Development of a real-time PCR assay to detect the fusion gene of the D26 strain of a commercial avian avulavirus 1

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PLAGIARISM DECLARATION

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DEDICATION

I dedicate this work to my parents (Leshata Thomas Phala and my late mom Lebogang Agnes Phala) for instilling in me a love and reverence for education. Thank you for your tireless efforts.

ABSTRACT

Newcastle disease (ND), caused by avian avulavirus 1 (AA1), an enveloped, negative sense, single stranded RNA virus belonging to the *Paramyxoviridae* family. ND is found world-wide and leads to severe economic losses from mortality and condemnation of carcasses. Virulent ND causes clinical signs such as respiratory distress, central nervous signs, drop in egg production, weakness, gastro-intestinal symptoms and death. The disease is listed by the World Organisation for Animal Health (OIE) and outbreaks require reporting to the OIE. The OIE requires a definitive diagnosis of virulent AA1 to enable effective control of an outbreak by strict control measures and trade restrictions.

Currently the real-time reverse transcription polymerase chain reaction (RRT-PCR) assay used to diagnose ND does not differentiate between field and vaccine strain. The aim of this study was to develop and optimise a real time RT-PCR assay that detects chickens vaccinated with Vectormune® HVT NDV vaccine based on the F gene of the D26 strain. NDV F gene sequences were downloaded from Genbank® and aligned. A region unique to the D26 strain, between nucleotides 69 to 131 (using accession number M24692 for numbering) was identified and a TaqMan® MGB™ assay was developed. Primer and probe concentrations were optimised at 200 nM.

Nucleic acid was purified using a MagMax™ Pathogen RNA/DNA extraction kit and a MagMax™ Express Magnetic Particle Processor (ThermoFisher Scientific). TaqMan Fast Advanced Master Mix PCR reagents were used to amplify the AA1 F gene with one StepOnePlus Real-time PCR system.

The PCR efficiency was calculated to be 81.8% with 0.9942 coefficient correlation (R^2). The 95% limit of detection was $10^{-1.31}$ plaque forming units per reaction. The assay was specific and did not detect any other AA1 isolates tested.

Twenty-four spleen impression smear field samples from chickens (12 Vectormune® HVT NDV vaccine samples and 12 vaccinated with ND virus conventional vaccine) preserved on Whatman® FTA cards, were collected between day 21 and 28 post vaccination. The assay detected only the D26 vaccine strain and was negative when tested on other field samples.

The developed real time PCR was sensitive, reliable and repeatable and will also be able to produce results rapidly as compared to other conventional methods.

Keywords: Real time polymerase chain reaction, cDNA, Vectormune® HVT NDV vaccine, F gene, D26 strain

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LIST OF ABBREVIATIONS

AA1	Avian avulavirus 1
AI	Avian influenza
E	Efficiency
EID_{50}	Embryo infective dose (50%)
EU	European Union
F	Fusion gene
F_0	Fusion protein precursor
HA	Hemagglutination
HI	Haemagglutinin inhibition test
HN_{0616}	Haemagglutinin protein precursor
HN	Haemagglutinin neuramidase
HVT	Herpes virus of turkey (meleagrid alphaherpesvirus 1)
ICPI	Intracerebral pathogenicity index
Kb	Kilobase
kDA	Kilodaltons
L	Large polymerase
M	Matrix gene
MD	Marek's disease
mRNA	Messenger ribonucleic acid
µl	Microliters
µM	Micrometre
nM	Nanometre
ND	Newcastle disease
NP	Nucleoprotein
OIE	World Organisation for Animal Health
ORF	Open reading frame
P	Phosphoprotein gene
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
vAA1	Virulent avulavirus 1

CHAPTER
1. LITERATURE REVIEW

1.1. History of Newcastle disease

Newcastle disease (ND) was previously called Ranihket disease named after a place of emergence in India. Other names used to describe the disease include Doyle's disease, tetelo disease, atypische geflugelpest, pseudo-fowl pest, pseudo Vogel-pest, pseudo poultry plague, Korean fowl plaque, avian pest, avian distemper and avian pneumoencephalitis (Heskett, 2003). The first reported cases of the disease in poultry were from Java, Indonesia in 1926 (Berinstein *et al.*, 2001; Ganar *et al.*, 2014; Seal, King & Sellers, 2000). In 1927, Doyle identified that ND is caused by a filterable virus that was different from fowl plaque and was later named Newcastle disease virus (NDV) (Ganar *et al.*, 2014). Severe cases were reported from 1926-1940 near the seaports of the Indian Ocean. The disease has a worldwide distribution, probably spread through the trade of live infected birds (Fringe *et al.*, 2012), although in some countries like Australia, only low virulent viruses for chicken have been reported (Aldous & Alexander, 2001; Berinstein *et al.*, 2001; Fringe *et al.*, 2012). AA1 occurs on at least six of the seven continents of the world.

Virulent AA1 (vAA1) strains are classified as selected agents in the United State of America. These strains are not present in United States poultry and therefore the disease caused by the virulent strain is commonly referred to as exotic ND (Miller *et al.*, 2009). Southern California experienced an outbreak of viscerotropic velogenic ND, due to illegal importation of psittacine birds in the early 1970s (Seal, King & Sellers, 2000). Other outbreaks of vAA1 isolates were reported from cormorants and gulls in Minnesota, Massachusetts, Maine, New Hampshire and Maryland.

Outbreaks of vAA1 continue to be a risk with clinical outbreaks confirmed in 2008 from the Dominican Republic, Belize, Peru, Finland, Germany and Japan and it is also possible that other outbreaks were not reported from countries throughout Africa and Asia where vAA1 is endemic (Chaka *et al.*, 2013; Miller, Decanini & Afonso, 2010).

After the initial 1930 vAA1 outbreak in Australia, the follow up outbreak of vAA1 was recorded in 1998 at Dean Park and New South Wales. Thereafter it spread rapidly at Peats Ridge, Mango Grove Mountain between 1998 and 2000 and again at Dean Park in 1999. In Orchard Hills, Llandilo, Marsden Park it occurred between January-February in 2000, in Tamworth and Rossmore in February 2000 and finally in Meredith, Victoria in 2002. Due to the presence of the vAA1 disease in most areas, control measures such as quarantine and slaughter policy were implemented to prevent the spread of the disease. The disease was only contained when a vaccination programme using the V4 vaccine strain was introduced (Aldous & Alexander,

2001; Kattenbelt, Stevens & Gould, 2006). The presence of an avirulent 'progenitor-like virus' was confirmed in all the areas where the outbreaks happened, except the first outbreaks at Dean Park and Meredith. Phylogenetic analysis of isolates was done and it was found that the progenitor virus and the progenitor-like viruses were endemic Australian viruses and that they were the reservoir from which the vAA1 had originated (Kattenbelt, Stevens & Gould, 2006). Before the outbreak, Australia had been declared free from vAA1 since the 1930s.

AA1 first appeared in China in 1946 and became endemic regionally. Implementation of an intensive vaccination programme in commercial and village poultry farming has reduced the number of ND episodes in the past three decades (Cai *et al.*, 2011). Despite the intense vaccination programme, there has been an epizootic occurrence of velogenic ND cases in vaccinated chicken flocks since the late 1990s in Southern China. Vaccination programme failure was suspected to be a cause of the sporadic virulent form of the disease. Factors such as incompatible circulating field and vaccine strains, inappropriate vaccine procedure and formation of novel genotypes under the condition of high immune pressure (Cai *et al.*, 2011).

In Kenya, the first report of the case was through the port of Mombasa during 1934 (Fringe *et al.*, 2012). The disease was diagnosed clinically in 1938 in Mozambique, although the virus was only isolated in 1946. The disease continues to cause major losses in rural poultry, in which chicken meat is still a main source of protein and plays an important part in poverty alleviation. The first reported case in Ethiopia was in 1971 from a small poultry farm in Asmara and it has since been considered the most devastating problem of chickens (Bwala, 2009; Chaka *et al.*, 2013).

In South Africa, the disease was first introduced in 1944 through the port of Durban and since then ND outbreaks occurred sporadically in South Africa until June 1993 when neuro/respirotropic AA1 was isolated near Pretoria (Abolnik *et al.*, 2004; Bwala *et al.*, 2009). The disease caused severe losses in all types of poultry and had spread throughout South Africa within six months. It was brought under control by vaccination and biosecurity measures. Another outbreak of ND was limited to village chickens in Kwazulu–Natal province and ended in 2000. It was speculated that village poultry were the source of AA1 (Abolnik *et al*., 2004; Bwala, 2009). Another virulent ND outbreak was experienced in North West Province in 2013 and 2014, and was limited to village chickens and chickens that were given to rural communities with the aim of poverty alleviation (personal observation).

1.2. Epidemiology of Newcastle disease

1.2.1. Distribution

Since the discovery of the disease in 1926 in Indonesia and England, isolations of AA1 have been made all over the world (Aldous *et al.,* 2001; Miller, Decanini & Afonso, 2010). The

widespread use of live vaccines has made it difficult to accurately assess the distribution of AA1 in the world (Aldous *et al.,* 2001). Newly isolated strains are being reported continuously from all over the world. Recently, AA1 outbreaks were reported from Vietnam, Indonesia, Malaysia, and Cambodia (Ganar *et al*., 2014). In 2011, an outbreak was reported from Israel in little owls and African penguins and 96 AA1 outbreaks were reported in poultry from Cameroon, Central African Republic, Cote d'Ivoire and Nigeria in 2013 (Ganar *et al.,* 2014). Phylogenetic analysis of AA1 isolated from different parts of the world could not provide a clear picture of how the virus crossed topographical barriers. It was shown that vAA1 isolated from Texas showed identity with the strains from the hot climatic zone, suggesting the prevalence of highly virulent strains in those areas. AA1 strains isolated from Egypt and central Africa showed high genomic identity with virulent strains such as Fontana and Texas GB (Ganar *et al.*, 2014). Occasionally AA1 has been isolated from non-avian species such as pigs and goats (Ganar *et al.*, 2014). The pigeon paramyxovirus 1 strain still represent a potential risk for poultry and has caused major outbreaks in commercial chickens by pigeon variants in 1984 (Barbezange & Jestin, 2002)

1.2.2. Hosts

The disease is known to infect over 250 species of birds, both wild and domestic (Farkas *et al*., 2009). The main reservoir of the AA1 are waterfowl and migratory birds (Rabalski *et al*., 2014). They normally do not show clinical signs with low pathogenic strains, and this allows the spread of the virus to go unnoticed. Turkeys (*Meleagris gallopavo*) are as susceptible to infections as chickens (*Gallus gallus*), but the clinical signs are less severe (Miller, 2008). Ostriches (*Struthio camelus*) are also susceptible to AA1 infection. Domesticated and feral pigeons (*Columba livia*) carry vAA1 and they have infected poultry and vice versa (Miller, 2008). The virus is maintained for a long period in the kidney tissue and has been isolated from cormorants and anhingas (*Anhinga anhinga*) (Miller, 2008).

Human infections are rare and usually exhibit as eye conjunctivitis.

1.2.3. Transmission, spread and maintenance

AA1 is spread horizontally via inhalation or ingestion of respiratory secretions and faecal matter from infected birds (Li *et al*., 2009; Miller, 2008; Sharif *et al*., 2014). The infective dose of AA1 per bird depends on the virus and susceptibility of the host. Overall, the infective dose of AA1 ranges between 10^3 to 10^4 median embryo infectious dose 50 (EID_{50}) (Miller, 2008). The virus can remain in the environment from contaminated tissues and faeces for days. People, equipment, pets, exotic birds, contaminated poultry feed or water, wild birds and insects are considered possible route of exposing poultry to AA1 (Heskett, 2003). There is evidence that the virus can spread through the air (Maraqa, 1996; Miller, 2008) and that the transmission of AA1 is dependent on relative humidity and ambient temperature (Miller,

2008). Wild birds are the reservoir of the virus and have been implicated in transmission of AA1 to poultry. There have been several outbreaks where wild birds were the cause of the disease, for example the case were imported psittacines infected with AA1 caused the California outbreak in 1971 (Miller, 2008). Another vNDV was isolated from turkeys that shared a common water source with cormorants. Insects as a route of transmission of AA1 has been investigated, small amounts of AA1 strain CA02 were isolated from flies collected from two places that kept backyard poultry in the CA02 outbreak (Miller, 2008). Based on these findings it is clear that pest control should be taken into consideration when biosecurity measures are planned for a facility. Vaccinated poultry can shed virus for at least nine days after vaccination (Miller, 2008).

1.3. Clinical Newcastle disease

The severity of the disease varies greatly: from per acute disease with almost 100% mortalities, to subclinical disease with no lesions (Musako & Abolnik, 2012). The severity of the disease depends on the species infected, age, immune status, virus strain and environmental stresses (Al-Habeeb, Mohamed & Sharawi, 2013; Chong, 2012; Farkas *et al*., 2009). The incubation period is usually five to six days, but can vary from two to fifteen and will depend on the species of the host, the virulence of the virus and immune status of the host (Miller, 2008). Most of wild bird populations seem to be carrier of ND without showing any clinical symptoms, although other host species are susceptible (Bwala, 2009). Virulent ND causes high morbidity and mortality. The visible symptoms caused by the disease depend on the predilection of the infecting virus strain for the respiratory, digestive or nervous system (Kim, Suarez & Afonso, 2008). The general clinical signs seen in infected birds are depression, loss of appetite, weakness, conjunctivitis and a drop in egg production. Respiratory signs associated with this disease may include sneezing, gasping for air, nasal discharge, while common intestinal symptoms are greenish and watery diarrhoea (Chong, 2012). Birds can also develop nervous symptoms such as paralysis of wings and legs, twisting of head and neck, or ataxia. Birds might die acutely without showing any clinical signs.

1.3.1. Pathotyping of AA1

The strains of AA1 were originally classified into four pathotypes, based on the severity of clinical disease. These were previously known as Doyle, Beach, Beaudette and Hitchner forms. Currently ND strains are grouped into five pathotypes based on the clinical signs induced in infected chickens. This classification is based on the results of the intracerebral pathogenicity index (ICPI) in day-old chicks (Brown, King & Seal, 1999; Cattoli *et al*., 2011; de Leeuw *et al*., 2005; Samal *et al*., 2011; Singh *et al*., 2005; Rabalski *et al*., 2014; Roy, 2012) (Table 1.1).

Table 1.1. Pathotypes and clinical signs of Newcastle disease in infected chickens, based on the intracerebral pathogenicity index (ICPI).

