of the virus, the nucleic acid nature of the virus, mode of propagation, virulence and nucleic acid sequence may be included. The techniques used in order to obtain this information on the specific virus may differ from laboratory to laboratory and from country to country. However, the basic principles of the methods remain the same. The aim of this dissertation is to establish a series of classical methods that will assist in the identification of an unknown virus.

1.7. Classical Investigative Methods

There are a range of classical techniques which can be used in order to determine whether or not one is working with NDV. These include: cultivation using SPF eggs via different inoculation routes, cell culturing, HA test, transmission electron microscopy (TEM) and MDT studies (Cunningham, 1966; Alexander, 2003a).

1.7.1. Inoculation routes of embryonated eggs

The major routes of inoculation and cultivation available in embryonated chicken eggs make it an important, efficient and globally used method for the isolation and production of poultry viruses and vaccines.

There are several routes of inoculation which can be used in the cultivation of viruses. The allantoic cavity (or chorio-allantoic cavity) route for inoculation uses embryonated eggs of 9 to12 days incubation. This route is mainly used for fowl plague, ND, IB, influenza, mumps and encephalitis (Villegas, 1989). It is the simplest route of inoculation and for the collection of virus and further can be used in chemical analysis, vaccine production and antigen production for serology. The allantoic cavity route is performed by inoculating the virus sample into the allantoic cavity through the chorio-allantoic membrane. There are two methods to inoculating via the allantoic cavity route. Method no.1 is by inoculating into the egg at a point free of large blood vessels below

the base of the air cell (Figure 1.3 A). Method no. 2 is the inoculation of the allantoic cavity through a point at the upper end of the shell over the air cell (Figure 1.3 A). Both methods inoculate the virus sample into the allantoic cavity of the egg. At this stage of the 9 to 12 days incubated embryos there is about 6 - 10 ml of allantoic fluid available (Cunningham, 1966) . Within the inoculated allantoic cavity, the virus particles enter the membranous cells, multiply and are released back into the allantoic fluid again.

The amnionic cavity inoculation route uses 7 to 15 day old embryonated eggs. The reason for the big age difference in eggs for inoculation via this route is that different viruses have a preference for eggs of different age. The slower growing viruses can take advantage of the longer incubation period. The method used in order to inoculate the amniotic cavity is by drawing a circle parallel to and 5 mm above the base of the air cell directly above the chicken embryo. The shell is cut around the drawn circle and the cap of the shell is removed to expose the shell membrane without breaching the membrane. A few drops of sterile saline solution are added to the membrane to make it transparent in order to make it easier to inoculate. The inoculating needle is then pushed through the hole made in the shell at the air sac end (Figure 1.3 B). The inner epithelial lining of the amnion and the epidermal epithelium of the embryo are inoculated with the virus sample. The virus is then swallowed by the embryos and the virus is allowed contact with the mucous membranes of the upper respiratory and gastrointestinal tracts. The viruses which are cultivated using this route include influenza and mumps viruses.

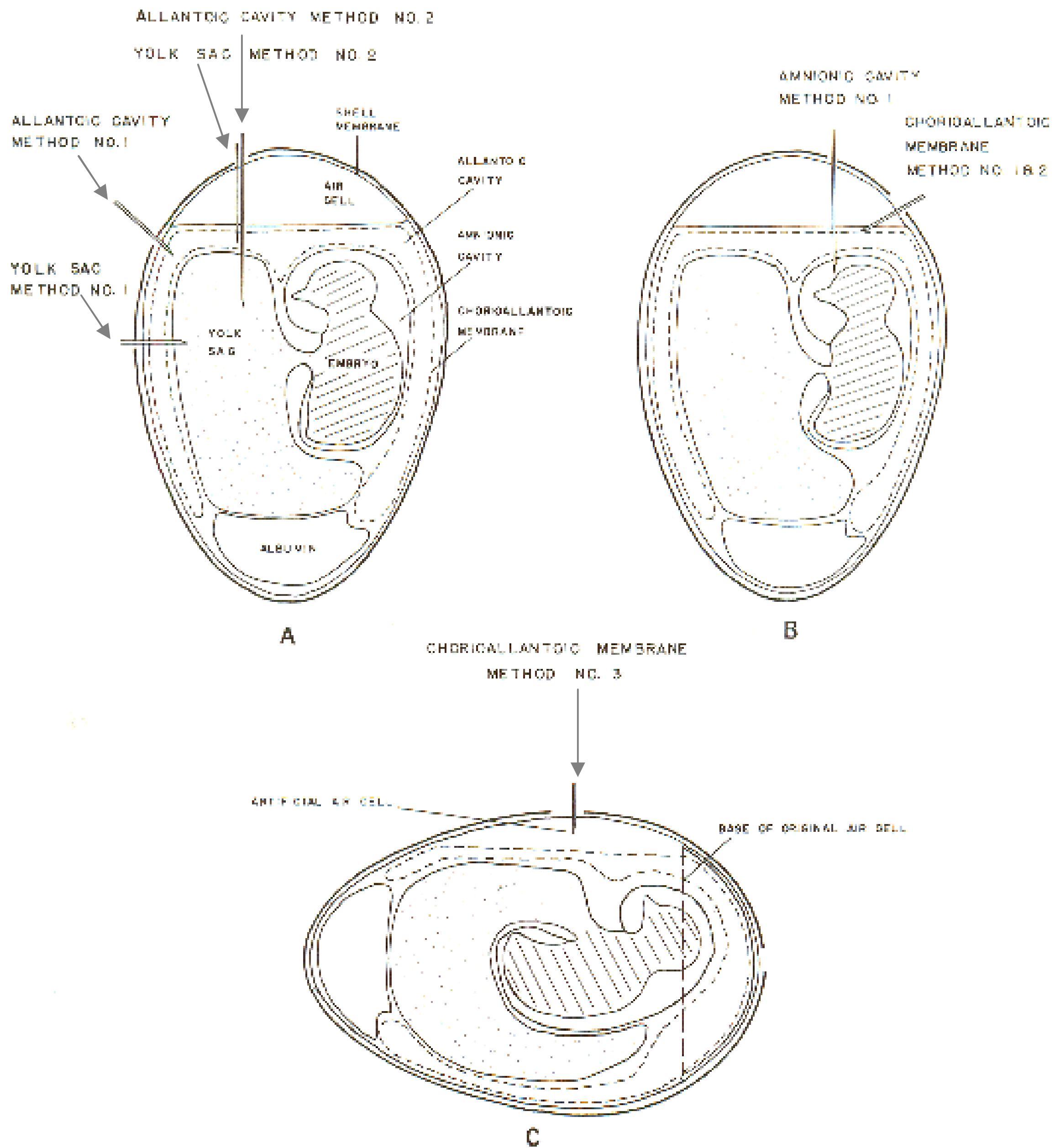

Figure 1.3. Routes of inoculation of embryonating chicken eggs. (A) Allantoic cavity method no. 1, allantoic cavity method no.2 and yolk sac method no.1. represented. (B) Amniotic cavity method no. 1 and the chorioallantoic membrane method no. 1 & 2. represented. (C) Chorioallantoic membrane method no. 3 represented. (Cunningham, 1966)

The chorio-allantoic membrane inoculation into 10 to 12 day old eggs is mainly used for the cultivation of viruses causing vaccinia, variola, fowl pox, LT of chickens and pseudorabies which show “pocks”. There are three different methods that can be used for the chorio-allantoic membrane route. Method no.1 is inoculation via a point cut in the egg shell about 3 mm above and parallel to the base of the air cell. The needle is inserted with the bevel edge down in between the chorio-allantoic membrane and the shell membrane and depositing the inoculum on several points across the chorio-allantoic membrane (Figure 1.3 B). Method no. 2 is the same as for the amnionic cavity route, but instead of depositing the inoculum into the amniotic cavity, the virus sample is inoculated on the chorio-allantoic membrane (Figure 1.3 B). With methods no. 1 and no. 2 it is possible that the viral inoculum may leak through the chorio-allantoic membrance into the allantoic or amnionic cavity. Method no. 3 is different from methods no. 1 and no. 2 for the chorio-allantoic membrane route in that method no. 3 uses an artificial air cell to inoculate along the chorio-allantoic membrane. This third method is used for ‘pock counts’ with viruses that produce gross lesions on the chorio-allantoic membrane. For method no. 3 the egg is candled and the position of the embryo is marked. The egg is placed horizontally so that the embryo is uppermost and a square area of 2, 4 cm^2 is cut on the side of the egg between the two ends of the egg without piercing the shell membrane. Thereafter, the shell is pierced at the end over the air cell as well as at the cut on the side of the egg. A vacuum is created by using a rubber bulb at the hole over the air cell. Air will pass through the opening in the shell membrane on the side of the egg and allows the chorio-allantoic membrane to drop from the shell membrane. The virus sample can is then inoculated, bevel down, onto the chorio-allantoic membrane through the cut on the side of the egg (Figure 1.3 C).

Inoculation into the yolk sac requires 5 to 8 day old embryos and is used to initially isolate mumps virus. Method no. 1 for inoculation into the yolk sac route requires the egg to be candled while in the horizontal position. The egg is candled and a point is marked about halfway from the small end of the egg to the apex of the curvature of the shell. A small hole is drilled through the shell at the mark without piercing the shell membrane. The virus sample is inoculated straight into the yolk sac via the drilled hole

(Figure 1.3 A). Method no. 2 for the yolk sac route is similar to the allantoic cavity route method no. 2 with the exception that the virus sample is inoculated into the yolk sac and not into the allantoic cavity route (Figure 1.3 A).

As the sample viruses were sent to our laboratory as NDV, it was logical to treat it as NDV and to try and cultivate it as such. The allantoic cavity route was used as it is generally the most employed and expedient method of cultivating NDV.

1.7.2. Cell Culturing

Cell cultures may be used as vehicles for *in vitro* propagation in virology as they allow for the study of the interaction between the host cell and the virus. In many cases they may even be used to inspect the effectiveness of antiviral drugs. They can also be used in the study of gene function in their natural environment, in the study of cell behaviour *in vitro*, in the study of cancer cells as well as a potential means for the mapping of genes on chromosomes by using human-mouse hybridomas. The expression of proteins and antibodies in cell cultures as a bio-medical technology. In other instances, the primary studies into drug safety and biocompatibility studies are performed on cell cultures. The most advanced use of cell cultures is in the development of techniques for tissue cultures/engineering (Ryan, 2008).

It was Willhelm Roux who first grew cell cultures from chick embryos in salt solutions in 1885. However it was only, due to Alan Parks, in 1949 that a protocol was developed which allows the cells to be frozen, stored at -196ºC for years and be revived completely. This technology is still used today albeit with modifications where necessary. The pinnacle of cell cultures to date is the famous HeLa cell line a derivative from the cancerous growth of Henrietta Lacks who died in 1951 of cervical cancer.

Some of the more common cell lines known and used globally are the 3t3 fibroblast cell lines which are of murine origin (rodent - mice and rats) and can make collagen if fed ascorbic acid. Another is the baby hamster kidney (BHK) fibroblast cell culture derived

from Syrian hamster can be used to isolate virus and maintain virus cultures e.g. adenovirus, the cell culture can be grown on solid substrate or in suspension. And, of course, the widely used HeLa cell line which is from human epithlia and is used in the research for cancer and other medical research and technology.

The substrate for the cells to grow in or on can be solid, semisolid or liquid. Solid surfaces include glass, plastic in the form of polystyrene and plastic coated with collagen. Semisolid substrates are the semisolid agars and matrices formed from collagen and cellulose sponges and other aids that have tube-like formation (Freshney, 2006). Liquid substrates simply refer to the suspension of cell cultures in liquid media. Generally, the chosen medium should supply nutrients, buffering for pH, sterility and an isotonic environment for the cells.

Balanced salt solutions can be used to support cell cultures, but not for an extended period of time. These solutions are usually just a simple mixture of salts with glucose and the choice of any salt solution depends on conditions like the carbon tension in the environment. For the support and increase in abundance of cells, a ‘complete’ medium must be used. Complete media are generally comprised of a pH buffer system like HEPES (N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid), additionally a pH indicator like phenol red, amino acids, vitamins, salts including; NaCl, KCl and CaCl, glucose and serum (Unchern, 1999; Ryan, 2008)

Serum refers to the surplus clear liquid after the removal of fibrin and cells from blood. The most commonly used sera for complete media and the general upkeep of cell cultures are calf/bovine, horse or foetal bovine sera (FBS). Serum is packed full of a large number of components. The function of the serum is to supply basic nutrients both in solution and protein bound and to serve as a source of growth factors and hormones. It also contains factors that promote attachment and spreading on artificial surfaces. Serum acts as a protease inhibitor which is important in inhibiting the actions of proteases such as trypsin from over hydrolyzing necessary proteins that help the cells to adhere to surfaces, as is necessary for the culture of monolayers.

The major problem with both complete media and serum is of contamination from bacteria, yeasts, fungi and mycoplasma. Antibiotics and fungicides may be added to the media in order to deter or inhibit contamination from bacteria and fungi respectively (Unchern, 1999).

The presence of cytopathic effects (CPEs) caused by a virus in chicken embryo fibroblast cells (CEFC) can be used as a quick *in vitro* method to differentiate between virulent and avirulent isolates. Pathogenic isolates are cytopathogenic for CEF and chicken kidney cells whereas non-pathogenic viruses cause CPE only in the latter (McFerran & McNulty, 1993).

1.7.3. Haemagglutination Assay

The haemagglutinating property of poultry viruses is not universal, but does occur in several types of virus. Within such virus species, this property is usually stable and characteristic of the virus. Haemagglutination (HA) does not only serve as a characteristic for a virus, but may be used further in distinguishing between the different serovars of a specific virus. This is due to variation in the structure of the haemagglutinin projections with even subtle differences in the haemagglutinin structure possibly leading to the serovars of a virus agglutinating a variety of avian or mammalian RBCs, or may even contribute to the changing strengths of HA that the serovars may possess. In general the Fowl adenovirus-1 (FAdV-1), for instance, is known to haemagglutiate only rat erythrocytes. However, FAdV-1 (Indiana C) strain was able to agglutinate sheep erythrocytes indicating a variation within the serovars (McFerran & McConnell Adair, 2003a).