Pathotype	Clinical signs
Viscerotropic velogenic (Doyle's form) - highest mortality rate and ICPI of > 1.5.	Acute lethal infection characterized by high mortality, usually with haemorrhagic lesions in the intestines of dead birds.
Neurotropic velogenic (Beach's form) - highest mortality rate and ICPI of > 1.5	High mortality following respiratory and neurological signs (head twitch, tremor, opisthosomas), but gut lesions are usually absent.
Mesogenic (Baudette's form) - low mortality rate but moderate signs from respiratory system, ICPI of 1.5 - 0.7.	Low mortality with respiratory infection and neurological signs. Death usually seen in young birds.
Lentogenic (Hitchner's form) - mild respiratory signs with no mortality, ICPI of < 0.7.	Causes mild or inapparent respiratory infection with no mortality.
Asymptomatic - no signs or subclinical enteric infections.	Avirulent infection with the virus replicating primarily in the gut.

In vivo tests are available to determine the virulence and pathotype grouping of AA1 and include ICPI in one day old chicks, mean death time (in hours) in embryonated chicken eggs for minimum lethal dose and intravenous pathogenicity index (IVPI) in six-week-old chickens. These tests may not give similar results due to some inconsistency of the tests (Chong, 2012; Roy, 2012). ICPI is considered the definitive assessment of AA1 virulence by the OIE. The process involves the injection of ten days old chicks with AA1 and daily observation of clinical signs for a period of eight days. The ICPI score ranges from 0.0-2.0, which represent mean score per bird observed over eight days of infection. Zero score represent negative results, a score of 1 is given to sick chicks and if dead the score is 2. An isolate with an ICPI of 0.7 or greater in day-old chicks is classified as vAA1 (Chong, 2012).

1.4. Avian avulavirus 1

Avian avulavirus 1 is a single stranded, non-segmented, negative- sense RNA virus in the genus *Avulavirus*, family *Paramyxoviridae,* order *Mononegavirales*. The size of the viral genome is approximately 15 kb (Gohm, Thur & Hofmann, 2000; de Leeuw *et al*. 2005; Miller, Decanini & Afonso, 2010; Munir *et al*., 2015; Nakaya *et al*., 2001). AA1 has been the best characterized compared to other avian avulaviruses, due to the devastating disease it cause in chickens. Avian avulavirus 2 (AA2) and 3 (AA3) have also been isolated from domestic poultry and have caused disease and economic losses in poultry industries (Chong, 2012; Fringe *et al*., 2012). There is little information known concerning replication and pathogenesis of avian avulavirus 2 to 9 (Kumar *et al*., 2011).

AA1 particles are pleomorphic, differ in diameter from 100 to 500 nm and consist of a host-derived lipid-containing envelope and a helical nucleocapsid complex, when viewed under negative electron microscopy (Chong, 2012).

1.4.1. Genomic organisation

The virus genome codes for six major structural proteins: fusion (F), haemagglutinin neuraminidase (HN), nucleoprotein (NP), phosphoprotein (P), matrix (M) and large protein (L), in the order 3'-NP-P-M-F-HN-L -5'(Al-Habeeb, Mohamed & Sharawi, 2013; Aldous *et al.*, 2001; Huang *et al.*, 2004; Liu *et al.*, 2011; Solomon *et al.*, 2012; Wise *et al.*, 2004) (Figure 1.1). RNA editing of the P gene results in two additional proteins (Zhang *et al.*, 2010). The six structural genes are separated by intergenic regions of different lengths (1 to 47 nucleotides), which could be involved in terminating mRNA transcription from the preceding gene, before initiating transcription of the next gene (Yusoff & Tan, 2015). AA1 contains six structural proteins (NP-P-M-F-HN-L), however avian avulavirus 6, in addition to the six proteins contains a small hydrophobic (SH) protein between F and HN genes (Kumar *et al.*, 2011).
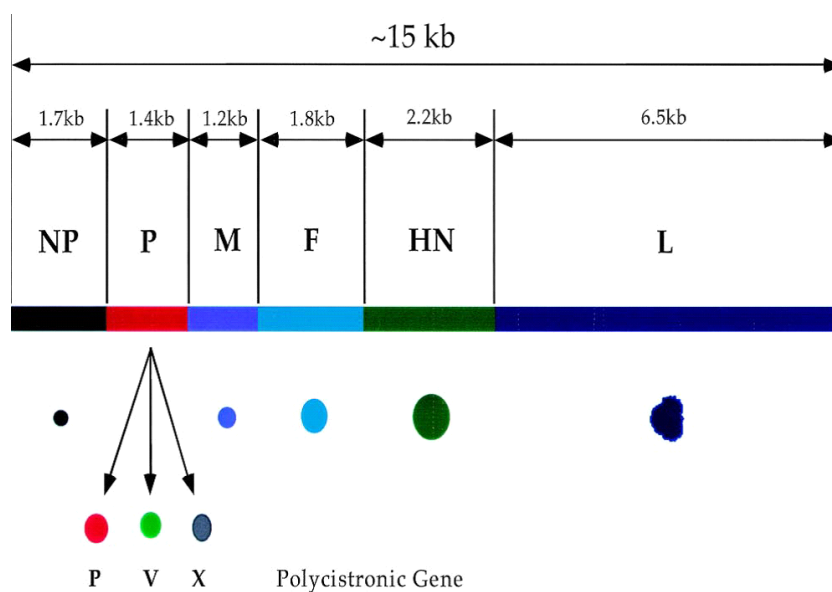
Figure 1.1. Genomic organisation of avian avulavirus 1 with relative gene sizes (from Seal, King & Sellers, 2000)**.**

The transmembrane glycoproteins HN and F are attached to the membrane and look like protruding spikes on the virion surface. The matrix (M) protein is non-glycosylated and is attached on the inner membrane. Nucleoprotein (NP), P and L proteins are packed with the RNA genomic strands to form ribonucleoprotein (RNP) complex (Kim *et al.*, 2013) (Figure 1.2).
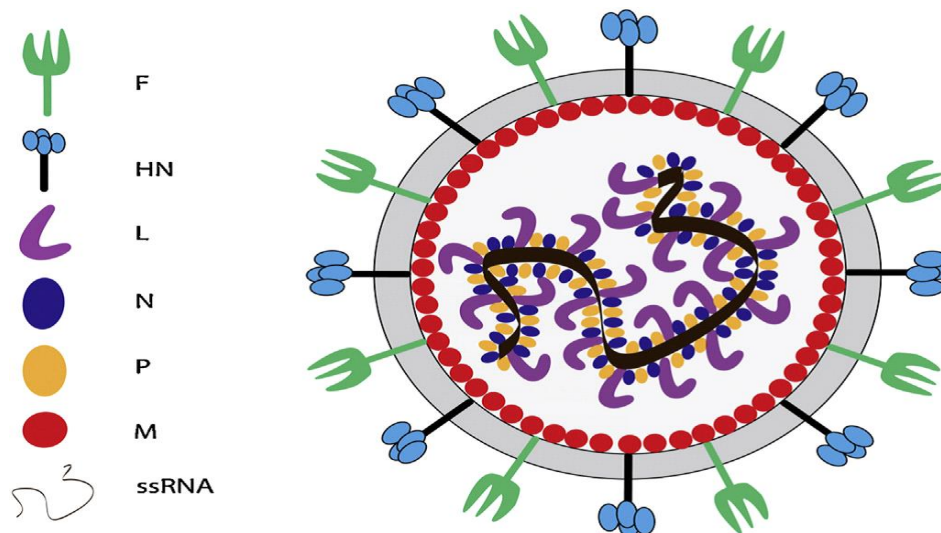
Figure 1.2. Schematic diagram of the structure of avian avulavirus 1 (Ganar *et al*., 2014).

1.4.1.1. Fusion glycoprotein (F)

The membrane of the virus contains glycosylated proteins and the fusion glycoprotein (F) is one of them. F forms a spike structure on the virion surface and plays a major role in the initiation of infection (McGinnes & Morrison, 1986). The protein occurs in an inactive form, F_0, which contains 553 amino acid, with a calculated molecular weight of 55 kDa. The cellular proteases aid in cleavage of the precursor F_0 at the peptide bond of residue 116 and 117, to yield two disulphide linked polypeptides: N-terminal F_2 (12.5KDa) and C-terminal F_1 (55kDa) (Aldous & Alexander, 2001; Samal, 2012; Hironori *et al*., 1987). The process takes place in the trans-Golgi network inside the mammalian cell. There is evidence that the cleavability of F_0 is a major determinant for virulence (Abolnik *et al*., 2004; Hines, 2012). The cleaved proteins are involved in the fusion and attachment of the virus and host membranes, which is required for infection and haemolysis to occur and for the virus to spread in the host. The cleavage specificity is determined by the amino acid sequence present at the cleavage site and varies with the type of the strain (Ganar *et al*., 2014). The cleavage of F gene is responsible for the systematic spread of AA1 and its virulence in a wide range of host tissues. F protein fuses with the host cell at neutral pH, causing multinucleate cells (syncytia) that result in tissue necrosis and virus spread. The F protein of a virulent AA1 strain contains lysine (K) and arginine (R) at the cleavage site (^{112}R-R-Q-R/K-R^{116}), and a phenylalanine at position 117 of F_1 (Rout, 2007, Nidzworski *et al*., 2013). The cleavage site is recognized by an intracellular protease furin that cleaves the polybasic cleavage site forming F1 subunits, which is suggested to be a contributor of neurological signs (Ganar *et al*., 2014). Glutamate is a neutral amino acid present at position 114 and it has been shown that substituting glutamate with a basic or acidic amino acid residue decreases the virulence of AA1. A neutral amino acid is required for proper binding of furin protease and its cleavage, altering host cell enzyme activity (Yusoff & Tan, 2015).

1.4.1.2. Haemagglutinin neuraminidase glycoprotein (HN)

Haemagglutinin neuraminidase (HN) is a surface glycoprotein with molecular weight of 74 kDa. It is a type II integral membrane protein, consisting of an uncleaved signal sequence near the amino–terminal end (Ganar *et al*., 2014). The whole HN gene consists of 2000 nucleotides that carry an open reading frame encoding 571, 577, 581, or 616 amino acids (Toyoda *et al*., 1989). The HN_{0616} amino acid is large compared to other viral proteins and it occurs in various lentogenic strains. It occurs in an inactive form and is converted to an active form by proteolytic cleavage. The other three translation products 571, 577 and 581 amino acids are already in their active form and are usually found in virulent strains (Toyoda *et al*., 1989). In principle, the length of the HN protein could be a pathogenic determinant of AA1. HN is responsible for viral attachment to sialic acid-containing host cell surface receptors. The cleavage of sialic acid residues from side sugars by neuraminidase activity possessed by the HN protein promotes the release of progeny virions from the surface of infected cell (Chong, 2012; de Leeuw *et al*., 2005; Loke *et al*., 2005; Kumar *et al*., 2011).

1.4.1.3. Matrix protein (M)

The matrix gene of many AA1 strains has been sequenced and its translated product contains 364 amino acids with a calculated molecular weight of approximately 40 kDa. The matrix protein (M) is highly conserved amongst the paramyxoviruses. It is also hydrophobic with no membrane spanning peptides and is present between the nucleocapsid and lipid membrane. M plays a vital role in the assembly of the virus through the interaction with the nucleocapsid, lipid bilayer, as well as the area of the glycoprotein that is exposed to the inner surface of the membrane. It also assists in the assembly of virions on the host cell membrane (Ganar *et al*., 2014; Yusoff & Tan, 2015). This finding can be used as a basis to classify different AA1 strains and isolates from different geographical locations. M has its own nuclear localization sequences and does not require other AA1 proteins to perform the nuclear localization function (Ganar *et al*., 2014).

1.4.1.4. Nucleocapsid protein (NP)

The nucleocapsid protein (NP) is a flexible helical structure with a diameter of about 18 nm and 1 µm in length. The essential subunits of the structures are a single polypeptide of 489 residues with a molecular weight of about 53 kDa (Bwala, 2009). Recent studies showed that several NP monomers form a ring like particle and many of these particles assemble to form a full length nucleocapsid. Viral RNA is located inside the central channel, surrounded by 2200 to 2600 NP subunits that protect it from nuclease activities (Yusoff & Tan, 2015). NP, together with L and P are suspected to be involved in replication and transcription of the viral genome. The role of NP and its function has not been extensively investigated (Chong, 2012).

1.4.1.5. Phosphoprotein and non-structural proteins (P, W and V)

The phosphoprotein gene codes for a protein of 395 amino acid residues with molecular weight of 42 kDa. The exact role of phosphoprotein (P) is not known. Together with L and NP, they form an active complex involved in genome replication and transcription (Hironori *et al.*, 1987). The phosphorylated P plays a vital role in RNA synthesis in other paramyxoviruses. It also acts as a gate keeper to prevent uncontrolled encapsulation of non-viral RNA by NP (Chong, 2012). Modifying transcript P mRNA at the editing site by insertion of one to four non-templated G nucleotides at position 484, permits potential translation of two non-structural proteins, V and W (Liang *et al.*, 2010; Rout, 2007). P, V and W occur in the approximate ratio of 7:3:1 in infected cells. A highly conserved motif resembling a zinc finger binding protein is contained by V and a cysteine-rich C terminal region, which are assumed to be involved in the replication and pathogenesis of the virus (Bwala, 2009).

1.4.1.6. Large protein (L)

The large protein (L) is the largest structural protein of AA1, consists of 2204 amino acids and has a molecular weight of approximately 249 kDa (Chong, 2012). L and P are involved in viral RNA synthesis. The exact functions of L are still not clear, and only cloning and expression of the 6.7 kb gene will provide a means to study the structure and functions of this protein (Yusoff & Tan, 2015).

1.4.2. Genotypes and lineages of AA1

Globally there are two different systems used to classify AA1, although there is no agreement on which system is the best (Miller, Decanini & Afonso, 2010). Aldous (2003) suggested a system that groups AA1 into six lineages (1-6) and 13 sub-lineages and an additional three sub lineages were added later (Miller, Decanini & Afonso, 2010). Avirulent viruses are grouped in lineages 1 and 6, while virulent viruses are grouped in lineages 3, 4 and 5. Lineage 2 contains both virulent and avirulent viruses. Some lineages tend to group together geographically, and others circulate worldwide (Herczeg *et al.*, 1999).

In 1999, a velogenic viscerotropic AA1, belonging to lineage 5d (goose paramyxovirus) was introduced in South Africa from the Far East and was responsible for the 1999/2000 outbreaks in Kwazulu-Natal province. In 2003, lineage 5d re-emerged and was responsible for one outbreak that infected chickens, peacocks, hadeda ibis chicks, geese, ostriches, pheasants and doves (Bwala *et al.*, 2009).

Two distinct classes, class I and II have been identified recently, after sequence analysis of L and F gene (Chaka *et al.*, 2013; Fringe *et al.*, 2012; Kim, Suarez & Afonso, 2008). Each of the classes contain at least nine genotypes, designated 1-9 and I-IX, respectively (Fringe *et al.*, 2012, Kim *et al.*, 2013). Low virulent AA1s predominate in class I and virulent viruses in class

II (Kim *et al.*, 2007). Class I viruses are the most genetically divergent, which makes the development of one rapid assay for the detection of all AA1's challenging , due to the genetic diversity present in the genome (Kim *et al.*, 2007). AA1 viruses have at least three genome lengths: 15 186, 15 192 and 15 198 nucleotides depending on the genotype (Miller, 2008; Nath, Barman & Kumar 2016; Rabalski *et al.*, 2014). Class I viruses are avirulent in chickens except for one known virus, and were previously recovered from waterfowls and shorebirds (Desingu *et al.*, 2015; Miller, 2008). This class has the longest genome at 15,198 nucleotides. Class II comprises ten genotypes. The following genotypes were discovered from 1930-1960: I II, III, IV, and IX contain 15 186 nucleotides, while the viruses which emerged after 1960 V, VI, VII, VIII and X contain 15 192 nucleotides (Rabalski *et al.*, 2014; Miller, Decanini & Afonso, 2010). Class II genotype I viruses are of low virulence and some are often used as live vaccines. Low virulent viruses used as vaccine viruses such as LaSota, B1 and VG/GA belong to class II genotype II. Circulating strains causing diseases outbreaks globally are associated mainly with genotypes V, VI, VII (Kim *et al.*, 2013). Genotype VII AA1 viruses have been suspected to be the cause of outbreaks in chicken flocks in China for the past decade. In 1997, an AA1 outbreak was reported in waterfowl geese, which caused severe clinical signs in two regions in Southern and Eastern China, which are the main suppliers of waterfowl in the country (Cai *et al.*, 2011).

1.4.3. Replication

AA1 binds respiratory epithelium cells via sialic acid–containing compounds, such as gangliosides and HN-glycoproteins receptors by its surface glycoprotein. AA1 infection occurs primarily via a pH-independent manner where, virus envelop fuses with host cell membrane (Ganar *et al.*, 2014; Gravel *et al.*, 2011; Samal, 2012). Infection can also occur by receptor-mediated endocytosis and sometimes through caveolae-dependent endocytosis (Figure 1.3). The negative sense RNA genome is transcribed into positive sense mRNA, which is then translated into viral protein, after entry into the host cell cytoplasm (Ganar *et al.,* 2014).
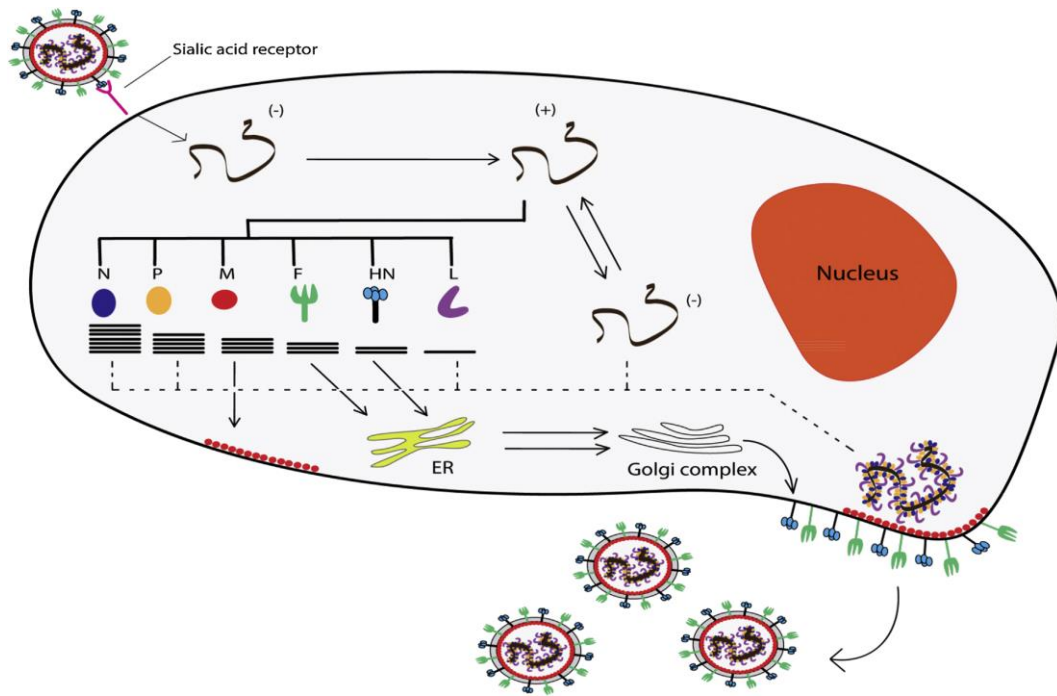
Figure 1.3. Schematic representation of avian avulavirus 1 replication. Entry of the virus into host cell system is mediated by the interaction of glycoproteins F & HN on the viral surface and binding to sialic acid-containing compounds such as gangliosides and N-glycoprotein receptors on the cell surface, which results in fusion of the virus to host cells. Adapted from (Ganar *et al*., 2014).

AA1 contains neuraminidase activity that infects a wide variety of cells containing sialic acid residues. It has been suggested that molecules containing sialic acid serve as receptors for the virus (Bwala, 2009; Rout, 2007). When the HN glycoprotein attaches to a receptor, a conformational change may occur in both proteins to disrupt the HN and F interaction, resulting in the exposure of the fusion peptide to the target membrane, which subsequently permits the fusion of the viral and cellular membranes (Rout, 2007). After fusion, there is dissociation of the M proteins underneath the membrane from the nucleocapsid through an unknown mechanism, releasing the viral nucleocapsid into the cytoplasm to begin replication and transcription. The active transcriptase complex consists of the NP, P and L proteins, as well as the encapsulated genomic RNA. It is assumed that the P and L proteins act as the viral RNA polymerase, transcribing the negative genomic RNA to produce the sub-genomic mRNAs that are required for the synthesis of the viral proteins. Genomic replication occurs by the synthesis of a full-length positive RNA, which in turn functions as a template for the production of negative genomic RNA (Ganar *et al*., 2014).

Glycoproteins HN_0 and F_0 are synthesized in the rough endoplasmic reticulum, whilst the rest of the viral structural proteins (NP, P, L and M) and the non-structural proteins (V and W) are produced in the cytoplasm. When transported across the endoplasmic and Golgi apparatus, these glycoproteins undergo glycosylation and formation of a disulphide bond. The F_0 cleavage into two disulphide-linked fragments, F_1 and F_2 occurs in the Golgi apparatus. The interaction

of HN and F_0 proteins prior to proteolytic cleavage of F_0, suggests that the two proteins interact in the rough endoplasmic reticulum. The M protein is vital for virion assembly and consequently involved in specific interaction with the nucleocapsid, plasma membrane and also the regions of the glycoproteins that are exposed on the inner surface of the membrane.

The F protein is regarded as an important pathogenic marker of NDV amongst the structural proteins (Mehrabanpour *et al.*, 2014).

1.4.4. Molecular basis for pathogenicity

The pathotype causing disease in chickens is based on the genetic determination of the F gene cleavage site (Chong, 2012; Roy, 2012) Recombinants generated by reverse genetics techniques have demonstrated that the F gene is not the only determinant of virulence, HN and P from AA1, together or individually can also contribute to viral virulence (Liang *et al.*, 2010).

There is variation in amino acid sequence surrounding the post-transitional cleavage site of the F protein (F_0) that cleaves into F_1 and F_2 proteins for each pathotype. The F_0 of lentogenic strains has two single basic amino acids at the cleavage site that can only be cleaved by trypsin-like enzymes, which are found in a few cells, such as the respiratory and intestinal tracts cells (Peeters *et al.*, 1999). The F_0 of the virulent strain has two pairs of basic amino acids at the cleavage site which can be cleaved by omnipotent protease that fuse with a wider range of cells and resulting in a fatal systematic infection (Gohm, Thur & Hofmann, 2000; Creelan, Graham & McCullough, 2015; Peeters *et al.*, 1999; Pham *et al.*, 2005).

Analysing the sequence of the F protein cleavage site can help in predicting pathogenicity and diagnosis of AA1 virulence. Virulent AA1 for chickens have the amino acid sequence '112-R/KR-Q-K/R-R-116' at the C-terminus of the F_2 protein and F (phenylalanine) at residue 117, the N-terminus of the F_1 protein. The viruses of low virulence on the other hand have sequences in the same region of '112-G/E-K/R-Q-G/E-R-116' and L (leucine) at residue 117 according to the definition of OIE (Aldous & Alexander, 2001; Berinstein *et al.*, 2001; Mehrabanpour *et al.*, 2014). When the amino acid sequence of the F protein cleavage site is compared to the ICPI for several AA1 it was shown that there were big differences in virulence that exist between strains with the same velogenic consensus sequence (de Leeuw *et al.*, 2005).

1.5. Diagnosis of avian avulavirus 1

The OIE Terrestrial Manual (www.oie.int/en/international-standard-setting/terrestrial-manual) prescribes AA1 isolation in embryonated chicken eggs, identification using haemagglutination (HA) and HA inhibition (HAI) tests with a AA1-monospecific antiserum and

this is considered to be the gold standard for diagnosis (Antal *et al*., 2007; Gohm, Thur & Hofmann, 2010; Mazumder *et al*., 2012). However, virus isolation and subsequent determination of ICPI is labour intensive and time consuming (Tiwari *et al*., 2004). It also requires a source of eggs and chickens which should preferably be from a specific pathogen free (SPF) flock (Aldous *et al.,* 2001).

Real-time RT-PCR offers increased sensitivity and specificity in a rapid format. There are different formats available for real-time RT-PCR (Al-Habeeb, Mahamed & Sharawi, 2013; Tan *et al*., 2009). The intercalating dyes (e.g. SYBR Green I) are cost effective and easier to establish compared to other detection formats, because target specific fluorogenic probes are not required. The major disadvantage with dyes is that they bind to nonspecific PCR products or primer dimers, which require melting curve analysis to distinguish the specificity of amplified fragments (Tan *et al*., 2009). Aldous and Wise described real-time PCR assays for AA1 detection and pathotype differentiation using several TaqMan® probes in 2001 and 2004 respectively. The primers and probes were designed to target specific conserved areas of various genes, as mismatches would lead to false-negative results (Miller, 2008). Unfortunately, there is no universal primer/probe set that can identify all genotypes of AA1 (Miller, 2008). The first SYBR Green I real-time PCR assay was described by Tan in 2004, but the assay failed to differentiate various pathotypes of AA1 (Tan *et al*., 2009). In 2005, Pham *et al*., (2005) described a SYBR Green I real-time PCR with melting curve analysis that detects and differentiates AA1 using a set of primers based on the F gene for amplification.

Recently the OIE has adopted an expanded definition of ND with the inclusion of additional criteria of virulence. This criteria is the demonstration (directly or by deduction) of a characteristic pattern of amino acid residues in the region of the fusion protein cleavage site, thereby enabling molecular–based techniques to be included into the diagnosis of ND (Creelan, Graham & McCullough, 2015). The F gene is an important determinant of pathogenicity of the virus and is used commonly for phylogenetic analysis (Wise *et al*., 2004). The highly virulent strains can cause severe economic impact and therefore early recognition and confirmation of the disease is of great importance (Cattoli *et al*., 2011). The most pathogenic virulent viruses have at least three arginine or lysine (multiple basic amino acids) between residues 113-116 at the C-terminus of F2 protein and phenylalanine at residue 117 in the N-terminus of the F1 protein (Roy, 2012). A real-time RT-PCR assay showed promising specificity and sensitivity to various pathotypes, but cannot replace virus isolation completely on an individual sample basis. However, real-time RT-PCR can be used for flock screening (Cui *et al*., 2007). Due to the highly contagious nature of AA1 and its clinical similarity to highly pathogenic avian influenza, accurate monitoring and rapid diagnosis of an outbreak are crucial to any control programme. It is challenging to detect and differentiate low pathogenic AA1 and vAA1 due to their broad genetic variability and because these viruses are serologically indistinguishable (Kim, Suarez & Afonso, 2008).

1.5.1. Limitations of molecular diagnostic assays

The OIE requires a definitive diagnosis of virulent AA1 to be able to effectively prevent ND outbreaks by strict control measures (Zhang *et al.*, 2010). Current diagnosis of AA1 is unable to differentiate virulent from avirulent AA1s in vaccinated chickens. Conventional *in vivo* methods are still used for pathotyping AA1 strains. Over the past years several molecular techniques have been developed to diagnose ND, such as triple one step RT-PCR, RT-nested PCR coupled with ELISA detection, real-time PCR, nucleic based amplification and a phage–capturing dot blot (Zhang *et al.*, 2010). The strains used in these assays include mainly class II types. Recently there have been two methods developed to detect both Class I and II AA1 isolates (Zhang *et al.*, 2010). There is still a need to develop an ordinary RT-PCR, which is less equipment-demanding for detecting class II AA1, as well as some prevalent class I strains. The gene targets used for the RT-PCR of AA1 include the F gene, M gene and L gene. A SYBR Green I real-time RT-PCR assay was developed to detect and differentiate AA1 velogenic and lentogenic strains, a based on primer designed for NP gene (Gopinath *et al.*, 2011)). It is still difficult to develop a universal assay to detect all the genetic groups of AA1, despite targeting the conserved genes such as M and L gene (Gopinath *et al.*, 2011).

1.6. Control of Newcastle disease

Control of ND can be complicated by various factors such as wide host range capable of infecting more than 250 species of birds, various routes of transmission, a virus that is stable at environmental temperatures (Munir *et al.*, 2015; Sharif *et al.*,2014; Roy, 2012). Psittacine birds harbour virulent AA1 without showing clinical signs and shed the virus in the droppings (Miller, 2008). Birds like the Amazon Parrot may act as carriers and shed the virus longer than one year. Conures can shed the virus up to 84 days after infection (Roy, 2012).Therefore the wild birds can infect and spread the virus to susceptible birds without showing clinical signs and making proper and effective control of the disease difficult. The virus can survive for more than eight weeks in hot dry tropical areas at an ambient temperature of 40°C and three months in temperatures ranging from 20 - 30°C. The birds carrying virulent AA1 pose a potential threat to susceptible commercial chickens (Roy, 2012). The control of the disease is based on several requirements (OIE, 2012), which include:

- Reporting an outbreak to OIE to ensure adequate measures are taken.
- Restriction of import and export of poultry and poultry products.
- Ensuring strict biosecurity measures in affected and non-affected farms.
- Proper disposal systems, restrictions of visitors and movement of personnel.
- Avoiding access of wild birds and other animals inside the farms.
- Strict sanitary measures, providing good quality feed and water, vaccination.