The HA test is, however, not a diagnostic test for serology. The result of the HA determines whether the virus can haemagglutinate and subsequently, what dilution of virus should be used in other serology tests, for example, VN test and HI test.

In some viruses, the ability to haemagglutinate spans across red blood cells from several different species. This is the case of *Egg Drop Syndrome virus* (EDS virus) which agglutinates erythrocytes of chickens, ducks, turkeys, geese, pigeons and peacocks, but cannot agglutinate rat, rabbit, horse, sheep, cattle, goat or pig erythrocytes.

Haemagglutinin, which is invariably a glycoprotein projection from the surface of virions, serves generally to adsorb to host cell receptors connected to glycoproteins with sialic acid. Upon this adsorption, the virion is then enclosed into the host cell via endocytosis which is triggered by the receptors. A good example of a virus which employs this function of haemagglutinin is the AI virus. In such cases, the HA gene is considered a crucial confirmation on whether a particular strain of virus is highly pathogenic or not (Swayne & Halvorson, 2003). This is due to the cleavage of the HA gene into HA1 and HA2 proteins enabling the virus to be infectious and venture into multiple replication cycles (Swayne & Halvorson, 2003).

Although the HA assay may not give exact correlations between a virus and its level of pathogenicity, it is important to note that the HA reading may possibly be a good indication to the haemagglutinating efficiency of that particular sample at a certain concentration. Primarily, the HA test is used in order to find the haemagglutinating titre or a virus which is able to haemagglutinate, which can then be used in the HI test, where virus specific antibodies can be used to identify the virus. Some of the avian viruses which carry this property are NDV, EDSV, AI virus and avian bronchitis virus. Once NDV has been isolated, the HA and HI tests are used to confirm for the presence of the virus in the culture.

1.7.4. Transmission Electron Microscopy

The word microscopy is derived from the Greek *mikros* (small) and *skopeo* (to look at). Electron microscopes include the family of instruments which produce magnified images by the use of electrostatic or electromagnetic lenses with fast-moving electrons as

illumination (Watt, 1997). These microscopes are capable of producing images of high resolution and into a practical depth of field. Electron microscopes are generally able to either look at the internal structure of semi–transparent specimens or visualise the external outline of structures. The former group of electron microscopes is represented by the TEM and the latter group of microscopes is the scanning electron microscope (SEM).

Due to viruses being classified based on the nature of their genetic material, either DNA or RNA, and their morphology i.e. size, shape and appearance. In most cases the viruses have a satisfactorily dissimilar morphology and this is often used to differentiate between the different virus groups. The members of a family of viruses usually have commonality in their morphology. However, there are exceptions in such cases as the families *Poxviridae*, *Papovaviridae* and *Reoviridae* where even within these families there are genera with identifiable differences (Doane & Anderson, 1987).

Due to the very high resolution of the TEM, it is an obvious choice in terms of looking at the ultrastructure of viruses. The TEM was chosen as an instrument for investigation into the ultrastructure of the sample viruses in the present study.

1.7.5. The Mean Death Time

The significance of viruses on any society lies in the virulence of the virus itself. Whether a virus is capable of perpetually propagating itself is dependant on whether or not its virulence is low or high. A simple depiction of such a distinction is in the case of the difference between HPAI and the regular AI.

The OIE that codifies sanitary and health standards, and is affiliated to the World Trade Organization (WTO), has included HPAI as a List A reportable disease (OIE, 2007). According to their standards, an AI virus that kills 75% of 4 to 6 week-old chickens within 10 days of an intravenous inoculation with 0,1 ml of a 1:10 dilution of virus in a bacteria-free, allantoic fluid; has a polybasic amino acid region at the haemagglutinin

cleavage site and is of the H5 or H7 serovars; or is not an H5 or H7 virus but kills 1 to 5 chickens and grows in cell culture in the absence of trypsin is to be considered an HPAI.

Determination of the pathogenicity of the isolated virus, such as establishment of the MDT, Intracerebral pathogenicity index or Intravenous pathogenicity index helps to identify the virus (CEI, 2004). Avirulent strains tend to kill embryos slowly or not at all. The time taken for an isolate to kill the embryos is related to its pathogenicity for chickens (McFerran & McNulty, 1993). Detection of NDV antibodies to the virus is possible using single radial immunodiffusion, single radial haemolysis, virus neutralization (VN) and enzyme linked immunosorbent assay (ELISA). However, these cannot by themselves be enough to determine whether there is ND infection. This is simply because vaccinations against NDV are very widespread and frequently applied meaning that antibodies against NDV are found often even in places devoid of infection.

Along with the complications of detection of ND infection mentioned above, the deviation of virulence of NDV indicates that detection of NDV infection alone is inadequate as a diagnostic approach. This stems from the difference in control measures which are used for avirulent viruses compared to those used for virulent viruses. Therefore, diagnosis is dependent on further characterization of the virus especially in terms of whether the virus is virulent or not. As the clinical signs are not pathognomonic (accurately diagnostic of a disease) even in cases with most virulent viruses, other evaluations and assessments with regards to virulence are necessary (Aldous & Alexander, 2001).

There is a particular significance as to how viruses should be classified according to their relevant virulence. Among the various methods used to ascertain the level of virulence or pathogencity of a virus is the MDT. This simple method yields a number that assists in the measure of a virus's virulence. There are however, standards that need to be set in order to use the results of an MDT test. As the two sample viruses, D1446/05 and 834/05 were initially thought to be NDV their results are compared to the standard MDT values of NDV.

1.8. Molecular Investigative Methods

In the 1940s, sometime after the established discovery of the virus, there was an observed inherited alteration in some of the viruses. Scientists were very unconvinced at the time to accept this as mutations within the viral genetic code. Instead, they preferred to think of it as an adaptation by free will of the virus. Max Delbrück and S.E. Luria in 1943, which led to their later award of the Nobel Prize in physiology or medicine in 1969 (http://nobelprize.org/nobel_prizes/medicine/laureates/1969), demonstrated that bacteria that were exposed to phage and survived the lysing of the bacteriophages produced resistant bacteria progeny. They proved that the bacteria were mutants of the original culture and that they were selected and not induced by the virus. Thereafter, a number of the geneticists of that time were able to think in terms of mutation and selection and not just adaptation. Thus, the viruses that are able to change virulence toward a given organism display a change in genetic property that can be regarded as mutation and selection (Fraser, 1967).

From these observations and discoveries regarding virus genetics there have been progressive molecular methods and manipulations in the further studies of virus genetics. Many of these were developed using bacteriophages as the model, due simply to their high infectivity, ease of starting synchronous infection, the ability to follow the development of new virus components chemically and microscopically and most importantly because the bacterial host cell and virus are agreeable to genetic analysis (Hayes, 1968). One of the earlier tools used in phage genetics was the conditional lethal mutants. These mutants are usually temperature-sensitive that are able to grow normally at 25 - 30°C, but are unable to develop at 42°C. The advantages of the conditional lethal mutants are that their mutations may be isolated in any gene making it possible to construct a complete genetic map of the organism. Mutants with different functional genes can be differentiated in complementation tests so that the physiological genetics of viruses such as RNA phages can be studied, and the ability to isolate mutants which are defective in essential functions allow these functions to be analysed

under restrictive conditions and observe what synthetic or morphogenetic step is blocked (Hayes, 1968).

Since early tools such as the conditional lethal mutants, there have been many more discoveries that are helping to unlock the mystery of the genetics of viruses. Several improvements of these earlier investigations and discoveries into virus genetics have resulted in new methods of molecular biology as technology and production of molecular tools progresses.

Molecular techniques and approaches to determining the nature of a virus, cell or virtually any nucleic acid coded organism are various and technologically advanced. The most important asset of molecular techniques is the way in which they gather the most accurate information on a micro-molecular level.

Currently, molecular identification as well as molecular investigation at the genetic level relies mostly on the polymerase chain reaction (PCR) that was accidentally discovered by Kary Mullis in 1980. This relatively recently developed system resulted in the *in vitro* exponential amplification of DNA (Gibbs, 1990).

DNA amplification by PCR *in vitro,* as opposed to *in vivo*, has since simplified many of the standard procedures for cloning, analyzing, and modifying of nucleic acids. The power of PCR lies in the fact that using the tools to retrieve the genes of interest, there can be almost unlimited copies of that gene amplified to be used in various molecular techniques.

In examining the general principles of PCR, the starting point is at the designing of the oligonucleotide primers that are first designed to be complementary to the ends of the sequence that is of interest. This is then added to the DNA template and deoxyribonucleotides in an accommodating buffer mixture that includes certain minerals and components to ensure the appropriate working conditions for the reaction to take place. At the beginning of the process, high heat is used to denature the original

template strands thereafter cooling is employed to support primer annealing to the denatured template strands (Gibbs, 1990). The primers will be ideally positioned so that as they are extended by the DNA polymerase; they will create newly synthesized strands that overlap the binding site of the opposite oligonucleotide (Erlich *et al.*, 1991). Through the continuous repetitive procedure of denaturation followed by annealing and polymerase extension, the primers will bind to the original DNA template as well as the complementary sites in the newly synthesized strands. This results in an exponential increase in the total number of DNA fragments, which consist of the sequences between the PCR primers.

The PCR has given access into several genetic investigations that were, up until its discovery, very difficult or impossible due to the insufficient amounts of genetic material available. Another aspect of PCR, besides the ability to amplify a specific gene of interest, is that the primers can be designed to be as stringent or as universal as is needed either for looking for specific genes of interest or in order to amplify any available genetic material.

In order to amplify RNA, the normal PCR reaction has been modified to form the RT-PCR. The RT-PCR is the ideal technique used to detect and quantify RNA. RT-PCR is also able to be used in cloning, constructing a cDNA library, amplifying signal during *in situ* hybridisations and synthesising probes that are specific for certain genes.

Chapter 2. Cultivation of virus

2.1. Introduction

The two methods of virus cultivation and virus isolation used in this study were SPF eggs and cell cultures. The SPF eggs are quite readily supplied and are a simpler route to use in terms of ease of inoculation and viral replication. Cell cultures are becoming a preferred method of virus cultivation especially for vaccine production. The use of cell cultures has been pronounced as a progressive technology and it bypasses the use of SPF eggs in their large quantities. The main aim of this chapter is to attempt to grow the two known virus samples in both SPF eggs, via the allantoic route as well as in primary cell culture. Efforts to propagate the two unknown viruses are the first steps in attempting to determine the nature of these two virus strains.

2.2. Materials and Methods

2.2.1. Inoculation of virus in SPF eggs

The virus samples, D1446/95 and 834/05, were provided by the OVI. These viruses were two amongst a series of NDV viruses provided by the OVI to the Veterinary Biotechnology Laboratory of the UFS to be used in a study conducted by Boucher in 2006 that focused on the oncolytic activity of NDV and the possible treatment of cancer in the medical field. They were described as NDV however; Boucher (2006) found discrepancies between viruses D1446/95 and 834/05 compared to NDV. The results of HA tests run by Boucher (2006) revealed that the two virus samples were unable to agglutinate RBCs. In addition, it was observed that primers specific for the F-gene of NDV were unable to amplify the two virus samples. Taking into account that the presence of both haemagglutination and the F-protein are characteristic of NDV, the virus samples D1446/95 and 834/05 were thereafter considered as “unknown” viruses. The control classical NDV was provided to this study by Ms C. Boucher.

The SPF eggs were supplied by Glen Agricultural Research College (ARC) of the Free State Province. They were all bred from SPF hens and roosters and were delivered as 9 to 10 day old embryos. The eggs were stored and handled in the 37°C incubators of the Veterinary Biotechnology Laboratory (UFS). The eggs were incubated at 37°C in order to maintain the internal temperature of the eggs. They were constantly kept humid by spraying a thin layer of distilled water on the surface of the eggs. As the allantoic route was used for the inoculation of the virus samples, the eggs that were inoculated were either 9 or 10 day old eggs.

The point of inoculation was marked out on the egg by candling the eggs. Candling is the method of illuminating embryonated eggs using a candling lamp in a dark room. While candling, a section of the chorio-allantoic membrane which is away from the embryo and the amnionic cavity, and free of large blood vessels was found and was marked with a pencil about 3 mm above the membrane which was then the chosen point of inoculation. The eggs were completely sterilized on the surface by spraying of 70% ethanol. This was to ensure that no infection should occur during the procedure of inoculation.

Once the inoculation area was determined, a small hole was drilled into the shell without breaking the membrane. This was done using an egg puncher which had been sterilized by flaming with 100% ethanol and further spraying with 70% ethanol. Thereafter, a 100 μμl sample of the two different virus samples were injected into the egg at a 45° angle to ensure that the embryo and yolk sac are not punctured. The hole was plugged with wood glue in order to ensure that the embryo was in a closed, bacteria-free environment once again. In instances where multiple eggs were inoculated with the same virus sample, the total required amount was drawn into the syringe. This was then used to inoculate several eggs which had already been sterilized and drilled in order to shorten the amount of exposure time for the eggs to possible infection.

After inoculation the eggs were incubated at 37°C and were periodically kept moist by spraying a fine mist of distilled water. This ensured that the egg shells remained moist enough to allow for efficient gas exchange across the usually hard and dry shell. Without regular moistening, the embryo would die from carbon dioxide poisoning and lack of oxygen. Normally the roosting of the hens on the eggs would allow for ideal temperature and humidity regulation. However, the in-house incubation facilities although adequately regulating temperature could not meet with the needs of high humidity as the climate in the region is dry.

The eggs were observed daily and embryo deaths were recorded appropriately. Once the embryo had died, the egg was marked and placed into the 4°C fridge for a period of 4 to 24 hours. Although the embryo may have died, the blood within the veins does not clot immediately upon embryonic death and the veins themselves are still easily perforated. As the veins are growing along most of the inner surface of the shell, it is important that they do not perforate otherwise the blood would hinder the harvesting process. Keeping the eggs at 4°C minimizes the haemorrhaging from the veins during harvesting and makes it easier to conduct due to better visibility.