In most developing countries the disease is controlled by vaccination. The disease is present in bird populations in most countries, with outbreaks occurring sporadically and this makes implementation of an eradication policy difficult (Roy, 2012). Most of these countries, which keep the commercial poultry and where the disease is endemic, rely on vaccination and good biosecurity practices to keep the disease under control (Palya *et al.*, 2014).

Current vaccination programmes for AA1 include the use of low-virulent live virus, or virus of moderate virulence (mesogenic) and inactivated vaccines, designed to control against endemic, low virulence field strain (OIE, 2012). The main purpose of the vaccination procedures is to provide protective immunity to the poultry population, without causing major side effects (Kapczynski & King, 2005; Palya *et al.,* 2014). Although the efficiency of current available vaccine against velogenic strain is widely accepted, there are still sporadic outbreaks occurring globally. The exotic AA1 that caused a major outbreak in commercial farms and backyard poultry in California and adjacent states during 2002-2003 raised concerns regarding the protective immunity of commercially available vaccines for prevention and control of the virus in poultry (Palya *et al.,* 2014; Pham *et al.,* 2005; Kaczynski & King, 2005).

Research has shown that the live and inactivated vaccine do provide protection against exotic AA1, but did not prevent infection and virus shedding (Miller, 2008; Kaczynski & King, 2005). The amount of virus shed due to vaccination depends on several factors such as: the amount and virulence of the challenge virus, type of AA1 vaccine, the host species infected and immunity of the host and the time between vaccination and challenge (Miller, 2008).

1.6.1. Recombinant vaccines

The existence of recombinant DNA technology has resulted in the development of novel AA1 vaccines. Vector vaccines use a recombinant herpes virus of turkey (rHVT) (now known as meleagrid alphaherpesvirus 1 – MeHV-1) that express one or more immunogenic AA1 proteins (usually HN and/or F) that induces an immune response against both AA1 and the vector virus itself (Heskett, 2003; Maraqa, 1996). MeHV-1 is an example of a vector that is used widely as both a live vaccine and as a recombinant polyvalent vaccine in the poultry industry (Heiden *et al*., 2014 ; Palya *et al*., 2014). The immune response induced by the MeHV-1/F combination seems to be less sensitive to interference from maternally derived antibodies, which is a useful characteristic (Palya *et al*., 2014). To date, reverse genetics systems have been described only for AA1 strains, which has benefited the understanding of AA1 replication and pathogenicity and led to studies aimed at using AA1 as a vaccine vector for both veterinary and human use. Reverse genetic systems for other AA-2 to -9 are not available yet (Kumar *et al.*, 2011).

Vectormune®HVT NDV vaccine is a genetically engineered serotype 3 Marek's disease vaccine (MeHV-1) expressing an AA1 key protective antigen. The vaccine is recommended for the protection against infection of ND and Marek's disease and is recommended for use in healthy

one day old chicks or in 18 to 19 day old embryonated chicken eggs. The vaccine is stored in a frozen cell-associated form in liquid nitrogen. The cells and virus particles are fragile and require careful handling to prevent damage or loss of titre in order to achieve optimum efficacy (Yu *et al*., 2014). The vaccine is given subcutaneously and *in ovo*. In 2014, Yu and colleagues (2014) demonstrated that Vectomune® HVT NDV has a high level of safety in chickens. No adverse vaccine reactions, or vaccine induced mortality or clinical signs of MD and AA1 were observed in experimental chickens inoculated with the vaccine. The MeHV-1/F vector vaccine has less effect on hatchability and it induces lifetime protection against Marek's disease with just one vaccination (Heskett, 2003; Palya *et al*., 2014; Reddy *et al*., 1996). The vector has a natural host range limited to avian species and is safe for other domestic animals and people working in the poultry industry (Sonoda *et al*., 2000).

The vaccine possesses ideal characteristics for use as recombinant vaccine vector. The MeHV-1 genome is approximately 159 kb and of sufficient size to insert multiple foreign genes into its genome. It is non-pathogenic to chickens and other animals. The vaccine is stable and persistent in its host, offering long-term protective immunity against pathogens. It is available in a cell–free dry lyophilized form, which can be more effectively transported and stored. It can also be used to prevent and control both MD and ND via simple injection compared to other conventional vaccines (Yu *et al*., 2014).

1.7. Economic impact of Newcastle disease

The disease is present globally and affects many species of birds, causing severe losses in the poultry sector (Musako & Abolnik, 2012; Palya *et al.,* 2014). ND is considered to be one of the two most important poultry diseases (the other is avian influenza) that cause huge economic losses in poultry production (Rabalski *et al.,* 2014). In developing countries where the majority of chickens are reared under "backyard" subsistence conditions, disease occurrence has robbed households of a cheap source of protein and loss of income (Cattoli *et al*., 2011). In developing and developed countries, the disease has a negative impact on commercial farming, due to the effort required in controlling the disease and loss of trade (Cattoli *et al*., 2011). In California in 2002-2003, more than 2,500 premises were depopulated (4 million birds) at a cost of US$162 million. In the United States the highly virulent form is considered exotic and if outbreaks occur, strict control measures are undertaken to eradicate the disease (Brown, King & Seal, 1999; Wise *et al*., 2004). Mildly virulent strains are endemic and circulate on a regular basis in many poultry populations. The virus causes reduced productivity and economic losses due to respiratory infections. Amongst the AA1 strains, the virulent F form imposes a major economic concern for poultry producers worldwide.

1.8. Surveillance

In South Africa, the recombinant Vectormune® HVT NDV vaccine is used widely at hatcheries and given to day-old chicks and 18 to 19 day old chicken embryos for prevention against Marek's disease and Newcastle disease. This vaccine is preferred because of its added advantages, such as long-term immunity and reduced vaccine induced mortality, or reduction in the severity of clinical signs.

There is a need for rapid, reliable tests that will be able to differentiate the Vectormune HVT NDV from the field strains during outbreaks and for monitoring purposes. The aim of developing this assay is to be able to monitor vaccine efficacy in poultry vaccinated with Vectormune® HVT NDV at the commercial level.

1.9. Aim of this study

To develop a PCR assay that detects Vectormune® HVT NDV, a recombinant vector vaccine for AA1 from Ceva Santé Animale, that can be used to monitor vaccine efficacy and differentiate the vaccine strain from field infections and other HVT AA1 vaccines on the market.

1.10. Objectives of this study

- Identify unique sequences in the Vectormune® HVT NDV genome.
- Develop a PCR assay that targets unique sequences in the Vectormune® HVT NDV genome.
- Optimise the assay and test the assay on field samples.

CHAPTER

2. MATERIALS AND METHODS

2.1. Assay design

Vectormune® HVT NDV vaccine, is a recombinant vaccine that contains the F gene of the D26 strain of AA1 inserted into, MeHV-1 genome of FC126 strain. Complete coding sequences of the F gene of AA1 were searched for in Genbank® (www.ncbi.nlm.nlh.gov) using the term "("Newcastle disease virus"[Organism] OR "Newcastle disease virus"[All Fields]) AND ("f gene"[All Fields] OR "fusion gene"[All fields]) AND "complete cds"[All Fields] NOT genome [All Fields] AND ("1"[SLEN]"2000"[SLEN])". A total of 844 sequences were downloaded. Sequences labelled as pigeon paramyxovirus were deleted, which left 832 sequences (see Appendix 6.2). The sequences were edited in Bio Edit (Hall, 1999), aligned online using MAFFT version 7 with automatic settings (Katoh & Standley, 2013), and duplicates removed using DAMBE software (Xia, 2013). An AA1 D26 F gene sequence was identified (M24692) and used as a reference sequence to design the assay. Primers and a TaqMan® MGB™ hydrolysis probe specific for the F gene of the D26 strain of AA1 were designed with Primer Express 3.0 software (Applied Biosystems).

2.2. Nucleic acid purification

Nucleic acid was purified from the Vectormune® HVT NDV vaccine (Ceva Santé Animale) with a serial no: 372874, using a MagMax™ Pathogen RNA/DNA extraction kit (ThermoFisher Scientific) according to the manufacturer's instructions. The sample (50 µl) was added to 65 µl lysis binding buffer, 65 µl isopropanol, 20 µl bead mix (10 µl lysis binding enhancer added to 10 µl nucleic acid binding beads). The samples were placed in MagMax™ Express Magnetic Particle Processor (ThermoFisher Scientific) and run using a custom protocol (see Appendix 6.1). The magnetic beads were washed twice with Wash Solution 1 and twice with Wash Solution 2 before elution in 50 µl Elution Buffer. The purified nucleic acid was transferred to a 1.5 ml microcentrifuge tube and stored at -20°C until used.

2.3. Real-time polymerase chain reaction

The assay is specific for Vectormune® HVT NDV vaccine, which is a DNA virus. There was therefore no need to do reverse transcription, even though AA1 is a RNA virus. This allows the assay to differentiate the vaccine from field samples.

TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific) was used as follows: 10 µl of master mix, 0.2 µl 20 µM forward and reverse primer (200 nM final concentration of each),

0.2 µl 20 µM probe (200 nM final concentration), 7.4 µl nuclease-free water and 2 µl of template, to obtain a final total volume of 20 µl. The sample was centrifuged briefly and analysed to run on a StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific) using the following programme: 50°C for 2 min, 95°C for 20 sec, 40 cycles of 95°C for 1 sec, 60°C for 20 sec.

2.4. Assay optimisation

Specific primer concentration was optimised by running three replicates of the assay with a constant probe concentration (120 nM) and testing final primer concentration of 50, 100, 200, 400 and 800 nM in the PCR reaction.

After the primer concentration was optimised, the probe concentration was optimised by keeping the primer concentration constant at 200 nM, and testing four replicates of final probe concentrations of 30, 60, 120, 250, 500 nM in the PCR reaction.

2.5. Assay characteristics

2.5.1. Linear range and efficiency

The Vectormune®HVT NDV vaccine has a stated concentration of 3420 plaque forming units (PFU) at release and 2280 PFU at expiration, to make a tenfold serial dilution from 10^0 to 10^{-7}. The nucleic acid was purified using the protocol described in 2.2 and the real time PCR run in triplicate for each dilution, using the protocol described in 2.3. The cycle threshold (C_T) value obtained from each dilution was plotted against the logarithm of minimum plaque forming units (PFU) (as described on the vaccine bottle). The efficiency of the assay was calculated by the slope of the regression line using Microsoft® Excel®, with the formula: PCR efficiency (%) = $100 \times (10^{1/slope} - 1)$.

2.5.2. Analytical sensitivity and variation.

A ten-fold serial dilution of Vectormune® HVT NDV was prepared from 10^{-1} to 10^{-3} with diluent (Ceva) and two-fold dilution of $10^{-3.3}$ to $10^{-5.0}$ (Table 2.1). Nucleic acid extractions of each two-fold dilution ($10^{-3.3}$ to $10^{-5.0}$) were performed on five separate occasions and five replicates of each extraction assayed (i.e. each dilution was tested 25 times).

Table 2.1. Dilution of Vectormune® HVT NDV to determine the limit of detection of a TaqMan® MGB assay targeting this vaccine.

Dilution	PFU/reaction	Vaccine (µl)	Diluent (µl)	Total mixture (µl)
$10^{-1.0}$	570	10	90	100
$10^{-2.0}$	57	50	450	500
$10^{-3.0}$	5.7	100	900	1000
$10^{-3.3}$	0.57	500	500	1000
$10^{-3.8}$	0.912	320	680	1000
$10^{-4.1}$	0.456	500	500	1000
$10^{-4.4}$	0.228	500	500	1000
$10^{-4.7}$	0.114	500	500	1000
$10^{-5.0}$	0.057	500	500	1000

SPSS software (IBM Analytics) was used to determine the 95% limit of detection (LOD) by probit analysis. The inter and intra-run standard deviation (SD), standard deviation of the means of all runs, total SD, standard deviation of all replicates and the coefficient of variation (CV) was calculated in Microsoft® Excel®.

2.5.3. Analytical specificity

The assay was tested on freeze-dried AA1 isolates provided by the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria. The freeze-dried samples were reconstituted with 500 µl of phosphate buffered saline (PBS) and tested, as described (2.2 and 2.3).

To confirm the viability of the DVTD isolates for use in molecular testing, a RT-PCR was performed on the isolates, using a published assay targeting the M gene (Wise *et al.*, 2004) that was modified into a TaqMan® MGB assay format (Table 2.2). Nucleic acid extraction was performed, as described (2.2). TaqMan® Fast Virus 1-step Master Mix kit (ThermoFisher Scientific) was used as follows: 5 µl of 4x master mix, 0.2 µl 20 µM forward and reverse primer (200 nM final concentration of each), 0.2 µl 20 µM probe, 12.4 µl nuclease-free water and 2 µl of template, to obtain a final total volume of 20 µl. The sample was centrifuged briefly and analysed on a StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific) using the following programme: 50°C for 5 min, 95°C for 20 sec, 40 cycles of 95°C for 1 sec and 60°C for 20 sec.

Table 2.2. Real-time PCR primers and probe sequence of Matrix gene-based assay, using Genbank® accession number NC_002617 for numbering. T_m – melting temperature, bp – base pairs.

Name	Sequence (5' to 3')	Start	Stop	bp	T_m	% GC
AA1_GroupF	CAGTGATGTGCTCGGACCTTC	4099	4119	21	59.3	57
AA1_GroupR	CCTGAGGAGAGGCATTTGCTA	4200	4220	21	58.2	52
AA1_GroupP	AGCAGTGGGACAGCC	4175	4189	15	69	67

2.6. Testing of field samples

Spleen impression smear samples from chicken, vaccinated with Vectormune® HVT NDV and an other live, low virulent conventional Newcastle disease vaccine, were preserved on Whatman® FTA cards (Sigma-Aldrich). The vaccinated chicken samples were collected between day 21 and 28 post vaccination. A total of 24 samples (12 Vectormune® HVT NDV and 12 conventional Newcastle disease vaccine) were analysed.