2.2.2. Harvesting of virus from SPF eggs

Harvesting was performed using two sterile tweezers (70% ethanol and autoclaved), an alcohol-flame sterilized egg-puncher, sterile 2,5 ml or 5 ml syringes and sterile falcon tubes. The surfaces of the eggs were sterilized with 70% ethanol when removed from the 4°C fridge. Throughout the procedure gloves were worn to further ensure the sterility during harvesting. Upon the evaporation of the ethanol, the eggs were placed into the biohazard cabinet. The egg shells were carefully drilled into using the sterilized egg-puncher and the chorio-allantoic membrane was slit with the sterile tweezers and removed without disturbing the contents of the allantoic cavity. Using either a 2,5 ml or 5 ml sterile syringe, the contents of the allantoic cavity was slowly and cautiously aspirated being careful not to perforate the flimsy inner amnionic membrane and consequently, aspirate the contents of its cavity. The collected allantoic fluid was

transferred to sterile falcon tubes and stored at -20°C as working cultures. All surplus eggs, materials, fluids and virus samples that were used or acquired were discarded as biohazard waste material. This meant that they were collected into biohazard autoclaveable bags, autoclaved at 121°C for 15 minutes and discarded along with other biological waste material.

2.2.3. Production of primary CEF cell culture and inoculation with virus

The cell cultures were produced in-house at the Veterinary Biotechnology Laboratory (UFS) and were prepared specifically for this study alone. The media and disposable flasks used for the growth of the cell cultures were supplied by the National Control Laboratories (NCL) situated in the UFS.

A total of three to five 9 to 11 day old SPF embryos were used for the production of chicken embryo fibroblast (CEF) cells. The eggs were sprayed with both 70% ethanol and 1% Virukill™ disinfectant to ensure sterility. The eggshells and chorio-allantoic membrane were carefully removed using sterile tweezers and the embryos were then taken out from the eggs. The embryos were placed in Petri dishes and heads, limbs and viscera were removed using sterile autoclaved surgical apparatus. Thereafter, the bodies were transferred to a new Petri dish containing phosphate buffered saline [PBS] (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.47 mM KH_2PO_4) without calcium or magnesium. The bodies were cut into small fragments using sterile scissors. The chopped pieces were put into a 50 ml falcon tube and washed 3 to 4 times with PBS until clear to remove the RBCs.

Tissue fragments were poured into a sterile 250 ml Erlenmeyer flask with the addition of 30 ml of pre-warmed 10% trypsin solution (5 ml trypsin diluted into 45 ml sterile distilled water). The flask’s contents were stirred slowly using a magnetic stirrer in a 37°C incubator for 15 minutes. A drop of supernatant was put on a glass slide and observed under a microscope. The supernatant was decanted if many single cells and small clumps of 2 to 10 cells were observed. If the cells were in large clumps of more than 10

cells, the flask was stirred for a further 10 minutes. The cloudy supernatant was poured through sterile gauze covering a 50 ml graduated falcon centrifuge tube containing 5 ml of 5% cold heat-inactivated calf serum. This tube was set in a pan of ice in order to keep the calf serum cold. The cells were centrifuged at 1000*g* for 10 minutes using a JA 21 Beckman rotor at 4°C.

After decanting the trypsin solution, the cells were resuspended in 3 – 5 ml Minimal Essential Medium (MEM). The contents of the MEM used included: MEM with Earle's salts and L-Glutamine (Gibco), 10% FBS (Delta Bioproducts); 100 ng/ml Streptomycin; 200 U/ml Penicillin; 0,002 ng/ml Fungizone (Highveld, Biological) and the pH of the MEM was adjusted to 7.6 (media provided by NCL at UFS). The cells were further diluted by filling up with 50 ml MEM with 10% calf serum. Resuspension was done by swirling the falcon tube gently – it was important not to resuspend using great force e.g. shaking the tube vigorously. This would ensure that the cells would not be damaged and ultimately that the chance of confluency of cells would be greater. After complete resuspension, the cells were divided into four separate sterile cell-culture flasks (Nunclon™ 25 cm^3).

The flasks were incubated at 37°C and were frequently observed, using an inverted microscope, for a) contamination from bacteria, b) depletion of nutrients in the media, c) confluency and d) 'floaters' or cells which had not adhered to the flask's surface. In the case of bacterial contamination, the media would turn cloudy and the flask was disposed of. The depleted medium (indicated by a change in colour from pink to orange/yellow) was decanted and replaced under sterile conditions. This was repeated until 80% confluency was observed. Upon confluency (which took between overnight to 2 days to reach), 500 µl of harvested virus D1446/95 or 834/05 was added, incubated and maintained at 37°C until possible CPEs were observed or until the cells lost confluency. The CPEs of the respective viruses were recorded as at 72 hours post inoculation (PI).

2.3. Results

2.3.1. Inoculation and harvesting of virus in/from SPF eggs

The embryos of the SPF eggs usually died 2 to 3 days after successful inoculation when inoculated with D1446/95 (Table 2.1) and after 3 to 4 days with 834/05 (Table 2.2).

Table 2.1. Results of cultivation of virus sample D1446/95 in SPF embryonated chicken eggs.

Virus	Egg no.	Signs* and Daily Observation period						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
D1446/95	1	L	L	L	L	D	D	D
D1446/95	2	L	D	D	D	D	D	D
D1446/95	3	L	D	D	D	D	D	D
D1446/95	4	L	L	L	D	D	D	D
D1446/95	5	L	L	L	D	D	D	D
D1446/95	6	L	D	D	D	D	D	D
D1446/95	7	L	D	D	D	D	D	D
D1446/95	8	L	L	L	D	D	D	D
D1446/95	9	D	D	D	D	D	D	D
D1446/95	10	L	L	L	D	D	D	D
D1446/95	11	L	D	D	D	D	D	D
D1446/95	12	L	D	D	D	D	D	D
D1446/95	13	L	L	L	L	D	D	D
D1446/95	14	L	L	L	D	D	D	D
D1446/95	15	L	D	D	D	D	D	D
D1446/95	16	L	L	L	D	D	D	D
D1446/95	17	L	L	L	D	D	D	D
D1446/95	18	L	L	L	D	D	D	D
D1446/95	19	L	L	L	L	D	D	D
D1446/95	20	L	L	L	L	D	D	D
D1446/95	21	D	D	D	D	D	D	D
D1446/95	22	L	L	L	D	D	D	D
D1446/95	23	L	L	L	D	D	D	D
D1446/95	24	L	L	L	L	D	D	D
D1446/95	25	L	L	L	D	D	D	D
D1446/95	26	L	L	L	D	D	D	D
D1446/95	27	L	L	L	D	D	D	D
D1446/95	28	L	L	L	D	D	D	D
D1446/95	29	L	L	L	D	D	D	D
D1446/95	30	L	L	L	D	D	D	D

* L – Live; D – Dead

Table 2.2. Results of cultivation of virus sample 834/05 in SPF embryonated chicken eggs.

Virus	Egg no.	Signs* and Daily Observation period						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
834/05	1	L	L	L	D	D	D	D
834/05	2	L	L	L	D	D	D	D
834/05	3	L	L	L	L	D	D	D
834/05	4	L	L	D	D	D	D	D
834/05	5	L	L	L	D	D	D	D
834/05	6	D	D	D	D	D	D	D
834/05	7	L	L	L	D	D	D	D
834/05	8	D	D	D	D	D	D	D
834/05	9	L	L	L	L	D	D	D
834/05	10	L	L	L	D	D	D	D
834/05	11	L	L	L	L	D	D	D
834/05	12	L	L	L	L	D	D	D
834/05	13	L	L	L	L	D	D	D
834/05	14	L	L	L	D	D	D	D
834/05	15	L	L	L	D	D	D	D
834/05	16	L	L	L	D	D	D	D
834/05	17	L	L	L	D	D	D	D
834/05	18	L	L	L	L	D	D	D
834/05	19	L	L	L	L	D	D	D
834/05	20	L	L	L	L	D	D	D
834/05	21	L	L	L	D	D	D	D
834/05	22	L	L	L	D	D	D	D
834/05	23	L	L	L	D	D	D	D
834/05	24	L	L	L	D	D	D	D
834/05	25	L	L	L	L	D	D	D
834/05	26	L	L	L	D	D	D	D
834/05	27	D	D	D	D	D	D	D
834/05	28	L	L	L	D	D	D	D
834/05	29	L	L	L	D	D	D	D
834/05	30	L	L	L	D	D	D	D

* L – Live; D – Dead

Eggs that were successfully inoculated yielded 2,5 – 7 ml of allantoic fluid per SPF egg depending on which stage of development the embryo was at.

2.3.2 Production of primary CEF cell culture and inoculation with virus

The cell cultures made from 9 -11 day old SPF embryos yielded monolayers that were able to be inoculated with the virus samples. Some of the flasks inoculated showed CPE on these monolayers and these CPEs were recorded and analyzed as rounding of cells, plaque formation, enlarging and formation of giant cells, and aggregation of cells into clusters (Figure 2.1).

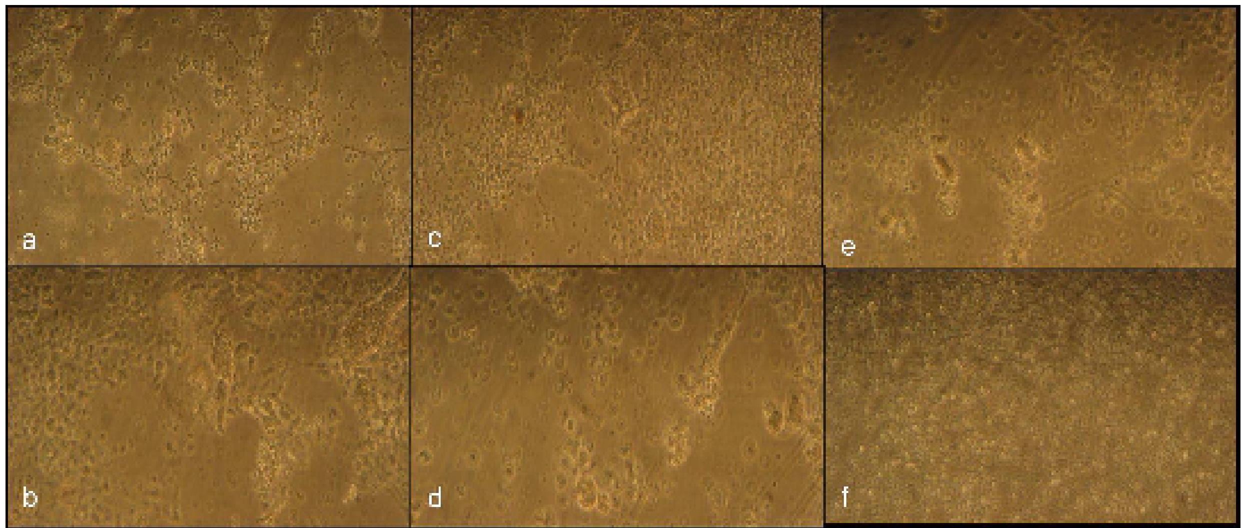

Figure 2.1. Cell cultures showing CPE of D1446/95 and 834/05. (a-c) CPE of D1446/95. (d-e) CPE of 834/05 and (f) Control monolayer of chicken embryo fibroblast cell culture.

2.4. Discussion

2.4.1. Inoculation and harvesting of virus in/from SPF eggs

All the harvested allantoic fluid samples were tested for bacterial contamination prior to inoculation. This was done 24 hours before inoculation by streaking out the samples onto tryptone soy agar (TSA) and incubating it at 37°C overnight. The streaked out plates were observed for bacterial colonies the following day in order to determine whether or not there was any contamination. Allantoic fluid samples which were free of

contamination were immediately used for inoculation in the SPF eggs. Afterwards, the filtered samples were streaked onto TSA and incubated at 37°C overnight in order to determine whether or not the inoculum was successfully salvaged.

The previously contaminated samples, which were cleared of bacterial contamination via filtration, were used for inoculation. However, those whose contamination was persistent were discarded so as not to contaminate other harvested allantoic fluid samples and so that they would not mistakenly be used for inoculation.

Some of the harvested allantoic fluid had unwanted substance present such as, organic tissue and cell debris. These would present problems later on by being obstructions in preparation for viewing the ultrastructure of the virus and by encouraging and providing for bacterial contamination with all the organic acids and nutrients available.

Thus, the harvested allantoic fluids that were found to be dense with the abovementioned materials were centrifuged at 1500g for 30 minutes using a JA 21 Beckman rotor at 4°C. Thereafter the supernatant was transferred, using strict sterile techniques, to a new sterilized falcon tube and stored at -20°C. The precipitated materials were discarded as biohazard waste. All harvested allantoic fluid was tested for contamination as mentioned above and thereafter was treated as inoculum for the next batch of SPF eggs.

2.4.2 Production of primary CEF cell culture and inoculation with virus

Positive growth results were observed in the flasks due to the change in pH (colour changed from pink to orange/yellow) as a result of the depletion of nutrients. The acidity of the medium is indicated by the phenol red indicator as the flask turns from reddish pink to yellow due to the liberation of carbon dioxide.

The carbon dioxide interacts with and dissolves into the water and forms carbonic acid (H_2CO_3). Hydrogen ions liberated from the acid lowers the pH of the medium and once

the pH drops below 6.8 the indicator turns yellow. This change in colour of the phenol red primarily indicates the acidity of the medium, but indirectly also signals the depletion of nutrients in the medium. This is simply due to the amount of cells present in order for such a change in pH to occur. For these cells to mature, maintain and propagate they would be consuming the nutrients available in the medium.

From the comparison of the monolayer of the control cell culture, the cell cultures of both samples indicate aberrations from the control monolayer indicative of CPEs. In the flask inoculated with sample 834/05 there are clear areas where the cells have deteriorated and disintegrated leaving plaques, enlarging and formation of giant cells, and aggregation of cells into clusters. Generally the deterioration of the monolayer in the flask inoculated with 834/05 as compared with the control flask showed a clear difference between normal monolayer CEFC and the infected CEFC. D1446/95 showed similar CPE including plaque-formation (clear areas or holes forming on the monolayer of CEFC), rounding of cells, enlarging of cells, clustering of cells, and strings of cells.

From literature, the CPE of NDV inoculated onto Vero cell line include the formation of giant cells with aggregated nuclei, followed by dendritic shaped cells and stellate shaped cells. These culminate in the ultimate degeneration of cells and perforations in the monolayer which are known as plaque (Ahamed *et al.*, 2004).

The virus samples in this experiment show a few correlations with the CPEs of those seen in cell cultures inoculated with known NDV. However, the failure of CPE of cell cultures to be diagnostic means that any suppositions cannot be conclusive in terms of the samples being positively identified as NDV. Nevertheless, the CPEs observed with both virus samples affirm their ability to replicate and propagate in CEFC cultures as well as their ability to form visible signs in terms of the deterioration of the monolayer cell culture.

In terms of the efficiency of cell cultures as a supply of replication site for virus and resource for virus harvest and manufacture of inoculum, there are several advantages

and disadvantages. An obvious advantage is the progress of virus production that can be monitored and easily detected. Due to CPEs that are easily examined, virus production (or lack thereof) can be confirmed daily. This ultimately reduces the time determining whether or not the virus has replicated. In SPF eggs the embryo death does not automatically mean that the virus has replicated.

Possibilities of embryo death include: embryonic shock, bacterial contamination and naturally weak embryos. As such, virus harvested from SPF eggs usually need to be tested for bacterial contamination and viral infectivity. It is also easier to detect bacterial contamination with cell cultures as the media becomes turbid and can be seen through the transparent flasks. When contamination is found in cell cultures, the flask may be discarded upon detection. However, this is not possible with SPF eggs and they have to be opened and streaked out in order to determine whether or not the sample is contaminated or not.

Regarding disadvantages of cell cultures, the practical intricacies found with the preparation of primary cultures are difficult and time consuming. If cell lines are used instead, this can be a considered method of virus manufacture and observation.

In view of the experience of primary cell culture manufacture, the effect of the trypsinization and centrifugation steps on the success of the cell cultures were noteworthy. If the trypsinization process took longer than 35 minutes in total, the cells of the monolayer either did not attach themselves to the bottom wall of the flask or when the monolayer began forming, it would completely detach from the flask wall. This is due to the overextended time period of trypsinization and in the same manner that the enzyme trypsin is good for releasing cells from monolayer cultures; it may easily result in the cells not attaching to or detaching from the artificial surface. Conversely, if the trypsinization step was too short and clumps of cells were distributed into flasks, cells would begin to grow, but would be unable to form monolayers. This was observed to be due to the proliferation of the cells in groups which adhered to the surface by a few cells. Eventually, the few cells that were adhering to the surface would no longer be

able to hold on due to the mass of divided cells and would be drawn away and the mass of cells would be suspended in the media leading to a failure to generate a monolayer.

It can be concluded from this work that both strain D1446/95 and strain 834/05 are capable of growing in both SPF eggs and in primary cells cultures. The growth characteristics of both of these viruses cannot be used for identification purposes, but it can be seen from this work that there are some similarities in the CPE recorded with these two virus strain and the description of CPE caused by NDV.

Chapter 3. Haemagglutination, virus ultrastructure & mean death time

3.1. Introduction

Usually viruses are initially identified and characterised using classical diagnostic methods and techniques.

A few of the classical methods used include the investigation of the ability of the virus to agglutinate red blood cells and subsequent haemagglutination inhibition using virus specific antibodies if the virus can haemagglutinate, the ultrastructure of virus and the level of virulence of the virus. An aspect of NDV that is used to preliminarily identify it is whether or not it haemagglutinates RBCs as previously discussed in Chapter 1. Thus the HA test is used in order to prove the haemagglutinating ability of a virus. If the virus is able to haemagglutinate, the next step would be to perform the haemagglutination inhibition test, using ND specific antibodies. If the HI test is positive, then the virus can be conclusively identified as NDV.

The virus ultrastructure is an important part of identifying a virus. The investigation into the virus ultrastructure helps to compare it to similar structured viruses and to group it into similar taxa and allow for easier initial identification by association of known virus families. Through the discovery of the ultrastructure of a virus it is sometimes possible to connect, for example the mode of viral entry into and exit out of the host cell. If the virus is found to be an enveloped virus it is known to enter into the cell by various means of fusion of the cell membrane and a type of endocytosis and exits by being surrounded by the cell membrane of the host which forms the envelope of the virus. Thus knowing the virus ultrastructure may not only enable the grouping of the virus with other known viruses, but also may give clues or confirmation to different modes of interaction between the virus and host cell.

The virulence of a virus is highly important for the investigation into how to handle a specific virus. Knowing the difference in virulence may provide information into the nature of the virus, but most importantly how to prepare vaccines and how to administer such vaccines. Understanding the difference in virulence between different viruses as well as between different serotypes of the same virus can indicate the viruses' effect on the host. This is very important for identifying the effect poultry viruses will have on the economy of the poultry market and can easily be determined using MDT. The MDT is a simple yet effective method that can be used to observe the virulence of a virus by inoculating SPF eggs with the virus sample and observing and recording the time of death of the embryo. A shorter MDT implies a higher virulence while a longer MDT means the virus is less virulent or avirulent.

3.2. Materials and Methods

3.2.1 Chicken Red Blood Cell Collection

The blood was collected from randomly selected chickens that were chosen from a large group of birds at Glen (Free State). The total collected chicken blood was washed as according to the procedure outlined by Villegas (1989). The blood was mixed thoroughly and then sterilely aliquoted into 1 ml volumes each into a graduated 1,5 ml microcentrifuge tube. The tubes were centrifuged at 100*g* (Eppendorf 5415D) for 4 minutes. Thereafter, the supernatant was carefully removed and discarded. The red blood cells (RBCs) were resuspended to the original volume using PBS without calcium and magnesium. The tubes were mixed well and centrifuged again. This process was repeated for an average of 3 times until the supernatant of the centrifuged RBCs was clear and without visible blood plasma. The RBCs were diluted to make a 25% working stock solution and refrigerated. In general the washed RBCs were useable for 5 to 7 days. During the HA tests, the 25% stock solution was diluted to a 0,5% working solution.

3.2.2. Haemagglutination Test

Using a P200 multichannel autopipette, 50 μl of PBS (with calcium and magnesium) was added to all the wells to be used in a V-bottomed 96-well microtiter plate (Greiner Bio-one). Thereafter, 50 μl of the virus allantoic fluid sample, which had been harvested from the SPF cultivation routes, was added to the first wells of the plate using a P200 autopipette. The virus was serially diluted in a 1:2 dilution by transferring 50 μl of solution from one well to the next in a consecutive manner. It was important that 50 μl from the final well of the row be discarded as this would ensure that all the wells of the microtiter plate were equal in volume. It was also important to use new sterile pipette tips for the transfer of the solution from one well to the next, otherwise the results of the HA could possibly be negated in view of the fact that the 1:2 dilution of the virus was not accurate. Upon the completion of the 1:2 dilution, 50 μl of 0,5% RBCs was added to all wells using a sterile pipette tip for each well, and the contents of the wells were mixed well.

The RBC controls was set up by adding 50 μl of 0,5% RBCs and 50 μl of PBS to a well. On average, a total of 4 – 6 wells of a row were set up as RBC controls. The plate was covered and incubated at room temperature for 45 minutes to an hour to allow for HA. The RBC control is expected to form a 'button' of erythrocytes at the bottom of the V-shaped wells. This is due to the RBCs settling in the conical V-shape of the wells. Any negative results for HA will show similar buttons to the RBC controls and positive HA results are expected to be in the form of opaque wells without buttons.

HA tests were performed regularly to examine the haemagglutinating properties of the two virus samples.

3.2.3. Ultracentrifugation

Initially, the harvested allantoic fluid sample was centrifuged in falcon tubes at 1500*g* for 30 minutes at 4°C using Beckman JA 21 rotor to sediment the heavier cells and larger debris (Villegas, 1989). Once the supernatant was transferred to a new centrifuge tube, the precipitate was discarded. The supernatant was centrifuged at 7310*g* for 45 minutes at 4°C using a Beckman JA 21 rotor. This allowed for further sedimentation of medium–to–heavy density particles. Afterwards, the precipitate was once again discarded and the supernatant collected into a new Beckman centrifuge tube. This was centrifuged at 25 000*g* for 2 hours at 4°C using the Beckman SW 28 rotor. After the supernatant was discarded, the thin whitish opaque precipitate was resuspended into 200 – 600 μl of sterile distilled water (SdH_2O).

3.2.4. Specimen Preparation and Staining for Transmission Electron Microscopy

The previously concentrated samples of D1446/95 and 834/05, were used for the investigation of the virus ultrastructure under the TEM. A Formvar-coated 200 mesh copper specimen grid (Athene Grids®) was used. The samples were prepared as according to the standard procedure for TEM specimen staining (provided by the CCEM at UFS) and were stained with 2% aqueous uranyl acetate.The specimen was viewed under the TEM at 100 000 – 130 000 times magnification at the Centre for Confocal and Electron Microscopy (CCEM), UFS with the assistance of Prof. Pieter van Wyk.

3.2.5. Mean Death Time

Infective allantoic fluid was diluted in sterile saline PBS in tenfold dilution series between 10^{-6} and 10^{-9}. Inoculations of 100 μl of each dilution were inoculated into each of five 9 to 10 day old embryonated SPF chicken eggs. These were then incubated at 37°C. The remaining virus was kept at 4°C and used 8 hours later in an identical inoculation of five 9 to 10 day old embryonated SPF chicken eggs. All the eggs were

examined twice daily for 7 days in order to record embryo deaths. The eggs were recorded according to the hours after which the embryos were found dead. Eggs that were found dead were labelled with the date and time and placed at 4°C. Of the eggs that were dead, those that had died within 24 hours of the inoculation had their allantoic fluid harvested and checked for contamination. Those that died between 48 to 72 hours were recorded as part of the MDT. The minimum lethal dose is the highest virus dilution that results in the death of all the embryos of that highest dilution. The MDT is calculated by the mean time in hours for the minimum lethal dose to kill all the inoculated embryos.

3.3. Results

3.3.1. Haemagglutination tests

The HA test performed on 31st March 2007 for the two virus samples indicated a proper HA profile for 834/05 and an obscure HA with D1446/95 (Table 3.1). The obscure HA of D1446/95 in Figure 3.1 is the absence of HA in the first four wells which are the 1 through to 1/16th dilution, thereafter HA is seen from the fifth well or the 1/32nd dilution. This result misled to the speculation that the sample 834/05 could most likely be NDV.

Table 3.1. HA test dates and results for D1446/95 and 834/05.

	Date	Result
HA test 1	19/09/2006	No haemagglutination for D1446/95 or 834/05
HA test 2	21/09/2006	No haemagglutination for D1446/95 or 834/05
HA test 3	05/10/2006	No haemagglutination for D1446/95 or 834/05
HA test 4	16/10/2006	No haemagglutination for D1446/95 or 834/05
HA test 5	03/03/2007	Obscure haemagglutination for D1446/95 and proper HA profile for 834/05
HA test 6	31/03/2007	Obscure haemagglutination for D1446/95 and proper HA profile for 834/05
HA test 7	15/04/2007	No haemagglutination for D1446/95 or 834/05
HA test 8	20/05/2007	No haemagglutination for D1446/95 or 834/05
HA test 9	05/06/2007	No haemagglutination for D1446/95 or 834/05
HA test 10	30/06/2007	No haemagglutination for D1446/95 or 834/06

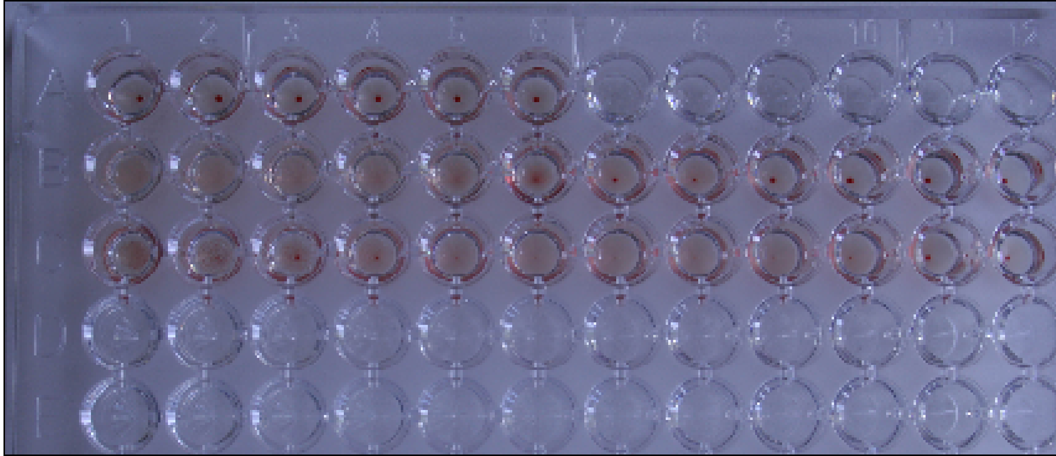

Figure 3.1. HA results from 31st March 2007. 1st row being RBC controls, 2nd row 834/05 virus sample and 3rd row D1446/95 virus sample. HA being a 1:2 dilution.

However, the most recent HA from the 30th June 2007 revealed that the viruses do not haemagglutinate as they formed red buttons similar to the RBC controls (Figure 3.2), and therefore did not fit the profile of being NDV. This most recent HA result of both samples being unable to haemagglutinate correlated to the initial HA test results of the samples after they were received at the beginning (Table 3.1).

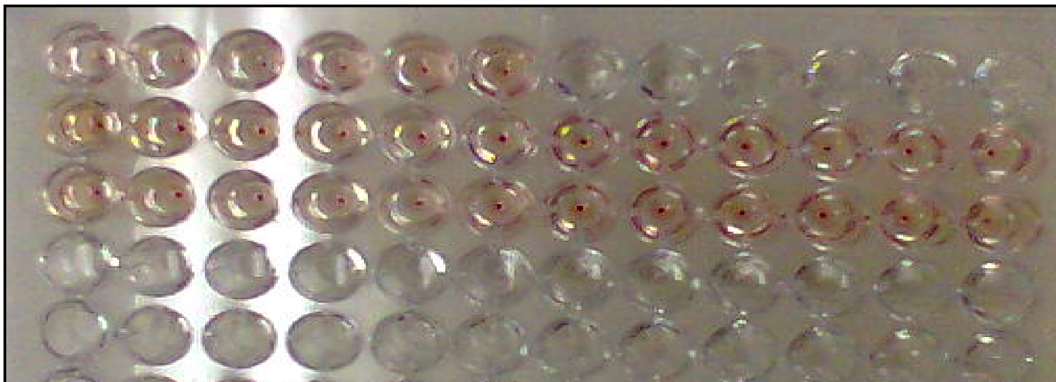

Figure 3.2. HA results from 30th June 2007. 1st row being RBC controls, 2nd row 834/05 virus sample and 3rd row D1446/95 virus sample. HA being a 1:2 dilution.