The protocol developed for nucleic acid purification on the MagMax® Express Particle Processor was not suitable for the extraction of nucleic acid from FTA cards. An in-house protocol, using a QIAamp DNA mini kit (Qiagen, Germany) was used instead: a rice grain sized section of the FTA card was cut out with a clean scalpel blade and placed in a microcentrifuge tube. ATL buffer (180 µl) was added to the tube and the sample incubated at 85°C for 10 min. The sample was centrifuged briefly for 2 - 3 sec, before adding 20 µl proteinase K and the tube vortexed. The sample was incubated at 56°C for 1 hour. Thereafter, 200 µl of AL buffer was added, the sample vortexed and incubated at 70°C for 10 min and centrifuged briefly for 2-4 sec. Ethanol (96 - 100%, 200 µl) was added to the sample and vortexed. The sample mixture was transferred to a spin-column and centrifuged at 8000 rpm for one min. The through-flow was discarded, 500 µl of AW1 buffer added to the column and centrifuged at 8 000 rpm for one min, and the through-flow discarded. Buffer AW2 (500 µl) was added to the column and centrifuged for 3 min at 14 000 rpm and the flow-through discarded. The column was placed in a new collection tube and centrifuged at 14 000 rpm for 1 min to remove residual AW2 buffer. The column was placed in a 1.5 ml microcentrifuge tube and 100 µl buffer AE added to the column. The column was incubated at room temperature for two min and centrifuged at 8 000 rpm for 1 min to elute the nucleic acid. The sample were stored at -20°C.

CHAPTER

3. RESULTS

3.1. Assay design

In the Genbank® database, 364 unique AA1 sequences in the target region (F gene) of this assay were identified. A unique region of the D26 sequence was identified and used to design a TaqMan® MGB assay (Table 3.1).

Table 3.1. Sequence and characteristics of real-time PCR primers and probe targeting the AA1 F gene, Genbank® accession number M24692 was used for numbering. T_m – melting temperature, bp – base pairs.

Name	Sequence (5' to 3')	Start	Stop	bp	T_m	% GC
AA1_D26F	GGATCCCAGTACCTCTGATGCT	69	90	22	58	55
AA1_D26R	TCGGACAGACGCAACTCAGT	112	131	20	58	55
AA1_D26P	CCGTCCGAATCAT	93	115	13	69	54

AA1 is a RNA virus and the assay designed targets the DNA of Vectormune® HVT NDV vaccine. As there is no reverse transcription step in the assay, it is highly unlikely that the assay will detect a field virus. In addition, there was a high level of variation in the region that the assay targets.

Alignment of the sequences showed that there was only one sequence (AY427817) that had an identical sequence in the target region of the assay, and it was identified as a "Heb02 isolate" submitted to Genbank® by authors from China. The sequence was not referenced in a published journal and it was therefore not possible to determine if this was a field or vaccine virus sequence (Figure 3.1).
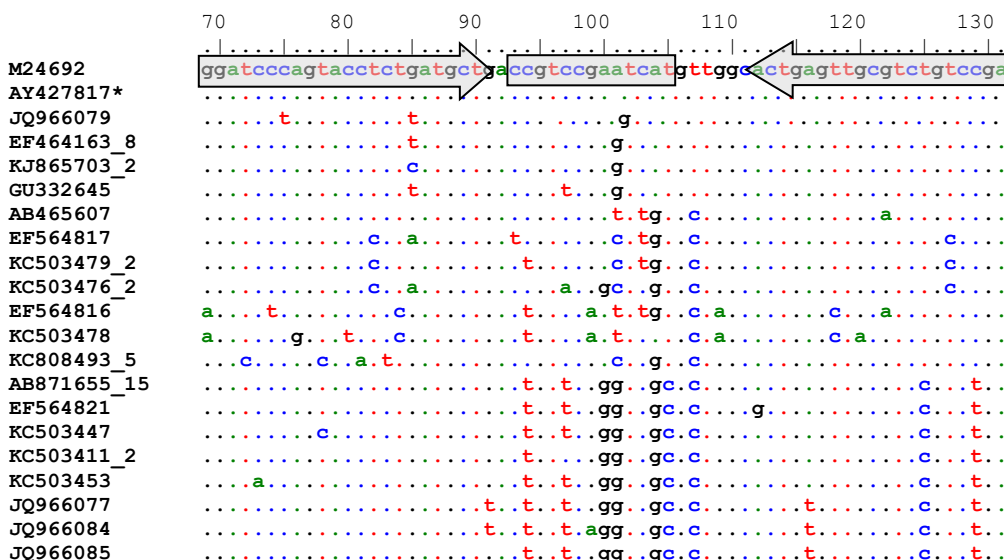
```
                        70        80        90        100       110       120       130
                        |    |    |    |    |    |    |    |    |    |    |    |    |    |
M24692        ggatcccagtacctctgatgctgaccgtccgaatcatgttggcactgagttgcgtctgtccga
AY427817*     ..............................................................
KC750152_2    ........................t..t..gg..gc.c.....t..t........c...t.
KT380032      .....................a..t..t..gg..gc.c.....t........c...t.
JQ966078      .a...................t.....gg..gc.c.....t........c...t.
KC503413_7    .a...................t..t..gg..gc.c.....t........c...t.
KT381597      .a....g..............t..t..gg..gcac.......ta.....c...t.
KT381598_2    .a....g..............t..t..gg..gcac.......ta.....c...t.
KC503482      .....................t..t..gg..gc.c....t..t......ac...t.
KC750151      .................t....t..t..gg..gc.c.....tt........c...t.
EF564826      .........g...............gg..gcac..a....a.....a..c...t.
EF564832      .....................a....gg.tgcac..a....a....a..c...t.
KR869090      a..a.....c....a........t..t..gg..gc.c.....t........c...t.
AY727881      ....t..ga................a...gg..gcac.....a...........t.g
AY727882      a...t..ga...............gg..gcac.....a........c.c...t.g
AF079172_11   atg......c.............a.g..tgc.c.....c.t..c.t..
JN698890      atg......c.............a.g..tgc.....c.t..c.t..
AF079322      atg......c.............a.g..t.c.c.....c.t..c.t..
EF589135_2    .........c.............a.g..tgc.c.....c.t..c.t..
FJ665433      ..................t.a....gg.tgc.c..a....c.t..tc..t..
AB465605_13   a..a.....c....a...ta.....g..gc.c...t.........a........g
GQ245770_2    a..a.....c....a........t..gg..gc.c...t.........a........g
FJ969393_2    a..a.t...c....a........t....g..gc.c...t.........a........g
JX244795_10   a..a.....c....a........t....gg..gc.c...t.......c.a........g
AY727883_47   a..a.....c....a...ta.....gg.tgc.c...t.........a........g
DQ195265      a..a.....c....a...c...ta.....gg.tgc.c...t.........a........g
EU239663      a..a.....c....a........ta.....gg.tgc.c...t.........a.c......g
KJ525703      a..a.....c....a.a........ta.....gg.tgc.c...t.........a........g
EU315123      ..a..a...c....a........ta.....gg.tgc.c...t.........a........g
EU330230      a..a....tc....a....tta.....gg.tgc.c...t.........a........g
EF589138      .....g...c....a........ta.....gg.tgc.c...t.........a........g
JQ013872      .........c............ta.....gg.tgc.c...t.........a........g
JN942033      a..a.....c....a.....a.....t...a..g..gc.c....t.t...c...a......g
HQ917083      .........c.....t....tta.....gg.tg..c...t.t..g...ta........g
DQ485257_3    ...............a.......a.a..g....c.c..........at...c...t..
KR869091      a..a.....c....a.....ta.....g..t...c..at.t..ac...a..c...t.
AB465606      .....tt........c......a.ttac........c....at.t...c..ta.....t.
EF589137      ...a...c.....c.......t.ac.a.....c..at.t...c..ta.....t.
AF109885      .................ca....t.ac....ct..c..at.t...c...a....c....
HM776583_2    .................t.act....t...c..at....c...a...c....
JX244794_4    .................c......t.ac.......c..at.t...c...a...c....
JX901312      a..c..t........c......t.ac.......c..at.t...c...a...c.a.
JX901318      ...c......g...c......t.ac.......c..at.t...c...a...c.a.
JX901341      ...c..........c......taac......c..at.....c...a...c.a.
AY734535      ................tgcat..gt.ac......c...c..at.t...c...a...c...
AF048763      ........ac..........t.ac...g..t.c.c..at......c..ta.....t..
KC808512      .....t............t.a....g..t..c..at.......c..ta.....t..
KC808508_2    .....t............t.ac...g..t...c..at......c..ta.....t..
KC808510_2    ..................t.ac...g.ct..c..at......c..ta.....t..
JN967789      .....t............t..c...g..t...c..at......c..ta.....a..
KF767468      .....t............ttac.a.g..t.c.c..at......c..ta.....t..
KF767469      .....t............ttact..g..t....tt......c..ta.....t..
JN942028      .....t............t.act.ag.c...c..at......c..ta...
JN942031_2    .....t............t.act..g.c...c..at......c..ta...
JN942039      .....t............a.t.act..g.c...c..at......c..ta...
JN942032      .....t............t.act..g.g...c..at......c..ta...
JN942040      ..................t.actt.g.c...c..at......c..ta.....a..
KF767470      .....t............t.ac..g.c.c.c..at......cc.ta.....a..
JN942027      ........c.........t.actt.g.c..c..at......c..ta...c....
EU140950      ......c.......a.....ac..g..t.c.c..at.t...c..ta...
AB853329_6    ......c...........t.act..g..t..cc.at.t...c..ta..c...t.
DQ485256_2    ......c...........t.act..g..t...c..at.t...c..ta..c...t.
AY325799      ......c...........t.act..g..t...c..at.t.a..c..ta..c...t.
GU332647      .....t..c.........t.act..g..t...c..at.t....c..ta..c...t.
KC750150      ......c...........t.act..g..t...c..at.t..g.c..ta..c...t.
KU200246      ......c...........t.act..g..t.c....at.t...c..ta..c...t.
KU200244_2    ......c...........t.act..g..t...c..at.t...c..ta..c...t.
KU200252      ......c.......a...t.a.t..g..t...c..at.t...c..ta..c...t.
GQ245801      a.....c.......a...t.a.t..g..t...c..at.t...c..ta..c...t.
JX244806      ......c.......a.c...t.act..g..t...c..at.t...c..ta..c...t.
EF589133_17   ......c.......a...t.act..g..t...c..at.t...c..ta..c...t.
GQ245798      ......c.......a...t.act..g..t.c.c..at.t...c..ta..c...t.
JQ013874_3    ......c.......a...t.act..g..t..ac..at.t...c..ta..c...t.
KJ525687      ......c.......a...t.act..g..t...c..at.t...ac.ta..c...t.
HM188398_6    ......c.......a...t.act..g..t...c..at.t....c..ta..c...t.g
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                    70        80        90       100       110       120       130
                    |····|····|····|····|····|····|····|····|····|····|····|····|····|····
M24692              ggatcccagtacctctgatgctgaccgtccgaatcatgttggcactgagttgcgtctgtccga
AY427817*           ..............................................................
GQ245796            .........c......a.......t.act..g..t...c..at.t.....c..ta..c.....g
GQ245797_3          .........c......a.......t.ac...g.ct..c..at.t.....c..ta..c...t..
GQ245813            .........c......a.......t.ac...g.ct.ac.at.t.....c..ta..c...t..
GQ245816_4          .........c......a.......t.act..g.ct..c..at.t.....c..ta..c...t..
GQ245808            .........c....ca.......t.act..g..t...c..at.t.....c..ta..c...t..
GQ245805_4          .........c....ca.......t.act..g.ct..c..at.t.....c..ta..c...t..
KJ525678            .........c....ca.......t.act..ggct..c..at.t.....c..ta..c...t..
KJ525709_2          .........c....ca.......t.act..g..t...c..at.t.....c..ta..c...t..
AY338284            .........c....ca.......t.act..g..t...c.aat.t.....c..ta..c...t..
JQ013857            .........c......a.......t.act..g..t..cat.t.....c..ta..c.....
JQ013869            .........c..............t.ac...g..t......at.t.....c..ta..c.....
JQ013858            .........c......t.......t.ac...g..t......at.t.....c..ta..c.....
JQ013855_3          .........c.............ttact..g..t..ac.at.t.....c..ta..c...t..
JQ013879            .........c..............t.act..g..t..ac.at.t.....c..ta..c...t..
JQ013866_2          .........c..............t.ac...g..t..ac.at.t.....c..ta..c...t..
KJ525697            .........c............gt.ac...g..t...c..at.t.....c..ta..c...t..
EF540729            .........c............gt.ac...g..t...c..at.t..g.c..ta..c.....
AF456438_10         .........c..............t.ac...g..t...c..at.t..g.c..ta..c.....
AF456442            .........c..............t.ac...g..t...c..atgt..g.c..ta..c.....
EF592504_2          .........c..............t.ac...g..t...c..atgt..g.c..ta........
KU295454_2          .........c..............t.ac...g..t...cc.at.t..g.c..ta........
GQ245793_4          .........c..............t.ac...g..t...c..at.t..g.c..ta........
GU227738_3          .........c..............t.ac...g..t...cc.at.t.....c..ta........
KU295452_2          .........c..............t.ac...g..t...cc.at.t..g.c..ta..c.....
KJ450979            .........c..............t.ac...g..t...c..at.t..gac..ta..c.....
KJ525680            .........c..............t.ac...g..t...c..at.t..gac..ta..c.....
EU140947_2          .........c..............t.ac...g..t..c.c.at.t....c..ta..c.....
EU140948            ....t....c............ac...g..t.c.c..at.t.....c..ta..c.....
KF208469            .........c..............t.ac...g.c....c..at.t..g.c..ta..c.....
HM188394            .........c..............t.ac...g..t...c..aa.t..g.c..ta..c.....
DQ485269            .....a...c..............t.ac...g..t...c..at.t..g.c..ta..c.....
KJ525704            ....t....c......a.....a.t.act..g..t...c.a..t.....c...a..c...t..
JQ013878_19         .........c......a.....a.t.act..g..t...c.a..t.....c...a..c...t..
KJ525688_2          .........c......a....agt.act..g..t...c.a..t.....c...a..c...t..
KJ525705            .........c......a.....a.t.act..g......c.a..t.....c...a..c...t..
JQ013873            .........c..............t.act..g..t...c.a..t.....c...a..c...t..
JQ013877            .........c..............t.ac...g..t...c.a..t.....c...a..c...t..
JQ013870_2          .........c..............t.act..g......c.a..t.....c...a..c...t..
KM016457_5          ......t..c......a.....a.t.act..g..t...c.a..t.....c...a..c...t..
KJ525686_2          .........c......a.....a.t.act..g..t...c.a..t.....c...a..c...t..
JQ013860            .........c..............t.t.act..g..t...c.a..t.....c...a..c...t..
KF442615            .........c..............t.act..g..t...c..at.t.....c...a..c.....
GQ245800            .........c......a.......t.act..g......c..at.t.....c...a..c.c...
EF592502_8          .........c..............t.ac...g..t...c..atgt.cg.c..ta........
HM188396            .........c..............t.ac...g..t...c..atgt.cg.c..ta........
DQ485274_4          .........c......a.......t.act..g..t...c..at.a....c..ta..c...t..
KC750157            .........c......a.......t.act.ag..t...c..at.a....c..ta..c...t..
GQ245819            .........c......a.......t.act.ag..t...c..at.t....c..ta..c.c.t..
KT381593_2          .........c..a...a.......t.act..g..t...c..at.t.....c..ta..c...t..
JX244790_2          ..t......c......a.......t.act..g..t...c..at.t.....c..ta..c...t..
KU200247            .........c........t.....t.act..g..t......at.t.....c..ta..c.....
DQ485261            .........c......a.......t.a.t..g..t...c..at.t.....ca.ta..c...t..
DQ485271            .........c......a.c.....t.act.....t...c..at.t.....c..ta......t..
KU200243            .........c..............t.a...ag..t......at.t.....c..ta..c...t..
JQ013863            .........c...............act..g..t..ac.at.t.....c..ta.tc...t..
DQ485260            .........c...c..c.......t.act..g..tt..c..at.t.....c..ta..c...t..
AY028995            .........c.....c........t.act..g..t...c..at.t.....c..ta......t.g
GU332646            ......c..t..............taact..g..t...c..at.t.....c..ta..c...t..
EF592500_16         ......c......a..at...t.act..g..t...c..at.t.....c..ta..ca..t..
FJ480782            ......g.c....a..at...tt.act..g..t...c..at.t.....c..ta..ca..t..
EF592501            .........c...c..a..at...t.act..g..t...c..at.t.....c..ta..ca..t..
FJ882014            ..............ag....a.t.cct..c..t...c..a..t.....c..ga..c...t..
JQ013868            ..............ag....a.t.cct..g......c..a..t.....c...a..c...t..
JQ013865            ..............ag....a.t.cct..g..t...c..a..t.....c...a..c...t..
JQ013856            ..............ag....t.t.act..g..t...c..a..t.....c...a..c...t..
KJ525689_2          ......g...t....a.c...a.t.act..g..t...c..at.t...ac...a..c...t..
KJ525693            ......g...a....a.c...a.t.act..g..t...c..at.t...ac...a..c...t..
KJ136258_3          ...............a....a.t.act..g..t...c..at.t...ac...a..c...t..
KJ136259            ...............a....a.t.act..g..t...c..at.t...ac...ac.c...t..
KJ450978            ...............a....a.t.act..g..t...c..at.t...ac...a..c.....