3.3.2. Transmission electron micrographs

The only micrographs taken of the virus samples that yielded clear pictures were those of the D1446/95 specimen (Figure 3.3 a & b); the specimen grids observed under the TEM of the 834/05 specimen did not show any viral particles. A micrograph of NDV, obtained from literature can be seen in Figure 1.2. This can be used to compare the ultrastructure observed for strain D1446/95 to that of published micrographs of ND virus.

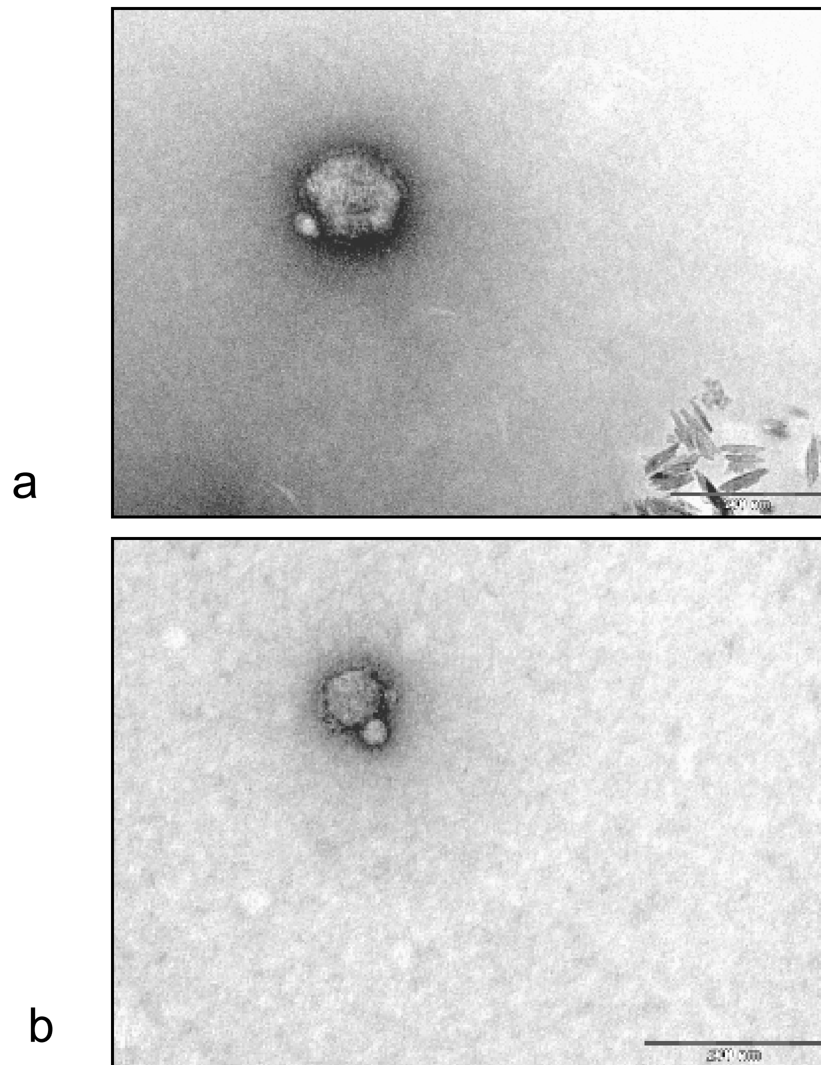

Figure 3.3. a & b. Electron micrographs of virus sample specimen D1446/95. Taken at 100 000 – 130 000 times magnification. Both inserted bars measure 200 nm. Micrograph (a) reveals a virus particle of 150 nm in diameter and (b) measures approximately 100 nm in diameter.

3.3.3. Mean death time profile

Both virus samples had a minimum lethal dose of 10^{-6} (Table 3.2). The MDT of D1446/95 was ~72 hours and that of 834/05 was 60 hours.

Table 3.2. The average calculated MDT for samples D1446/95 and 834/05.

	MDT 1		MDT 2		Average MDT
	Dilution	Hours	Dilution	Hours	Hours
D1446/95	10^{-6}	76	10^{-6}	67,5	71,75
834/05	10^{-6}	59	10^{-6}	61	60

3.4. Discussion

3.4.1. Haemagglutination characteristics of D1446/95 and 834/05

Properties like haemagglutination are characteristic to a virus and are not known to be discarded and acquired as easily as what bacteria may likely do with plasmids conferring resistance. This is simply because, as explained in Chapter 1, the role of haemagglutination for a virus is important for the propagation of the virus. Without which, the virus would no longer be able to adhere to the host cell and in turn terminate its survival.

Thus, it is difficult to give reasons for the virus samples 834/05 or D1446/95 seemingly giving false positive results in the previous HA tests. Furthermore, because of the structural specificity of haemagglutinin, it would seem highly unlikely that other structural projections on the virus samples would be able to unexpectedly agglutinate chicken erythrocytes when they previously could not do so. A possibility could be the

contamination of the samples with a haemagglutinating virus or antigen during virus isolation, or even during the HA tests. However, all work was carried out methodically and as sterilely as possible to ensure that contamination did not occur and that virus samples were correctly labelled and stored. All tests were performed in a biohazard cabinet and no other work in the laboratory on other agglutinating viruses was being performed during the course of this study, thus making the possibilities of contamination unlikely.

It may also be possible that the variable HA profiles were as a result of false-positive reactions. The harvested allantoic fluid samples used to perform the HA tests may have included additional factors such as unwanted substances, cells and cell debris which may possibly be able to cross-react with RBCs resulting in false positive HA as indicated in Figure 3.1. False positive reactions can occur in both the HA and HI tests which may be alleviated by heat-inactivation although not all cross-reacting antigens can be destroyed through this process.

An event that may cause a lack of HA for organisms that carry haemagglutinin would be the destruction of the protein by heat, acid or enzyme treatment which is not a highly probable occurrence. Any of these would be highly unlikely as the samples were always handled with extreme care and were not in direct contact with extreme heat, acid solutions or enzyme stocks. Moreover, most haemagglutinins are able to withstand heat, for example the EDSV haemagglutinin has been recorded to resist heat even up to 56°C and even remaining stable for 4 days thereafter (McFerran & McConnell Adair, 2003b). However, there was a reported fourfold drop in HA titer after 16 hours of heating and no detectable haemagglutinin activity could be measured after 8 days of heating at 5°C (McFerran & McConnell Adair, 2003b). As this was not the case for the virus samples in question, high heat is most likely not a possible reason for the puzzling differences in HA profiles given by the previous and recent HA test results (Figure 3.1 and 3.2).

As has been mentioned previously, there are possibilities of viruses agglutinating different RBC of different fowl or mammalian species. There are instances where a virus can agglutinate RBC of, for example, mammalian species over that of chicken RBC. A recent case study into *Goose paramyxovirus* has shown that a new and different strain of NDV may possibly bind to different sialic acid receptor-binding sites from usual NDV strains (Xu *et al.*, 2008). The study found an outbreak of infectious disease in geese that resulted in high morbidity and mortality. The agent of this disease was found to be a serotype 1 *Avian paramyxovirus*. NDV is also a serotype 1 *Avian paramyxovirus*. This is a startling discovery as NDV, even velogenic strains, have previously not been able to infect waterfowl such as geese. This led to the investigation into the route and mechanism behind the transmission between chicken and geese.

After much genetic investigation the analysis of the information gathered through the study indicated that the goose NDV probably evolved from the standard ND virus strain Herts/33 (Xu *et al.*, 2008) as well as the differences in the binding of the goose NDV to different sialic acid receptors compared to normal standard ND virus strain. What the study found was that the genome of the NDV strain NA-1 had 6 more repeats of a conserved region (UGGGAG) when compared to other NDV genome sequences found in GenBank, for example Herts/33, LaSota/46, Ulster and Aus/32. The genome difference between NA-1 and other NDV genome nucleotide ranges were from 83,3 to 87,7% (Xu *et al.*, 2008). This showed a substantial difference between goose NDV and the standard ND virus strain. Within these differences, the HN of goose NDV was found to be more hydrophilic when compared to other NDV strains. This difference was speculated to lead to the HN proteins binding to different sialic acid receptors as compared to standard ND virus strain.

What can be derived from the HA tests is that the virus samples are unable to haemagglutinate. This conclusion is not derived solely from a single test, but from multiple HA tests which were performed on the two virus samples. These tests all revealed that the virus samples did not show any signs of HA. However, future investigation into the use of other mammalian and avian erythrocytes should be

performed in order to determine whether or not the haemagglutinating properties are suited to agglutinate other erythrocytes than those of chickens and to better understand the range of HA.

It is also quite possible that the HN proteins of these specific virus samples are perhaps activated under certain very specific circumstances. It may be that although there are HN-like proteins on the surface of the two virus samples they may be inactive under normal circumstances. It is however, rather difficult to suggest what these circumstances could be. Perhaps a certain salt concentration or pH will provide the necessary change in protein structure that would result in a haemagglutinating response.

The final possibility would be that the virus samples are perhaps NDV of different species, for example goose NDV as was investigated by Xu and colleagues (2008). At this point, it seems most likely that the two virus samples, D1446/95 and 834/05, are most probably viruses that have HN modifications that allow them to haemagglutinate RBC of species other than chicken. This may also explain the obscure results of the HA test of the 3rd March 2007 and 31st March 2007.

3.4.2. Transmission electron micrograph of ultrastructures

Allantoic fluid that was harvested from the SPF eggs contained a lot of non-virus particles being: free chicken embryo cells, proteins and debris. These particles, although generally not deterring the growth of the virus, were obstacles for viewing under the electron microscope.

The family of *Paramyxoviridae* virus has ultrastructures that are described to be pleomorphic, enveloped virions with ranging diameters of between 150 - 300 nm. This description includes ND as it is the chief model of the *Paramyxoviridae*. In comparison to the brief description given of the ultrastructure of *Paramyxoviridae*, the sample virus D1446/95 may possibly also be grouped to this family.

D1446/95 shows itself to range in diameter between 100 nm to 150 nm as seen in the electron micrographs (Figure 3.3 a & b). It is also pleomorphic as can be deducted from the absence of rigid icosahedral structures. However, due to the insufficient magnification, details such as a nucleocapsid with "herringbone" pattern that is typical of paramyxovirus cannot be seen (Figure 1.2, Chapter 1). This also makes it difficult to determine whether or not there are spike-like projections on the envelope.

What can be seen is that there is a "double layer" of the envelope of D1446/95 which appears similar to the spike-like projections which are expected on envelope of paramyxovirus. However, higher magnification and resolution of the image is required in order to determine whether or not this "double layer" is indeed such a projection. Unfortunately, difficulties were experienced when the magnification was increased beyond 130 000 times. The image would blur beyond recognition and move out of focus which could not be remedied using the intensity of the electron beam alone. Other problems included the loss of high resolution at increased magnifications which did not help in achieving the purpose of seeing detail on the envelope. In some cases, the high intensity of the electron beam would simply increase the cracks in damaged Formvar thin films and result in the loss of the specimen. Limitations were therefore set on the amount of magnification permitted for the viewing of the virus sample.

Other than the comparison between virus sample D1446/95 and avian or standard ND virus strain, it has similarities with some of the descriptions of other common poultry viruses. Some of the viruses that are similar in the description of their ultrastructures include APMV, APV, LTV, IBV, AIV and the Coronavirus that are all described as being pleomorphic and enveloped (Table 3.3).

An example of how comparisons of the ultrastructure of a virus can be used as a preliminary grouping of viruses according to structural similarities is the clear differences between D1446/95 compared with IBDV, AdV and CAV that have an icosahedral structure and are non-enveloped viruses (Table 3.3). This clear distinction between the

structure of D1446/95 and IBDV, AdV and CAV already eliminates these viruses as possible identities for D1446/95.

Virus sample 834/05 did not show any viral particles under the TEM. This may have been due to the low numbers of the virus in the fluid even after concentration of the particles. Further concentration of the virus is crucial in order to attempt a visualization of the virion using the TEM. Time constraints as a result of complications with the harvesting of sufficient virus required for further concentration of the virus from large volumes of allantoic fluid led to the inability to perform further TEM to determine the ultrastructure of 834/05.

Table 3.3. A summary of the ultrastructures of some of the most common poultry viruses.

Virus	Shape	Size	Envelope	Details (where applicable)
APMV	Pleomorphic, rounded	100-500 nm in diameter	Present, surrounded by attached tightly packed rod-shaped spikes.	Helical nucleocapsid has the characteristic "herring bone"- like structure, 18nm in diameter.
APV	Pleomorphic, spherical	80-200 nm in diameter	Present, fringed.	Helical nucleocapsid, 14nm in diameter
IBV	Pleomorphic, rounded	120 nm in diameter	Present, with widely spaced club-shaped surface projections.	
LTV	Pleomorphic	195-250 nm in diameter	Present, irregular envelope with fine spiked surface projections.	Hexagonal icosahedral nucleo-capsid, 80-100nm in diameter. Similar to herpes simplex virus.
AIV	Pleomorphic, spherical	80-120 nm in diameter	Present, surrounded by rod-shaped and mushroom-shaped glycoprotein projections	
IBDV	Icosahedral	55-65 nm in diameter	Absent	Capsid symmetry is askew.
CAV	Icosahedral	25 nm in diameter	Absent	Capsid rotational symmetry is 3-fold or 5-fold.
Adenovirus (AdV)	Icosahedral	70-90 nm in diameter	Absent	Capsomeres arranged in triangular faces of six capsomeres along each edge. Vertex capsomeres have projections called fibers.
Fowlpox virus (FPV)	Brick shaped	±330 x 280 x 200 nm	Present, with surface tubules on the outer coat.	The virion consists of an electron-dense biconcave core flanked by two lateral bodies which is enveloped
Coronavirus	Pleomorphic, spherical	60-200 nm in diameter	Present, with widely spaced long club-shaped peplomers on the surface.	

Adapted and compiled from (Alexander, 2003a); (Gough, 2003); (Cavanagh & Naqi); 2003); (Guy & Bagust, 2003); (Swayne & Halvorsen, 2003); Lukert & Saif, 2003); (Schat, 2003); (McFerran & McConnell Adair, 2003a); (Tripathy & Reed, 2003) and (Guy, 2003).