KC489471            ...............a....a.t.act..g..t...c..at.t...ac.a.a..c...t..
KU200253            ..............a.c...a.t.act..c..t...c..at.t...ac...a..c...t..
JX840455            .........c......a....a.t.act..g..at..c..at.t...ac...a..c...t..
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                    70        80        90        100       110       120       130
                    |....|....|....|....|....|....|....|....|....|....|....|....|....|....

M24692              ggatcccagtacctctgatgctgaccgtccgaatcatgttggcactgagttgcgtctgtccga
AY427817*           ..............................................................
KJ525717            ........c.............t.ac...g..t.a.c..at.t.ag.c..ta..c......
KJ865713            .....a..c.............t.ac...g..t...c..at.t..g....ta..c......
FJ011441_14         ........c............gt.ac...g..t...c..at.t..g.c..ta.tc..t...
GQ245786            ...c....c............gt.ac...g..t...c..at.t..g.c..ta.tc..t..
GQ245788_2          ........c............gt.ac...g..t...c..at.t....c..ta.tc..t...
GQ245791_2          ........c............gt.ac...g..t...c..at.t..g.c..ta.t...t...
HM748945            ........c............gt.ac.t.g..t...c..at.t..g.c..ta.tc..t...
KJ525685            ........c............gt.ac...g.ct...c..at.t..g.c..ta.tca.t...
FJ011442_2          ........c............gt.ac...g..t...c..at.t..g.c..ta.ac..t...
GQ245784            ........c............gt.ac..g..t...c..at.t.ag.c..ta.tca.t..g
JX487185            ........c.....c.g.....gt.ac...gg.t...c..at.t..g.c..ta.tc..t...
GQ245785_2          ........c...t........gt.ac...g..t...c..at.t..g.c..ta.tc..g...
JQ013862            ................t.cc...g..t...c..at.t..g.c..ta..c.t...
JQ013861            ........c.......t.cc.g.g..t...c..at.t..gac..ta..c......
AY325909            ........c.........ac...g..tg.c..at.t..g.c..ta..c.t...
KU200251            ..g.....c............t.ac...g..t...c..at.t..g.c..ta.a......
KJ865717            .....t..c...c.....t.ac...g......c..at.t..g.c..ta......
KU200249            ..g.t...c......t.....t.ac...g..t...c..at.t..g.c..ta..c...
KJ865702_3          ...c.t..c......t.....t.ac..ag..t...c..at.t..g.c..ta..c...
GQ245790            ........c..a.........t.ac...g.c......at.t..g.c..ta..c...
KU295453            .....c......c......t.ac...g.c..at..c...g.c...a..c...
JF824013            ........c...ct...c.t...t..c.........c..at....c...a.tc...t..
KT965727_3          ........c...ct....t....t..c.........c..at....c..ta.tc...tt.
KT965728_3          ........c..c.c.....t....tt.c.........c..a....c...a.tc...t..
JF824032            ........c.......t....ac.........c..at....c...a.tc...t..
JQ039385            .a...t...c....c....t...t.ac.........c.c..at....c...a.t...t..
JQ039389            .a.....c...c......t...t.ac.........c.c..at....c...a.t...t..
JN255773_16         .a.c....c..c...c....t.ac...g.c..c.c..at.t..c...ta.t....t..
JN942020_2          .a.c....c..c........t.ac...g.c..c.c..at.t..ac..ta.t....t..
JN942029            ...c....c..c........t.ac...g.c..c..at.t..c...ta.....t..
JN942030            ...c....c..c........t.ac...g.c..c..at.t..c...ta.....t..
JN942024_2          ...c....c..c........t.ac...g.c..c..at.t..c...ta.....g..
JN255781            .a.c....ac..c...ct...t.ac...g.c..c.c..at....c...ta.t....t..
JN255785            .a.c....c..c...ct...t.ac...g.c..c.c..at....c...ta.t....t..
JN942023            .a.c.t..c..c........t.ac...g.c..c.c.tat.t..c...ta.....t..
JX915243            ........c........c...t.a....g.c..cac..at....c...ta......t..
EU477188_3          .......g.c.....a......t.ac......ctc..at....c...a...c.t..
EU477191            ........c.....a......t.ac......ctc..at....c...a...c.t..
JX901342            .....t.g.c...........t.ac...t.ctc..at....c...a...c.t..
JX901317            .......g.c...........t.ac...t.ctc..at....c...a...c.t..
JX901376_2          .......g.c...........t.ac...t.ctc..at....c...a...c.c.t..
JX901327_2          .....tg.c...........t.ac...t.ctc..at....c...a...c.c.t..
JX901329_3          .....tg.c...........t.ac...g..t.ctc..at....c...a...c.t..
JX901347            .....tg.c...........t.ac...g..t.ctc..at....ac...a...c.t..
JX901304_2          ......tg.cg..........t.ac...t.ctc..at....c...a...c.t..
EU477189_24         .....tg.c...........t.ac...t.ctc..at..c.tg...a...c.t..
JX901334_3          .....tg.c...........t.ac...t.ctc..at....c...a...c.t..
JX901328_2          .....tg.c...........t.ac...t.ctc..at....c...ta...c.t..
JX901332_7          .....tg.c...t...a....t.ac...t.ctc..at....c...a...c.t..
JX901354            .....tg.c...t........t.ac...t.ctc..at....c...a.tc...c.t..
JX901365            .....tg.c...t........t.ac.....ctc..at....c...a...c.t..
JX901352_2          ......g.c...c.....a...t.ac...t.ctc..at....c...a...c.t..
JX901375            .....tg.c...........t.ac...t.ctc..at.a...c...a...c.t..
GQ281085_4          ......c.............t.ac...t.ctc..at....c...a...c.t..
JX244789            ........c........c...t.ac...t.ctc..at....c...a...c.t..
JX244798            ........c.t..........t.ac...t.ctc..at....c...a...c.t..
JX244799            ........c............t.ac...g.t.ctc..at....c...a...c.t..
JX244800            ........c............ttac......t.ctc..t....c...a...c.t..
KT381606            ........c............t.ac......t.ctc.a.t....c...a...c.t..
JX901362            ....g.tg.c...........t.ac...g..t.ctc..at....c...a...c.t..
JX901335_4          .....tg.c...t........t.ac.....tgctc..at....c...a...c.tt.
JX901348            .......g.c...t........t.ac.....tgctc..at....c...a...c.tt.
GQ281086_3          ....t...c...........gt.ac......ctc....c...a...c.c.t..
JQ268609_2          ...t....c........c...t.ac......ctc....c...a...c.c.t..
KJ525672_3          ...ct...c...........gt.ac......ctc...t....ac..a...c.c.t..
KT381595            ...c....c........c...t.ac......ctc....c...a...c.c.t..
KF828884            ......g.c...t...gc...t.act......ctc...t....a...a...ct...
KJ600778_3          ......g.c.....c..c...t.act.a....ctc...t....c...a...ct...
KT381592_5          ......g.c.....c..c...t.act......ctc...t....c...a...ct...
AY734534            .....a..ac...........t.a....g..tt..c..at....c...ta......t..
EF589131_2          .....tc.c......c.....t.act..g.gt...c..at.t..c...ta..c...t.
FJ772466            ...t...........a......t.a.t..g.ct...c...t.t.a..c.........t..
JX546248            ....t...............t.a.t..g.ct...c..at.t.a..c.........t..
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                    70        80        90       100       110       120       130
                    |....|....|....|....|....|....|....|....|....|....|....|....|....|
M24692      ggatcccagtacctctgatgctgaccgtccgaatcatgttggcactgagttgcgtctgtccga
AY427817*   ................................................................
J772491_2   ..........c...c..........t.act..g..tg..c..rtct.a..c..ta.....t...
JN942034    ..........c..............t.act..gg.t...c..atct.a..c..ta.....t...
JN942041    ..........c..............t.act..gg.t...c..atct.a..c..ta......t..
JQ267579_3  ....t.....c...g......t.acta..g.t...c.aatct.a..c..ta......t...
JQ267581    ....t.....c...g......t.acta.g.t...c..atct.a..c..ta......t...
JQ267583_3  ....t.....c...g......t.acta.gg.t...c..atct.a..c..ta......t...
JN682184_11 .a..t.....c...gt....t.acta.gg.t..a...atct......c..ta......t...
JX436340_2  .a.at......t.c....gt....t.acta.gg.t..a...atct......c..ta......t..
JQ517285    .a..t.......ca...gt....t.a.ta.gg.t..a..aatct......c..ta......t...
JN942043_2  .......g.c...c..c.....t.act...g.t...c..attt.a..c.ata..c..t...
KF026013    ........ac...c.c.....t.act..g......c..at.t.a..c.tta....c.t.g
AY734536    .........c.a.........t.ac.a.g..t.c.c..at....c...a....t...
JQ039387_3  ........c.g.t........t.ac.a...t.c.c..atc....c..a....c....
JN942045_2  ....t.....a.........t.act..g.c...c..at...t..cc.ta...a....
KC568207    ......t......ct.....t.act.tg.ct...c..atg..a..c...a.a....t..
JQ039390    ......t......ct.....t.act.tg..t...c..atg..a..c...a.a....t..
JX546245    ......t......ct.....t.act.tg..t.c.c..atg..a..c...a.a....t..
KC568205_2  ......t.a....ct.....c...t.act.tg..t...c..atg.ca..c...a.a....t..
KC568206    ......t......ct.....t.ac..cg.c...c..atg..a..c...a.a....t..
FJ772452    ......t......ct...a.t...t..ct..g..t...c..ttg..a..c...a.a....t..
JQ039386    ......t......c......t...t..ct.gg.t...c..ttg......c...a.ac...t.g
KU594613_3  ....tt......c....ca.....t.act..g..tgc....at...a..c..ta....cat.
KU594617    ....tt......ct....ca.....t.act..g..tgc....at...a..c..ta....cat..
KU594616    ....tt......c....ca.....t.act..g..tgc....at...a..c..ta....c.t..
KU594618    ....at......ct...ca.....t.act.tg...gca...at...a..c..ta....c.t..
JN638234_2  ...c.....g..a..c.........t.ac.........c..atc...c......t...
FJ969394    ....t......t....t...t..ac..a.c.t..c..at.t...c...a.t......g
KJ958913    .a......c...g...c.....t.c..a...t......at.t...c..ta........
KJ958914    aa......c...g...c.....t.c..a...t......at.t...c..ta........
FJ772455    ..tca..t......c..a.....t.act..g..t...c..at.t.ag.c..ta......t..
KU200245    ...c.....cct..gga.......t.act..g..t......at.t...c..ta..c...t..
JN942035_4  ...c.....cg......g.....t..c...g.c.gc.....tgt..g.c..t.........g
KU200250    ...a......t.....t.....t..c...g....a.c..at.t...c..ta..c.g....
KT901462    ...c.....c..a...ag.......t.a......ctgca...a........c..ga..c...t.g
KT987209    a..c.....c..a...ag.......t.a......ctgca...a........c..ga..c...t.g
FJ772446_2  ...c......t.c..a....ct.t....ag..t...c....t..a..c..t....a.at..
FJ772449_11 ...ct......t.c......ct.tt....ag..t...c....t..a..c..t....a.at..
FJ772478_2  ...ct.t....t.c......ct.tt....ag..t...c....t..a..c..t....a.at..
JQ039393    ...ct......t.c......ct.tt..t.ag..t...c....t..a..c..t....a.at..
KC568204    ...ct......t......ct.tt....ag..t...c....t..a..c..t....a.at..
FJ772463    ...ct......t.c......ct.tt....ag..t...c....t..at.c..t....a.at..
FJ772475    ...ct......t.c......ct.tt....ag..t...a....t..a..c..t....a.at..
FJ772481    ...ct......t.c......ct.tt....ag..t..a....t..a..c..t..t.a.at..
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FJ487637    .ctatttgtacg.ct.tccat...t.tctatgg.t..cagtt..g.attcatg.ctg.gaaag
HQ997388    .ctatttgtacg.ct.tccat...t.tctatgg.t..cagtt..g.att.atg.ctg.gaaag
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26

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JX844030        .ccatttgcacg.ct.tcca....t.t..gtggctg.cagttt.g.attcatg.ctg.aag.g
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KC503444        .ctatttgcacg..t.tc.a....t.t..gtggct..ca.ttt.g.attcata.ctg.aag.g
EF564820_2      .ctatttgcatg....tcca....t.t..gtggct..ca.ttt.g.attcatg.ctg.aag.g
JN942015        .ctatttt.catg....tcca....t.t..gtggct..ca.ttt.g.attcatg.ctg.aag.g
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EF564824        .ctatgtgcaca..t.tcca....t.t..gtggct..cagttt.g.attcatg.ctg.aag.g
EU493451        .ccatttgcacg.ct.tcca....t.t..gtggct..cagttt.g.attcatg.cgg.aag.g
KC503435        .ctatttgcacg..t.tcca.a...t.t..gtggct..ca.tt..g.attcatg.ctg.aag.g
KC503424_4      .ctatttgcacg..t.ccca....t.t..gtggct..ca..tt.g.attcacg.ctg.aag.g
KC503442        .ctatttgcacg..t.ccc.....t.t..gtggct..ca..tt.g.attcacg.ctg.aag.g
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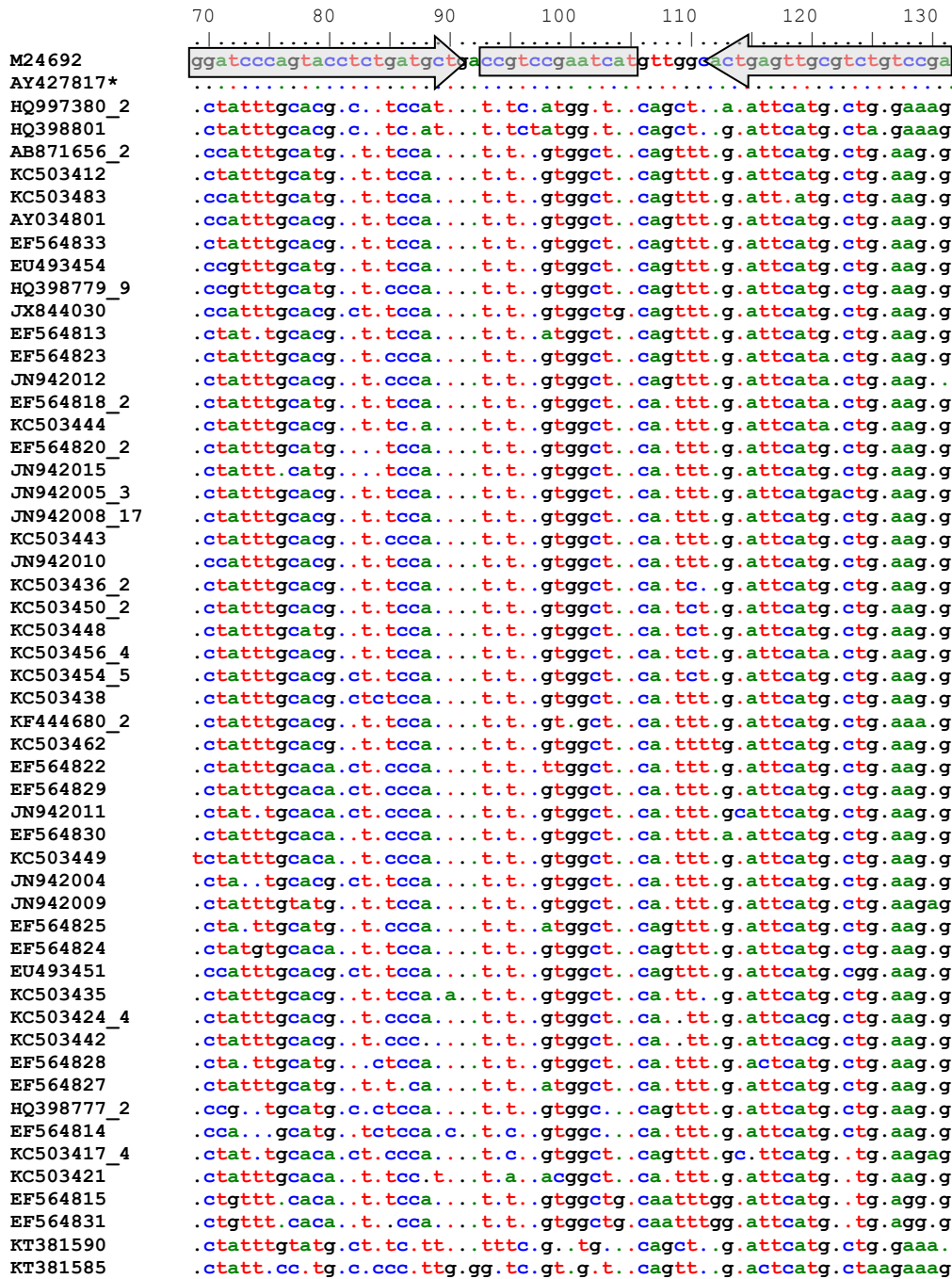
Figure 3.1. Sequence variation in the primers (indicated with grey arrows) and probe (indicated with a grey rectangle) region of a TaqMan® MGB assay to detect AA1 D26, using M24692 for numbering. Dots represent nucleotides that are identical to the first sequence. Sequences are identified by the Genbank® accession number, followed by the number of sequences that are identical. There are 364 unique sequences in the target region of the assay. *Not able to confirm if this is a field or vaccine virus sequence.

A possibility exists that the assay may detect other DNA vaccines that use the F gene in their construct. A vaccine strain – Clone 30, which is derived from a lentogenic AA1 strain was identified (Y18898) (Römer-Oberdorfer *et al.*, 1999). An alignment of the D26 and Clone 30 strains showed that there were numerous differences, especially in the probe region, between the two strains, so it is unlikely that the assay will detect Clone 30 (Figure 3.2).
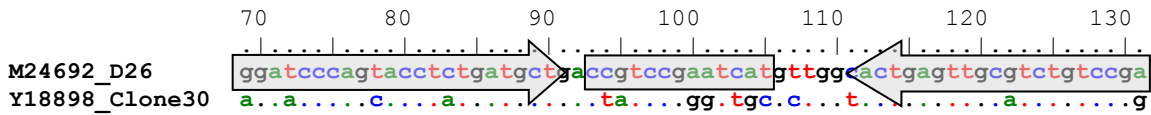