The negative staining solution is usually a heavy metal salt, phosphotungstic acid and uranyl acetate being two of the more commonly and frequently used ones. Since they are electron opaque, they are deposited around the relatively electron transparent virus particles and causing the virus particles to appear light against a darker background on the TEM screen. This can be seen as in the electron micrographs above which have been efficiently negatively stained.

One disadvantage of the negative staining method is that the virus may only possibly be identified to the family and additional clinical data would be needed in order for appropriate diagnostics. Another disadvantage of the negative staining method is that similar objects resembling quite a few of the classes of viruses may be present. Examples of such cases include bacteriophages which are common in faeces and have a similar appearance to herpesvirus. Another example is the membrane fragments which have surface morphology similar to several enveloped virus families which may make conclusive identification problematic.

3.4.3. Mean death time profile and virulence of D1446/95 and 834/05

According to the FAO (Food and Agriculture Organisation of the United Nations) and the OIE, the MDT used to classify NDV strains specify that velogenic strains take less than 60 hours to kill; mesogenic strains kill between 60 to 90 hours and lentogenic strains take longer than 90 hours to kill the SPF embryos (OIE 2004).

Thus sample 834/05 can be considered to be a velogenic strain while sample D1446/95 can be considered to be a mesogenic strain when compared to such standards. Although the two samples are not NDV, the importance of these results lies in the fact that of the two samples, 834/05 is a highly virulent virus while D1446/95 can be considered as a mildly virulent virus. The NDV MDT standards are used as a reference point, in this case, even though the two virus samples are not NDV.

Their virulence also accounts for the speedy killing of the embryos by both samples, which has previously added doubt as to the sterility of the samples during inoculation. During inoculation and harvesting of the allantoic virus (Chapter 2) there had often been doubts as to whether or not the samples were a) either contaminated before inoculation, b) during harvesting or c) post harvesting. As such, the samples were frequently checked for bacterial contamination before and after inoculation. It now seems that the quick deaths of the embryos can be attributed not to contamination (as was seen in the absence of it), but can be contributed to the relative virulence of the virus samples.

Even though D1446/95 killed the embryos within 2 to 3 days as opposed to 834/05 (which took 3 to 4 days) as reported in section 2.3.1 (Chapter 2), it is possible that this is due to the sheer number of viral particles in an undiluted sample of D1446/95 as opposed to the virulence of 834/05 that remains regardless of dilution. Inoculation of SPF eggs for virus cultivation and MDT was performed with an initial undetermined concentration of viral particles for both virus samples. Thus, the differences in the MDT and the killing of the embryos can be attributed to the undetermined initial concentration of the inocula.

It can be concluded from this chapter that strains D1446/95 and 834/05 did not consistently show HA activity. Sporadic HA activity was recorded during this experiment, but the HA was inconsistent, thus preventing the performing of HI tests. The fact that these viruses do no consistently show HA activity, rules out their identity as NDV. From the EM studies, it can be concluded that strain D1446/95 appears to show ultrastructure similarities to NDV which indicates that this virus bellows to the *Paramyxoviridae* family.

It can be concluded from the virulence studies, that both of these viruses can be seen as virulent based on their MDT in SPF eggs.

Chapter 4: Molecular Techniques

4.1. Introduction

The viruses were not amplified using RT-PCR with primers that were designed to be specific for NDV. These same primers were successfully used to perform RT-PCR tests on other strains of ND virus, including the La Sota vaccine strain (Boucher, 2006). In addition to using classical investigative techniques for the identification of viruses, the two samples thought to be NDV were further characterised using molecular techniques. The possibility of the two virus samples being a variant NDV strain was furthermore considered due to the investigation into goose NDV paramyxovirus serotype 1 found in China that reported both genetic similarities as well as differences to chicken NDV (Xu *et al*., 2008).

Thus, RT-PCR was used to investigate the genetic nature of the unknown virus samples D1446/95 and 834/05.

4.2. Materials and Methods

4.2.1. RNA Extraction and purification of RNA

Virus samples D1446/95 and 834/05 were cultivated in embryonated eggs according to the methods described in Chapter 2. Thereafter the total RNA from harvested allantoic fluid was extracted through the standard Trizol RNA extraction method.

4.2.2. RT-PCR performed on virus samples D1446/95 and 834/05

The extracted RNA samples of D1446/95 and 834/05 were used in RT-PCR reactions according to the Access RT-PCR System (Promega).

Table 4.1. Contents of the RT-PCR reactions as provided by Access RT-PCR System (Promega).

Component	Volume (μl)
Nuclease-Free Water	33,5
AMV/T*fl* 5X Reaction Buffer	10,0
10mM dNTP Mix	1,0
downstream primer	0,5
upstream primer	0,5
25mM $MgSO_4$	2,0
AMV Reverse Transcriptase	1,0
T*fl* DNA Polymerase	1,0
RNA sample or control	0,5
Final volume	50 ,0

The reactions were set up for D1446/95 and 834/05, as well as a positive NDV control and a negative control for amplification from a primer set used to amplify the F protein of NDV using a nested RT-PCR. The negative control was set up in the same manner with an addition of 0,5 μl nuclease-free water in the place of the RNA sample. The first set of degenerate primers, K1 (5'-gggraagaragtgacwtttgaca-3') and K2 (5'-tkggataawccryyrgtgacctc-3') were used to amplify a template fragment starting at nt 778 of the M gene to nt 523 of the F gene of NDV. Primer set MV1 (5'-ccyraatcayyrygryrcyrgataa-3') and B2 (5'-kcrgcrttytgkktgkctkgtat-3') was then used to amplify a 400 – 557 bp product starting at nt 1163 of the M gene to nt 492 of the F gene (Boucher, 2006). D1446/95 and 834/05 were additionally amplified using primer sets described for the amplification of goose NDV (Xu *et al.*, 2008). The primer sets used were specific for Goose paramyxovirus and can be seen in the table below (Table 4.1).

Table 4.2. Primer sets based on the genome sequence of paramyxovirus ZJ1 (Xu *et al.*, 2008)*.

Primer sets	Orientation	Sequence	Length (bp)	Tm min/max (°C)
NP	forward	5`- accaaacagagaatctgtgaggtacg-3`	26	64,59/64,59
	reverse	5`- caccaagttctctgttctcccttca -3`	25	64,58/64,58
P	forward	5`- atgcccagagcccaaggtattag -3`	23	64,55/64,55
	reverse	5`- cttacaagacacaggagacgggtca -3`	25	66,22/66,22
F	forward	5`- ccagcacattcaggacacgacat -3`	23	64,55/64,55
	reverse	5`- agtcggaattaaggcttcacaac -3`	23	60,99/60,99
HN	forward	5`- agtagaacggtcagaggagcc -3`	21	64,52/64,52
	reverse	5`- aaacaaccaacaggagagacc -3`	21	60,61/60,61
La	forward	5`- gagtcccgcccactatcactttat -3`	24	64,57/64,57
	reverse	5`- gacgcagttgcatcaagctagtga -3`	24	64,57/64,57
Lb	forward	5`- tttcaatcctccaagtgacccgac -3`	24	64,57/64,57
	reverse	5`- atcccaccccatcattcactcaag -3`	24	64,57/64,57
Lc	forward	5`- gatgtggtccgcctgtttgagtatg -3`	25	66,22/66,22
	reverse	5`- aagagtgcgcaaagtcgtcccta -3`	23	64,55/64,55

*The primer sets were designed according to the article by Xu and colleagues (2008).

The nested PCR was performed according to Boucher (2006). The prepared reactions were placed in thermocyclers and underwent reverse transcription according to the following program:

First strand cDNA Synthesis

	Temperature (°C)	Time (minutes)	Process
1 cycle	45	45	Reverse transcription
1 cycle	94	2	AMV RT inactivation and RNA/cDNA/primer denaturation

The first strand cDNA synthesis cycle is immediately followed by the second strand synthesis and amplification.

Second strand cDNA Synthesis and PCR Amplification

	Temperature (°C)	Time (minutes)	Process
40 cycles	94	0,5	Denaturation
	50	1	Annealing
	68	2	Extension
1 cycle	68	7	Final extension
1 cycle	4	8	Soak

Following cDNA amplification, nested PCR using primers MV1 and B2 were performed as follows:

Nested PCR Amplification

	Temperature (°C)	Time (minutes)	Process
1 cycle	94	2	Denaturation
5 cycles	94	0,5	Denaturation
	55	0,5	Annealing
	72	1.5	Extension
30 cycles	94	0,5	Denaturation
	48	1	Annealing
1 cycle	68	7	Final extension

The prepared reactions for amplification with primers specific for Goose paramyxovirus were placed in thermocyclers and underwent reverse transcription according to the following program:

First strand cDNA Synthesis

	Temperature (°C)	Time (minutes)	Process
1 cycle	45	60	Reverse transcription
1 cycle	94	2	AMV RT inactivation and RNA/cDNA/primer denaturation

The first strand cDNA synthesis cycle is immediately followed by the second strand synthesis and amplification.

Second strand cDNA Synthesis and PCR Amplification

	Temperature (°C)	Time (minutes)	Process
40 cycles	94	0,5	Denaturation
	60*	1	Annealing
	68	2	Extension
1 cycle	68	7	Final extension
1 cycle	4	8	Soak

*Annealing temperature was kept at 60°C for primer sets: NP, P, La, Lb and Lc. The annealing temperature was adjusted to 58°C for primer sets: M, F and HN.

4.3. Results

Nested RT-PCR amplification using the primer set MV1 and B2 resulted in amplification of a ~400 bp fragment from the positive NDV control while no amplification was observed for sample D1446/95 and 834/05 (Figure 4.1).

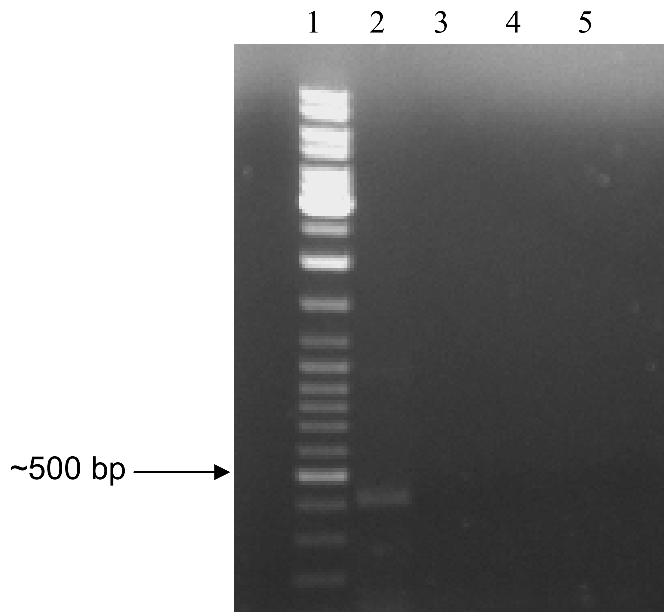

Figure 4.1. Gel electrophoresis of the RT-PCR product using the primer set MV1 and B2 run on a 1% agarose gel. Lane 1) O'GeneRuler™ DNA Ladder Mix, ready-to-use, Lane 2) Positive standard ND virus strain control, Lane 3) Negative control, Lane 4) D1446/95, Lane 5) 834/05.

Of the primer sets designed for amplification of Goose paramyxovirus, used in separate RT-PCR reactions, only primer sets NP and P showed amplification of the virus samples D1446/95 and 834/05. Primer sets F, HN, La, Lb and Lc did not give amplification of the virus samples, D1446/95 and 834/05, nor of the positive standard ND virus strain control (data not shown). There were multiple bands that were observed in both the D1446/95 and 834/05 RT-PCR products that were amplified using the NP primer set (lanes 5 and 6 respectively of Figure 4.2). These were different from the amplification profile that was observed from the positive standard ND virus strain.

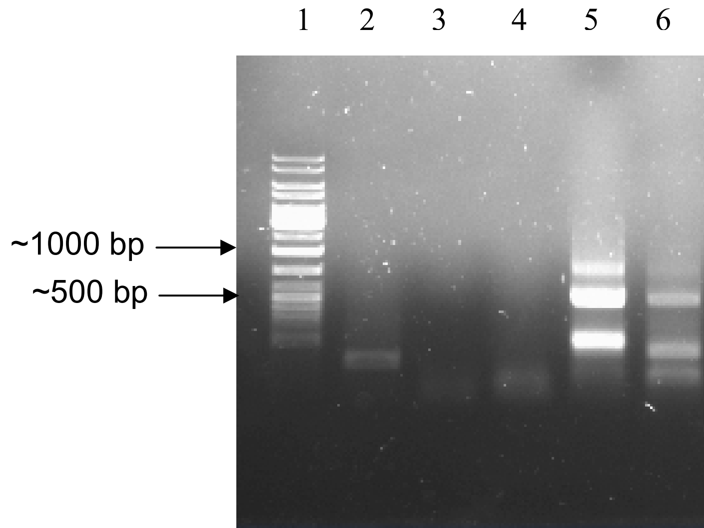

Figure 4.2. Gel electrophoresis of the RT-PCR product using the NP primer set run on a 1% agarose gel. Lane 1) O'GeneRuler^{TM} DNA Ladder Mix, ready-to-use, Lane 2) Positive RNA control, Lane 3) Negative control, Lane 4) Positive standard ND virus strain control, Lane 5) D1446/95, Lane 6) 834/05.

The RT-PCR products of the virus samples when amplified using primer set P resulted in a single highly intense band of similar fragment size and additionally with other faint bands seen in lane 5 (Figure 4.3).

Once again, the primer set P also gave a different amplification result with the positive standard ND virus strain sample as compared with both of the virus samples: D1446/95 and 834/05.

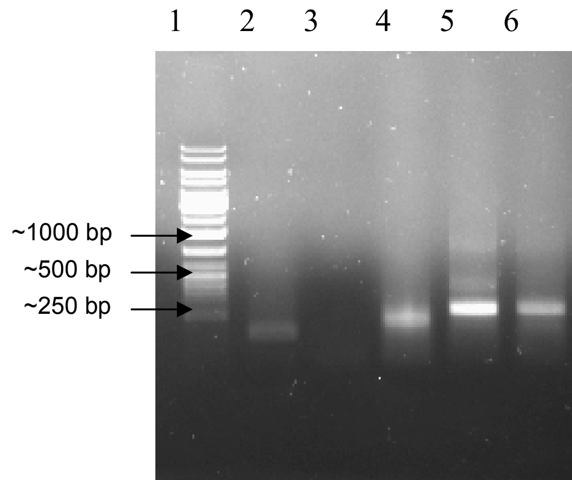

Figure 4.3. Gel electrophoresis of the RT-PCR product using the P primer set run on a 1% agarose gel. Lane 1) O'GeneRuler^{TM}DNA Ladder Mix, ready-to-use, Lane 2) Positive RNA control, Lane 3) Negative control, Lane 4) Positive standard ND virus strain control, Lane 5) D1446/95, Lane 6) 834/05.

4.4. Discussion

The nested RT-PCR resulted in amplification of the positive standard ND virus strain NDV control, which resulted in a fragment of approximately 400 bp and this is the expected fragment size for the F gene. This indicated that the nested RT-PCR was functional and therefore, it can be implied that samples D1446/95 and 834/05 are not the standard ND virus strain as this primer set did not show any bands with these strains. Alternatively, the lack of amplification of the F gene may be due to these samples having significant differences in this region that did not allow for primer binding. Although such an occurrence has not been previously reported for NDV, environmental pressures may lead to an adaptation of the virus F protein to ensure its survival. Hence, further investigation into the molecular characterisation of these samples is required to confirm or refute such a possibility.

The likelihood of D1446/95 and 834/05 not being NDV led to the investigation of these samples being NDV of other poultry species, specifically Goose paramyxovirus None of the primer sets for Goose paramyxovirus resulted in the amplification of the correct fragment sizes as expected according to their alignments on ClustalW, but there were several bands that were both clearly and faintly visible. The NP primer set yielded multiple bands in both D1446/95 and 834/05. None of the bands were of the expected size. However, the size ranges of the multiple bands found in the sample products ranged between 100 – 1000 bp thus separating them from being primer-dimers. This was also seen in the gel results of primer set P that showed multiple band fragments.

What is apparent from the results of the RT-PCR is that none of the primer sets were specific enough for the samples or the programs for the RT-PCR reactions were not optimal. Even the barely visible, faint bands observed for virus sample 834/05 of about 1000 bp using the NP primer set (Figure 4.2. lane 6) and for virus sample D1446/95 of about 1000 bp and again at <500 bp using the P primer set (Figure 4.3. lane 5) demonstrated the lack of optimal annealing between the sample template and the various primers. Additionally, though the results were not the expected sizes using the Goose paramyxovirus primers they could be an indication that samples D1446/95 and 834/05 may be an alternative paramyxovirus to either classical or Goose paramyxovirus Primer set P was the only set of primers that annealed with the positive control standard ND virus strain template. This may be an indication of similarities in the gene sequence of goose and standard ND virus strains, which has also been reported by Xu and colleagues (2008). The failure of amplification of the sample templates D1446/95 and 834/05 by the F, La, Lb, Lc and HN primer sets as well as that of the positive control standard NDV strain suggests, once again, that either not all of the primer sets were able to anneal to a sample template due to differences in the genome or that there was insufficient optimization of the RT-PCR program.

The RT-PCR products from the reactions using the P primer sets show bands of differing size fragments between the two samples and the positive control. A similar fragment of about 250 bp in length was observed in the two samples while the positive control yielded a fragment <250 bp in size. However, this similarity between the sizes of the two samples may suggest that although they are not similar to standard ND virus strain NDV, there may be certain gene similarities to standard ND virus strain that can be found in the samples. It is entirely possible that the two virus samples are indeed a variation of standard ND virus strain branching off into another host species as in the case of the Goose paramyxovirus that was found to have correlations with avian paramyxovirus. Perhaps it served as an adaptation to pressurised change according to environmental changes that threaten to deter the reproduction of the virus itself or worse, a way of shutting off the production and proliferation of the virus completely. A further reason for adapting to another host may simply be the availability of another host species in the immediate environment of the virus.

These scenarios are possible if the environment of the virus samples was such that different species of animals were kept in close proximity for an extended period of time. If such an environment is present, for example the growing of chickens, ducks, geese, pigs etc. in the same holding area for long periods of time as found on animal farms it could be the model system for such a mutation or adaptation to occur. This kind of genetic movement has been observed in the SARS (Severe Acute Respiratory Syndrome) crisis that occurred beginning in 2002 (Kamps – Hoffmann SARS Reference, *http://www.sarsreference.com/sarsreference.pdf*). Thus, if this situation was present at the source of the samples over a long period of time it is entirely possible that the virus samples adapted themselves to different hosts. In this way they could change their viral genome. Such a change, if present, would explain the difference between the expected amplification profiles of the virus samples and that of the positive NDV sample.

A probable development to the differences in the expected amplification profiles of the virus samples and that of the positive NDV sample may be differences of NDV that have been introduced from other countries and continents where they originate from, into South Africa. There is an indication of movement of ND virus into South Africa as was deduced from the study by Herczeg and colleagues (1999). During the period 1990 and 1995 there were found to be several strains of NDV that were isolated in South Africa and Mozambique as well as in Bulgaria and Turkey during 1995 to 1997. These different strains were identified using restriction enzyme and partial sequence analysis based on the F protein gene. Most of the virus isolates found in the southern Africa region, Bulgaria and Turkey were grouped into a novel group VIIb. This group VIIb was part of another genetic cluster VII. The cluster VII included NDV strains from the Far East and certain western European countries that had VIIa sub-clusters. There were also another cluster of genetically related NDV strains in South Africa that comprised the genotype VIII. In the study it was found that the genotype VIII NDV were indigenous and endemic to South Africa, while the VIIb group NDV were introduced into South Africa from outside of the country (Herczeg *et al.*, 1999). In this same manner, it is possible that the differences observed in D1446/95 and 834/05 to classical NDV may be attributed to a possible movement of genetic divergence from outside the country into South Africa.

In lieu of the fact, however, that the primers used in the RT-PCRs were designed from a goose paramxyovirus ZJ1, it is expected that the primer sets would not anneal to the positive NDV control template as effectively. This can be seen from the resulting gels, where there are several more DNA bands that are visible in the lanes for D1446/95 and 834/05 than there are for the positive NDV control. This speculatively seems to point in the direction of either the primers being inappropriate for the positive NDV control, that the RT-PCR reaction and program were not optimized for each set of primers or that the unknown virus samples, D1446/95 and 834/05, are perhaps a different Goose paramyxovirus or even a different NDV strain that infects other species of poultry besides goose or chicken.

The expected band sizes were not found in any of the RT-PCR products. However, there were bands that were moderately-to-very intense indicating amplification that had led to high copies of the same fragment size. It is possible that the genomes of the virus samples have several regions that are complementary to the respective primers enabling the amplification of multiple fragments. This could explain the resulting multiple bands observed on a gel that was from a reaction using a single primer set.

Although the positive standard ND virus strain has been previously characterised by Boucher (2006), it has not been amplified using the primer sets for goose NDV prior to this study and therefore an expected amplification profile has not been established. The difference between the amplification of the positive control NDV strain, or a closely related paramyxovirus, and that of the two virus samples does not necessarily exclude the virus samples from being NDV or a closely related paramyxovirus. It is possible that the positive control NDV strain used in this project may not give the same amplification profile as the expected fragment sizes as seen in the alignment results.

The differences between strains of the same virus family may result, although usually very unlikely, in extremely different fragment sizes when amplified using the same primer set. The nucleoprotein, which is the protein that the NP primer set targets, is a region that is in all NDV samples. However, this region is not completely conserved. There may be a few differences in the nucleotides, resulting in varying, perhaps without much difference, functional structural proteins for different strains of NDV. This is also possible in the case of the phosphoprotein which is targeted by the P primer set. Thus, the differences may lie in the nucleotide difference found in different NDV strains. Even though this is possible, it should also be maintained that genomic differences between two strains of the same virus family are usually found to have more similarities than differences. Thus, it is more likely that the differences found in amplification profiles are not very different from each other. In order to clarify this point, the genome of the different viruses should be sequenced, but this falls outside the scope of this project.

Chapter 5. Discussion and Conclusion

The usual diagnositic steps which are carried out on suspected NDV infections include the isolation of the virus from affected birds, either in embryonated eggs or in cell culture, the identification of the virus as NDV by performing serological tests and thereafter establishing if the isolated virus is a virulent virus or a live vaccine strain or avirulent virus.

Conventional techniques of diagnosis have been sufficient for the managing of ND in most cases. However, due to the need of SPF eggs and chickens from a single source flock, the diagnosis may be slow. Firstly this may be due to the inconstant availability and supply of the eggs. Furthermore, the determination of the pathogenicity index for the virus also takes several days, thus further slowing down the diagnostic process. There is also a concern as to some cases where the NDV may not show pathogenicity for chickens and eggs (Collins *et al.*, 1994). The identification of the virus as NDV together with the calculated pathogenicity may not be able to pinpoint the source of the virus nor its spread. In addition, the diagnosis of viruses can be simplified by using molecular diagnostic processes. Since molecular techniques focus on the genetic content, it is possible to identify viruses by using such techniques as PCR or RT-PCR, cloning and sequencing to determine the identity.

Poultry viruses cost the local and international poultry markets substantial amounts of money during disease outbreaks. Of these poultry viruses NDV is still known for its constant presence and difficulty to achieve complete eradication status in most countries where the virus is known to occur. This is also true for a number of other known poultry viruses including AIV, IBV, EDSV, LTV and IBDV.

With this in mind, the poultry market is appropriately concerned with the movement, emergence and monitoring of the abovementioned poultry viruses as well as novel viruses that may infect and affect poultry. The OVI, Poultry Section of the Faculty of

Veterinary Science at Onderstepoort and various regional government laboratories plan an important role in the diagnosis of various poultry pathogens. The Veterinary Biotechnology Research Group of UFS is undertaking various research projects on pathogens of avian species. One of these projects involved a study on the oncolytic activity found in NDV as possible treatment for cancer in humans (Boucher, 2006). Thus, this laboratory approached the OVI for several NDV samples in order to proceed with this study that was performed by Boucher (2006).

Out of the NDV samples that were sent to Boucher (2006), there were two virus samples, D1446/95 and 834/05, that did not appear to be NDV. This finding was based on the HA tests that revealed that the two virus samples could not haemagglutinate RBCs and also according to the RT-PCR that was unsuccessful at amplifying the two virus samples (Boucher, 2006). These samples were not used in the study that Boucher (2006) undertook as the main aim of that study incorporated only NDV and NDV-related oncolytic activity. These samples were then used for this project in the hope of identifying these “unknown” virus samples.

The aims of this study was thus to determine whether or not the virus samples 834/05 and D1446/95 are NDV, and if not, to further try to identify these viruses. using mainly classical methods and simple molecular methods. Some of the classical methods employed in order to reveal the nature of the viruses were virus cultivation in SPF embryonated eggs, production of primary cell cultures and virus cultivation in these cell cultures and observations of CPEs, HA tests, TEM ultrastructure observation and MDT relative pathogenicity examination. The simple molecular method used in this case was the RT-PCR using several different primer sets specific for NDV as well as primer sets based on the Goose paramyxovirus strain ZJ 1 genome sequence stored on GenBank (Xu *et al.*, 2008).

The “unknown” virus samples D1446/95 and 834/05 were found to be able to propagate and be cultivated successfully in embryonated SPF eggs via the allantoic cavity route. The techniques for the establishment of primary chicken embryo fibroblast cell cultures

were successfully established and the two virus samples also showed CPEs after being inoculated onto these cell cultures. When these viruses were compared to NDV regarding their cultivation characteristics in embryonated eggs and on CEF cell cultures, both virus samples shared the same ability with NDV to be cultivated in these culture systems.

In order to work with a virus sample for the duration of a project, it is necessary to have sufficient quantities of the virus sample available. Thus, the virus samples were inoculated into SPF eggs via the allantoic cavity route in order to have ample virus sample. The samples were successfully cultivated in SPF eggs and were harvested accordingly. The harvested virus samples were stored at -70°C in sterile falcon tubes and also in Vaccutainer™ tubes until required for further studies.

The cultivation of viruses in cell cultures, although being an appropriate method, was in this case used more for the observation of CPEs. The problems associated with the cultivation of monolayers of primary cell cultures, for example problems with bacterial contamination and the technical complexity and labour intensive nature of primary cell culture, made this technique difficult and the cultivation of the virus in embryonated SPF eggs was preferred.

The separate inoculation of CEF cell cultures with the virus samples was for the observation of CPE characteristics of the two virus samples. Both of the virus samples were found to induce CPE in CEF cells. Classical NDV is also known to cause CPE on cell cultures. However, it is not possible to diagnose a virus using the observed CPE on cell cultures. This is simply because CPE are not diagnostic and can change from virus to virus according to the type of cell culture as well as other parameters. However, there were some similarities in the CPE see for these two virus samples when compared to the reported CPE for NDV in CEF cells cultures.

There was conflicting and inconsistent results obtained when samples 834/05 and D1446/95 were tested in the HA test. The first time that the HA test was performed,

both of these virus samples did not agglutinate chicken red blood cells. These results confirmed the results obtained by Boucher (2006) who also reported on a lack of HA activity with these two virus samples. This is, in fact, one of the reasons that the virus samples were regarded as not being NDV. When the HA tests were repeated in 2007, an unusual haemagglutination reaction was recorded for strain D1446/95 (Figure 3.1) and a normal haemagglutination profile was recorded for 834/05. However, upon subsequent attempts to perform the HA test no haemagglutination could be recorded for either of these virus samples. The inconsistent and non-haemagglutinating nature of D1446/95 and 834/05 do not necessarily mean that they do not haemagglutinate at all. It merely means that they cannot agglutinate the chicken RBC used in these experiments. Haemagglutination was recorded for a period during this project, using chicken RBC's collected from the ARC poultry facility at Glen. Individual variation in the ability of RBC from individuals from the same species of birds have previously been reported on for *Beak and feather Disease virus* (Sanada & Sanada, 2000) were it was observed that the RBCs of the cockatoos were able to be used in HA assays for PBFD. However, there were differences between the RBCs of the different individuals of cockatoos belonging to the same species and RBC collected from some individuals did not agglutinate with BFDV.

Results on HA tests performed after the 31st March 2007 once again gave negative haemagglutination for both virus samples. This also points in the direction of the two virus samples possibly being a variant NDV or being related to the Goose paramyxovirus (Xu *et al.,* 2008) which was isolated in China.

It can be concluded from these results that the HA test is inconsistent with both of these virus samples. This is in clear contradiction with classical NDV virus which consistently shows haemagglutination abilities. A possible explanation of these variable results could be found in the fact that the same chickens were not used for the collection of blood for all of the HA tests performed.

Chicken blood was collected from Glen on a regular basis and birds which were used for the collection of blood were randomly selected from a larger population of birds. In other words, RBC from different individuals were used for the HA test.

From the TEM experimentation, it can be seen that D1446/95 may be structurally similar to NDV (Figure 3.3. a & b). Based on the TEM data, it could be suggested that D1446/95 could belong to the same virus family as NDV, based on the structural similarities between the viruses. TEM electronmicrographs clearly show that the virus D1446/95 has an enveloped ultrastructure and is not a naked virus. This characteristic will help to determine the nature of this virus sample in the future.

Using the MDT standards of classical NDV, the sample viruses 834/05 and D1446/95 were found to be highly virulent and moderately virulent respectively. Since the virus samples were not proven to be NDV the MDT standard used to categorize NDV could not formally be used for the virus samples. However, it was perhaps not too presumptuous to compare the virus samples to the NDV standard. This is arguably because both samples showed significant virulence when it came to killing the SPF eggs that they were inoculated into during virus cultivation. Thus the insight that the NDV MDT standard gave was perhaps relevant in this case. Nevertheless, the point that was made using the MDT results of the virus samples was to point out the virulence of the two virus samples, 834/05 and D1446/95, where it was demonstrated that both of these viruses have the capability of killing the embryos in the eggs in a relatively short time.

Thus far, it appears as though both of the unknown virus samples, D1446/95 and 834/05, may be related to classical NDV viruses that infect poultry. The possibility of this being the case is increased by the results found for the RT-PCR using primers based on the Goose paramyxovirus strain ZJ1. The designs of these primers were based on work that has been done on a Goose paramyxovirus found in China (Xu *et al.*, 2008). It could be seen from the preliminary molecular work that there is positive RT PCR results obtained with two of the primer sets based on the ZJ1 strain of Goose

paramyxovirus. Other primer sets for the ZJ1 strain did not react with strains D1446/95 and 834/05. These results indicate that the viruses may be paramyxoviruses other than strain ZJ1 Goose paramyxovirus or classical NDV. It is possible that they have similarities that are found within the paramyxovirus as they were amplified using some of the primer sets from the Goose paramyxovirus strain ZJ1 (Xu *et al.*, 2008).

It can be concluded from this work that strains D1446/95 and 834/05 are not classical NDV virus, based on the inconsistent HA activity and the negative RT-PCR results obtained with primer sets specific for NDV. It can further be concluded that these viruses show high to moderate levels of virulence to embryonated eggs and their virulence levels could be comparable to NDV. Based on the morphology of the virus, as seen under the TEM, there appears to be similarities between the ultrastructure of NDV and strain D1446/95, which indicates that strain D1446/95 could be a related paramyxovirus virus. It was demonstrated that both the D1446/95 and 834/05 strains resulted in positive RT-PCR results with some of the primer sets which had been designed from the Goose paramyxovirus ZJ1 strain. It was also seen that not all of the Goose paramyxovirus ZJ1 strain showed positive results with these two strains. These results indicate that these two unknown viruses are paramyxoviruses, probably more closely related to the Goose paramyxovirus strain ZJ1 then to classical NDV.

5.1. Future Work

Additional work which could be done on this project is a more in depth RT-PCR study of the viruses using the primer set designed from the Goose paramyxovirus ZJ1 strain. Optimization of the RT-PCR reactions could result in more fragments would be amplified. If fragments of expected size are found, these could be cut out and purified and further amplified in order to get sufficient nucleic acid to be sequenced. However, even fragments of unexpected sizes can be purified and sequenced. Thereafter the sequences can be BLASTed in order to determine what they are most related to. Hopefully this will give a better idea as to what the viruses are. If they are indeed NDV that have adapted or mutated along the way then they will display similar genes as

found in NDV. However, if they are not NDV nor match up with other viral sequences, then the samples will have to be further amplified using the results of the sequences to design new primers. Through a form of shotgun cloning is possible to hopefully unravel the identity of the sample viruses at the molecular level.

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Summary

The poultry industry suffers severely every year due to bacterial and viral infections. Hence, great effort has been incorporated into isolating and identifying the different microorganisms that afflict poultry with their disease. The efficiency of the vaccines provided against such infections is only as good as the research of the responsible bacterium or virus.

There are cases, however, when “unknown” viruses create havoc within industry. This is mainly due to the a) virulent nature of the virus, b) frequency of infection and c) lack of knowledge about the virus. The unknown virus may be a truly novel virus or an existing virus that has mutated whilst replicating. It is thus important to refine the procedure of isolation and identification of the poultry viruses in order for outbreaks of an unknown virus to be dealt with quickly and efficiently.

In this study, the isolation and passaging of two unknown virus samples as Newcastle disease virus (NDV) but failed to react with ND-specific PCR primers and did not haemagglutinate red blood cells were investigated These samples were two of a group of viruses submitted as NDV to the Veterinary Biotechnology Laboratory of the University of the Free State by the Onderstepoort Veterinary Institute.

The aim of this study was to investigate into the identity of the two unknown virus samples, D1446/95 and 834/05, using various methods of identification.

Procedures performed on this virus include:(presumed to be Newcastle propagation in embryonated SPF eggs and primary chicken embryo fibroblast (CEF) cell cultures; Disease Virus), purification of virus samples for ultrastructure identification (ultrastulu studies as by transmission electron microscopy (TEM) and negative staining;) serology work as per haemagglutination and mean death time (MDT) studies as well as restriction transcriptase polymerase chain reaction (RT-PCR) work.

The virus samples were cultivated in embryonated SPF eggs via the allantoic cavity route. The embryo morality was observed and recorded, and the allantoic fluid containing the virus was successfully harvested.

CEF primary cell cultures that were prepared in-house were inoculated with the individual samples separately. The virus samples showed cytopathic effects (CPE) after inoculation and incubation of the cell cultures. These CPE indicated that the virus samples infected the CEF monolayer and could be grown on CEF cell cultures as well. However, the cultivation of the virus samples in SPF eggs was preferred due to the difficulty that the primary cell cultures presented in a laboratory that is not directed at cell culture production and use.

Through haemagglutination (HA) tests the virus samples were found to be unable to haemagglutinate chicken red blood cells (RBCs). It is speculated through this result that the virus samples are not classical NDV. However, it is entirely possible that the viruses agglutinate the RBCs of other mammalian or avian species. It is possible that these viruses may be evolved from classical NDV into, for example, Goose paramyxovirus

The ultrastructure of the virus sample D1446/95 as observed through transmission electron microscopy, found an enveloped virus that measures about 100 nm to 150 nm in diameter. Although the ultrastructure of the virus does not conclusively identify the virus, it helps in preliminarily grouping it to other known viruses with similar ultrastructure and eliminating it from groups of viruses that have obvious differences in ultrastructure.

Investigation into the virulence of the virus samples using the mean death time (MDT) indicated that the virus sample D1446/95 was moderately virulent with an MDT of 71,75 hours and the sample 834/05 was highly virulent with an MDT of 60 hours. As NDV ranges from being highly virulent (velogenic strains) to having low virulent (lentogenic strains) the MDT results were compared to the standard of the MDT of NDV. The standard of MDT for NDV states that velogenic strains take less than 60 hours to kill;

mesogenic strains kill between 60 to 90 hours and lentogenic strains take longer than 90 hours to kill the SPF embryos.

After the extraction of RNA from the harvested virus sample using TRIzol reagent, the viral RNA of D1446/95 and 834/05 were amplified using RT-PCR, primer sets designed based on Goose paramyxovirus ZJ1 and the Access RT-PCR System (Promega). The RT-PCR products were run and observed on 1% gels using gel electrophoresis. The RT-PCR products of the two virus samples resulted in multiple bands for the two virus samples that indicated that they are probably not classical NDV, but may possibly be NDV of other species perhaps even of goose NDV. However, this is still to be investigated further.

Thus, through this study it was found that the unidentified virus samples D1446/95 and 834/05 are most likely not classical NDV, but rather could be a variation of classical NDV that may be branching off into paramyxovirus of other poultry or mammalian species as was found in the study of Goose paramyxovirus.

Key words: Newcastle disease virus, “Unknown” poultry virus, Virus cultivation, Haemagglutination assay, Transmission electron microscopy, Mean Death Time, RT-PCR.

Opsomming

Die pluimvee bedryf lei elke jaar groot verliese weens bakteriese en virale infeksies. As gevolg van hierdie verliese word navorsing toegespits op die isolasie en identifikasie van verskillende mikro-organismes wat pluimvee met siektes affekteer. Die doeltreffendheid van die entstowwe teen hierdie siektes is slegs so doeltreffend soos die navorsing wat gedoen word op die siekte-veroorsakende bakterieë of virusse.

Daar is wel gevalle waar onbekende viruses in die industrie verwoesting saai, dit is hoofsaaklik toe te skryf aan a) die virulente aard van die virus, b) gereeldheid van infeksie en c) die gebrek aan kennis oor die virus. Die onbekende virus kan dalk 'n "nuwe" virus wees of 'n reeds bestaande virus wat gemuteer het tydens replikasie. Dus is dit belangrik om die prosedure van isolasie en identifikasie van pluimvee virusse te verfyn sodat uitbreke van onbekende virus infeksies vinnig en doeltreffend opgelos kan word.

Vir hierdie studie is twee virusse ontvang wat as Newcastle disease virus (NDV) geïdentifiseer is, maar hierdie virusse het nie net ND-spesifieke PKR priemstukke vermeerder nie en het ook nie rooibloedselle gehemagglutineer nie. Hierdie monsters was twee van 'n groep virusse ontvang deur die Veeartsnykundige Biotegnologie laboratorium aan die Universiteit van die Vrystaat vanaf die Onderstepoort Veeartsnykundige Instituut.

Die doel van die studie was om ondersoek in te stel na die identiteit van die twee onbekende virusmonsters, D1446/95 en 834/05, met behulp van verskeie identifikasie metodes asook die isolasie en kweking van hierdie onbekende virusse.

Die prosedures wat op hierdie virusse uitgevoer is (aangeneem om NDV te wees) het ingesluit: kweking in embryo bevattende spesifieke patogeen vrye (SPV) eiers sowel as in primêre hoender embrio fibroblaste (HEF) selkulture, suiwering van die virus vir ultrastrukturele identifikasie met die gebruik van transmissie elektronmikroskopie (TEM),

serologiese studies wat HA en gemiddelde dodingstyd (GDT) ingesluit het. Laastens was tru-transkriptase (TT)-PKR uitgevoer op die isolate.

Die virale monsters is gekweek in ge-embrioneerde SPV eiers via die allantoïese roete. Embrio mortaliteit is waargeneem en die virus-bevattende allantoïese vloeistof is geoes.

Hoender embrio fibroblaste primêre selkulture is voorberei en geïnnokuleer met die individuele virus monsters. Die virus monsters het sitopatiese effekte (SPE) post-innokulasie en inkubasie van selkulture getoon. Hierdie SPE toon dat die virus die HEF monolaag geïnfekteer het en dus op die HEF selkulture kon groei. Dit was verkies om die virusse te kweek in SPV eiers. Weens die feit dat die laboratorium nie ingerig is vir die gebruik en produksie van selkulture nie.

Met gebruik van die HA toetse is bevind dat die virus monsters nie die hoender rooibloedselle kon agglutineer nie, dus het die moontlikheid ontstaan dat die virus monsters dalk nie klassieke NDV was nie, alhoewel dit wel moontlik is dat die virusse die rooibloedselle van ander soogdier of pluimvee spesies kon agglutineer. Dit is ook moontlik dat hierdie virusse gemuteer het vanaf klassieke NDV in byvoorbeeld Goose paramyxovirus.

Die ultrastruktuur van virusmonster D1446/95 was waargeneem met TEM. Die virus was omhul met ’n deursnee van ongeveer 100 – 150 nm. Alhoewel die ultrastruktuur van die virus nie sonder twyfel die virus kan identifiseer nie, kan die virus wel gegroepeer word met ander bekende virusse wat soortgelyke ultrastrukture het, wat kan bydra tot identifikasie.

Ondersoek in die virulensie van die virus monsters met gebruik van GDT het getoon dat die virus monster D1446/95 matig virulent was met ’n GDT van 71,75 ure en virus monster 834/05 was hoogs virulent met ’n GDT van 60 ure. Newcastle disease virus strek van hoogs virulent (velogeniese stamme) na lae virulensie (lentogeniese stamme). Die standaard GDT van NDV stipuleer dat velogeniese stamme minder as 60 ure neem

om dood te veroorsaak, mesogeniese stamme tussen 60 en 90 ure en lentogeniese stamme meer as 90 ure om die SPV embrios se dood te veroorsaak.

Na die ekstraksie van ribonukleïensuur (RNS) vanaf die geoesde virusmonster met behulp van TRIzol reagens, is die virale RNS van D1446/95 en 834/05 geamplifiseer met gebruik van TT-PKR, die priemstukke is gebaseer op die Goose paramyxovirus ZJ1 isolaat en die Access RT-PCR sisteem (Promega). Die TT-PKR produkte is gevisualiseer op ’n 1% agarose gel met behulp van ultra violet illuminasie. Die TT-PKR produkte van die twee virusmonsters het veelvuldige bandjies vir beide monsters getoon wat daarop gewys het dat hierdie monsters waarskynlik nie NDV is nie, maar moontlik NDV van ’n ander spesie of moontlik gans NDV. Hierdie resultate sal wel verder ondersoek moet word.

Dus, met hierdie studie is bevind dat die ongeïdentifiseerde virusmonsters, D1446/95 en 834/05 waarskynlik nie klassieke NDV is nie, maar eerder ’n variasie van klassieke NDV wat moontlik kan vertak in paramyxovirus of ander pluimvee of soogdier spesies soos gevind is in die studie gedoen op Goose paramyxovirus.