Figure 3.2. Alignment of AA1 D26 and a Clone 30 strain shows numerous differences between the two strains, especially in the probe region. Primers are indicated with grey arrows and the probe indicated with a grey rectangle. Dots represent nucleotides that are identical to the first sequence. Sequences are identified by the Genbank® accession number.

3.2. Assay optimisation

The lowest concentration of primer that yielded the lowest C_T and with a steep amplification slope was selected. The primer concentration was limited to allow for future multiplexing of the assay. A primer concentration of 200 nM was selected (Figure 3.3).
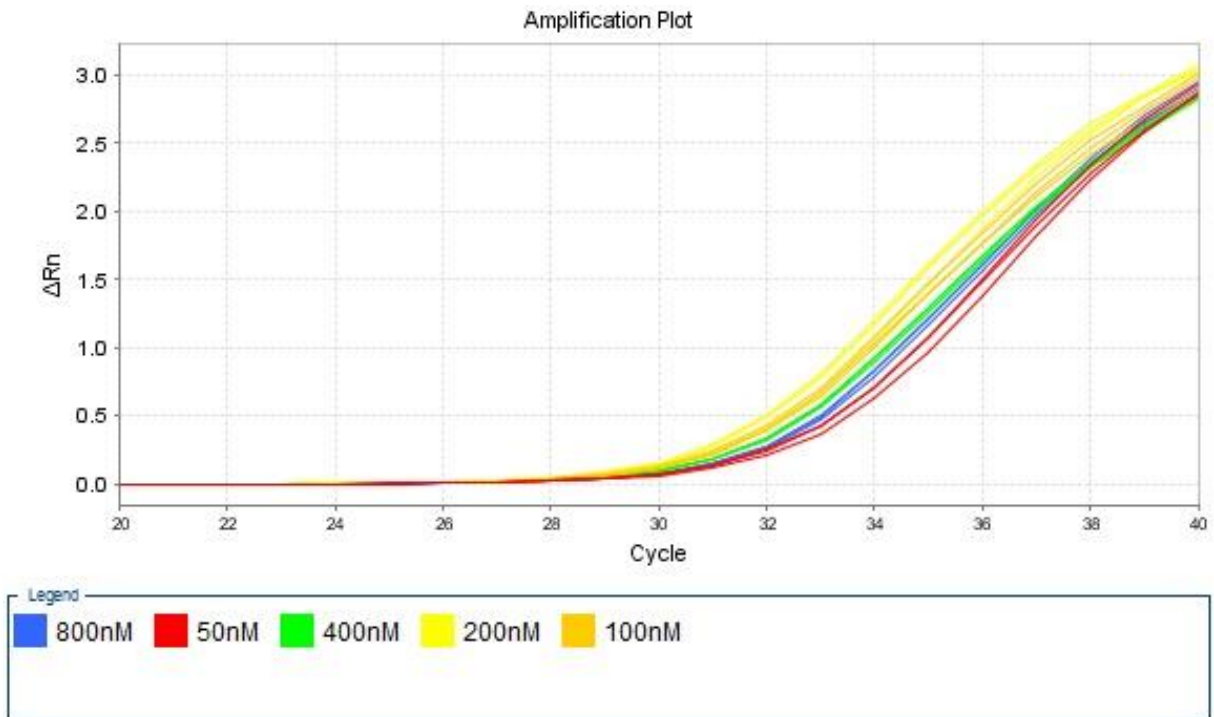

Figure 3.3. Primer concentration optimisation curve of a TaqMan® MGB assay to detect Vectormune® HVT NDV.

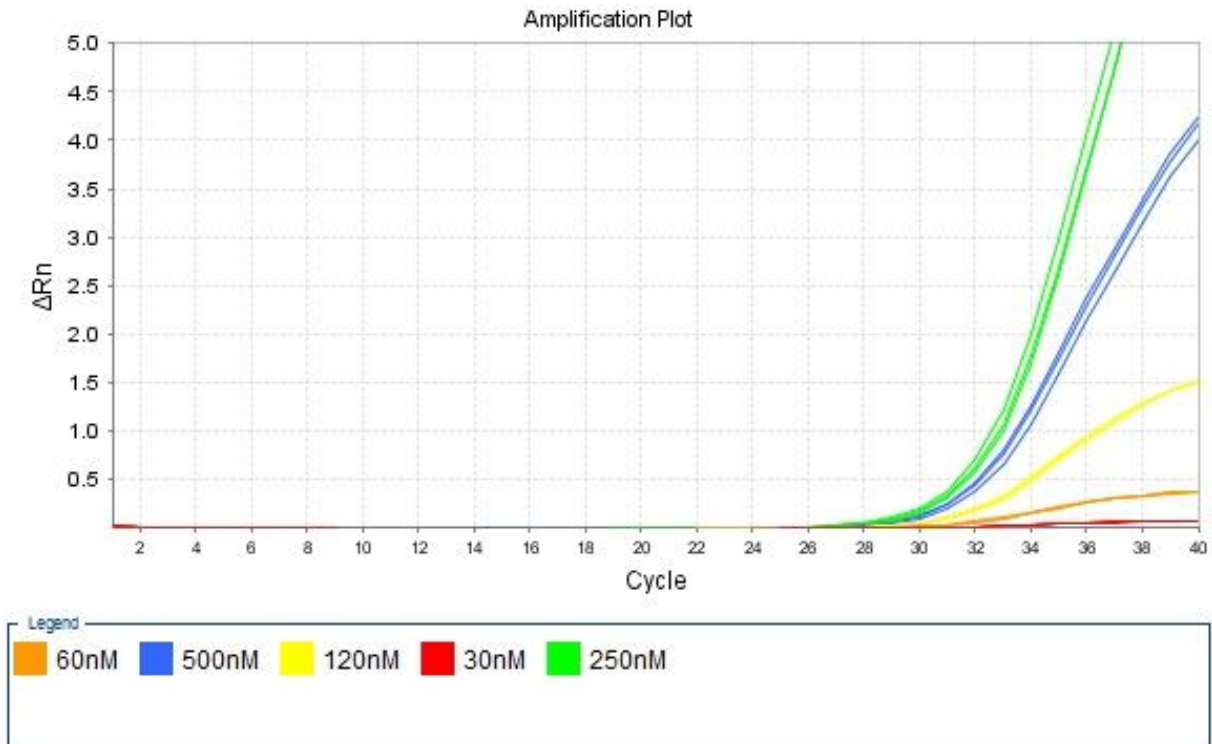An optimum probe concentration of 200 nM was selected (Figure 3.4).

Figure 3.4. Probe concentration optimisation curve of a TaqMan® MGB assay to detect Vectormune® HVT NDV.

3.3. Assay characteristics

3.3.1. Linear range and efficiency

To analyse the efficiency of the PCR assay developed, a standard curve was generated (Figure 3.5). The assay was linear between $10^{3.76}$ and $10^{-1.24}$ minimum PFU/reaction, a range of five logs. At a low concentration of the target ($10^{-2.24}$ minimum PFU/reaction), the assay appeared to be non-linear. The efficiency of the developed real time PCR assay was 81.8%. The coefficient of correlation (R2) that indicates the linear regression between the standard curve line and the individual C_T data points from the standard reaction was 0.9942.
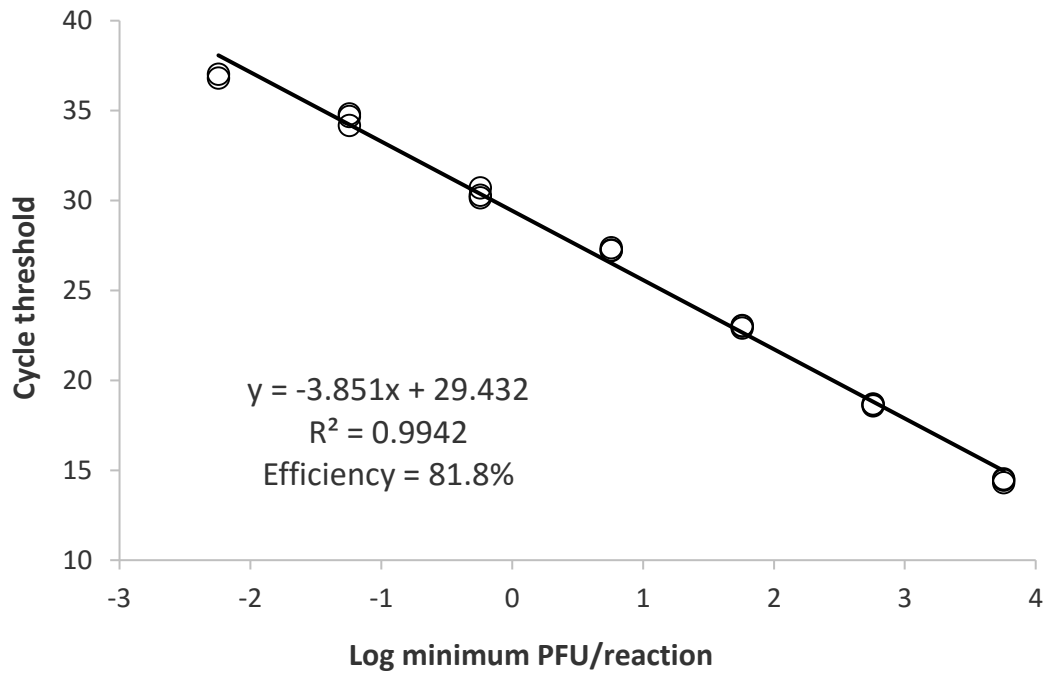
Figure 3.5. Standard curve of a TaqMan® MGB assay to detect Vectormune® HVT NDV. Cycle threshold (C_T) values from replicates plotted against logarithm minimum plaque forming units (PFU).

3.3.2. Analytical sensitivity

The two fold serial dilution using the $10^{-3.3}$ dilution of the vaccine was used as the starting point to determine the 95% LOD (Figure 3.6).

Figure 3.6. The 95% limit of detection of a TaqMan® MGB assay to detect Vectormune® HVT NDV.

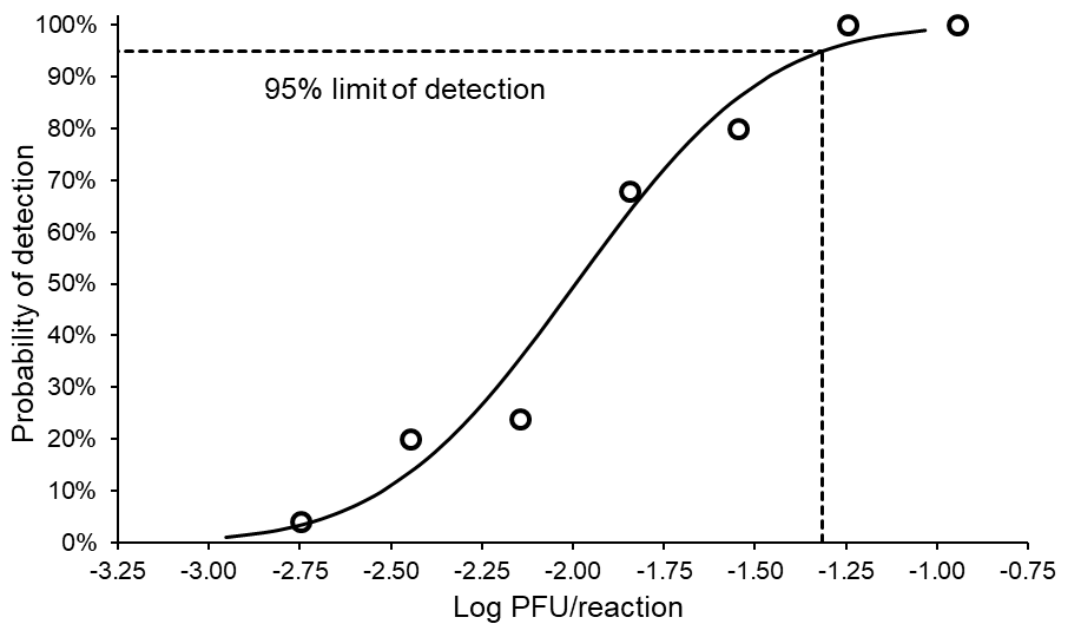The 95% LOD was $10^{-1.31}$ PFU/reaction (95% confidence interval: $10^{-1.47}$ - $10^{-1.07}$).

3.3.3. Analytical specificity

The developed TaqMan® MGB assay did not detect the nucleic acid of any isolates, with the exception of the D26 strain (Table 3.2). The isolates were detected when tested with a group-specific AA1 TaqMan® MGB assay, based on targeting the M gene (Wise *et al*., 2004). The isolates were not detected when analysed with the assay developed to detect Vectormune® HVT NDV, indicating the specificity of the assay.

Table 3.2. AA1 isolates detected when tested with a modified group-specific AA1 TaqMan® MGB assay* (Wise *et al*., 2004), but not when tested with a TaqMan® MGB assay developed to detect Vectormune® HVT NDV, with the exception of the D26 strain.

AA1 isolate identification	Storage	RT-PCR*	Real-time PCR
374-001	Lyophilised	18.90	undetermined
28/08-NDV 2V	Lyophilised	25.35	undetermined
ND 1996-983/001-26/6/001	Lyophilised	23.22	undetermined
ND 1996-903/001-26/6/001	Lyophilised	20.29	undetermined
ND 1996-776/001-26/6/001	Lyophilised	17.99	undetermined
Lasota 17/3/09	Frozen	12.67	undetermined
171/06 2AS 10/2/09	Frozen	19.41	undetermined
D26^-strain-Vectormune HVT-NDV vaccine	Frozen	35.04	15.25
Negative control	-	undetermined	undetermined

3.3.4. Variation

The inter-run and intra-run values were low, with the highest values of 0.5 and 1.73 respectively (Table 3.3). The coefficient variance ranged from 0.7% to 4.3%.

Table 3.3. Variation of TaqMan® MGB assay to detect Vectormune® HVT NDV. Nucleic acid from the samples were purified five times and each purified sample analysed five times (total of 25 analyses/sample). C_T - cycle threshold, CV - coefficient of variation, PFU – plaque forming units, SD - standard deviation.

Min PFU/reaction	Inter-run SD	Intra-run SD	Total SD	Total CV (%)
9.12×10^{-1}	0.10	0.42	0.41	1.4
4.56×10^{-1}	0.19	0.35	0.38	1.3
2.28×10^{-1}	0.34	0.37	0.46	1.5
1.14×10^{-1}	0.14	0.32	0.34	1.1
5.70×10^{-2}	0.22	0.66	0.65	2.0
2.85×10^{-2}	0.33	0.87	0.87	2.4
1.43×10^{-2}	0.50	1.73	1.56	4.3
7.13×10^{-3}	0.19	0.20	0.25	0.7
3.56×10^{-3}	0.29	0.12	0.26	0.7

3.4. Testing of field samples

The developed TaqMan® MGB assay specific for Vectormune® HVT NDV was specific, as it detected all the Vectormune® HVT NDV samples and there was no amplification of the samples with live, lentogenic vaccine (Table 3.4).

Table 3.4. Results from spleen impression smear samples collected on FTA cards from chickens vaccinated with either Vectormune® HVT NDV or ND conventional vaccine (NDV), and tested with a TaqMan® MGB assay to detect Vectormune® HVT NDV.

Sample	C_T value	PCR result
Live, low NDV vaccine 1	undetermined	negative
Live, low NDV vaccine 2	undetermined	negative
Live, low NDV vaccine 2	undetermined	negative
Live, low NDV vaccine 4	undetermined	negative
Live, low NDV vaccine 5	undetermined	negative
Live, low NDV vaccine 6	undetermined	negative
Live, low NDV vaccine 7	undetermined	negative
Live, low NDV vaccine 8	undetermined	negative
Live, low NDV vaccine 9	undetermined	negative
Live, low NDV vaccine 10	undetermined	negative
Live, low NDV vaccine 11	undetermined	negative
Live, low NDV vaccine 12	undetermined	negative
Negative control	undetermined	negative
Vectormune vaccine 1	34.95	positive
Vectormune vaccine 2	32.78	positive
Vectormune vaccine 3	30.25	positive
Vectormune vaccine 4	31.60	positive
Vectormune vaccine 5	34.45	positive
Vectormune vaccine 6	31.11	positive
Vectormune vaccine 7	32.08	positive
Vectormune vaccine 8	30.95	positive
Vectormune vaccine 9	28.06	positive
Vectormune vaccine 10	31.22	positive
Vectormune vaccine 11	30.98	positive
Vectormune vaccine 12	32.21	positive
Positive control	35.09	positive

CHAPTER
4. DISCUSSION

AA1 is a listed virus and requires reporting to the World Organisation for Animal Health in the event of an outbreak (OIE, 2012). The Newcastle disease has a major economic impact on the poultry industry worldwide and is kept under control by intense vaccination programmes, good biosecurity practices and regular surveillance done at the commercial level. Regardless of these efforts, the poultry industry experiences sporadic outbreaks, which are suspected to be caused by backyard poultry production units and wild birds.

The use of live vaccines without markers to differentiate infected from vaccinated (DIVA) birds complicates the control and diagnosis of the disease. Current real-time polymerase chain reaction assays cannot differentiate field infections and AA1 F gene, recombinant vaccines. This project focused on developing and optimising a reliable, quick and specific real-time PCR assay that detects Vectormune® HVT NDV vaccine.

The F gene sequence of the D26 strain of AA1 contained in the Vectormune® HVT NDV vaccine sequence was downloaded from Genbank® and used to design a TaqMan® MGB assay. The vaccine strain Clone 30 with accession no (Y18898) was identified. This strain is used in other Newcastle disease vaccines that use the F gene in their construct and is similar to Vectormune® HVT NDV. The possibility exists that the assay that was developed may cross-react with the Clone 30 vaccine, but there were various differences in a region of the probe between the sequences of the two vaccines when they were aligned (Figure 3.2), suggesting that it is unlikely that the assay will detect Clone 30. We were not able to test the Clone 30 vaccine as it is not currently available and not registered for use in the South African market.

The assay that was developed used a TaqMan® MGB probe, with a fluorescent reporter dye (FAM™) on the 5' end, a minor-groove binder moiety and a non-fluorescent quencher dye on the 3' end. TaqMan® MGB probes are short in length and enhance the melting temperature differentials between matched and mismatched probes. The main advantage of this type of probe is that they result in increased specificity of the PCR and they can be used in multiplex reactions. When using these probes, there is no need for post-PCR processing because the fluorogenic probes allow for the detection of specific amplification products, thus preventing the detection of non-specific PCR products (Applied Biosystems, 2004).

There are a few RT-PCR assays developed to detect AA1 field strains and differentiating virulent and avirulent NDV isolates that make use of a TaqMan® MGB hydrolysis probe. Most of these assays detect the fusion, matrix or haemagglutinin-neuraminidase genes (Aldous *et al*., 2001; Wise *et al*., 2004, Qin *et al*., 2008, Sato *et al*., 1987, Hua *et al*., 2007). Wise and colleagues (2004) focused on AA1 clinical samples and showed that their M gene assay was

more sensitive than a F gene assay. Occasionally, where not all isolates are identified, detection will rely on current in *vivo* methods used (ICPI) (Aldous *et al*., 2001).

The primers and probe were designed according to the following recommendations for probe and primer design (Primer Express guide, Applied Biosystems): the amplicon length was 63 bp long to ensure PCR efficiency (recommended 50 – 150 bp), the probe was 13 bp long (recommended 13-25 bp for a MGB probe and 13-30 bp for a TAMRA probe), the melting temperature was 69°C (recommended 68 - 70°C), the percentage GC was 54% (recommended 30 - 80%) and there was no G residue on the 5'-end, as this could interfere with the fluorescence of the reporter dye.

To determine the efficiency of the assay, the slope of the semi-log regression line plot of C_T value versus log of input nucleic acid was used (Bio-Rad manual, 2006). The efficiency of the developed assay was 81.8%, which was lower than the recommended PCR efficiency value between 98 and 100% (Tomás *et al*., 2012; Wang *et al*., 2017). The efficiency results obtained were similar to what Rathogwa and colleagues (2014) obtained when developing a real-time PCR assay that detects equine encephalosis virus. Low efficiency may be due to factors such as sample quality, primer quality, amplicon length, DNA concentration, PCR inhibitors, poor pipetting techniques or suboptimal reaction conditions (Bio-Rad manual, 2006; Raymaekers *et al.,* 2009). Dilutions were made initially with RNase-free water, but it appeared that the cDNA was not very stable. When dilution were repeated using sterile diluent (Ceva), much better results were obtained.

The assay was specific and did not show any cross reactivity, but there was a weak amplification of Vectormune® HVT NDV when tested with an assay that targets the M gene of AA1 (Wise *et al.,* 2004). The result may be a false positive result, but our negative control was consistently negative when the assay was repeated. It is also possible that the assay may have cross-reacted with HVT. The HVT genome consists of 79 putative genes and 73 of those are single copy, 66 are found within an unique long region and seven in an unique short region. HVT contains homologues found in herpes simplex virus type 1, six genes with homologues in MDV and two genes (HVT 068 and HVT 070 genes) that are unique to HVT (Afonso *et al.,* 2001; Hall *et al.,* 2015).

A total of 24 field samples (12 Vectormune® HVT NDV and 12 live, low virulent conventional Newcastle disease vaccine) preserved on FAT cards were tested. Different purification methods, such as soaking the FTA cards in lysis or TE buffer, incubation overnight or for 60 minutes, and at different temperatures (37°C and 56°C), did not give very good results. An in-house protocol using a QIAmp DNA mini kit that made use of proteinase K was used and gave better results. The assay detected all Vectormune® HVT NDV samples and there was no amplification of samples vaccinated with conventional NDV vaccine (Table 3.4).

In conclusion the developed real-time PCR assay in this study has been shown to be suitable for detecting Vectormune® HVT NDV vaccine. The assay is sensitive, specific reproducible and suitable for assaying large numbers of samples. However the reagents used for this type of assay are expensive and require the use of expensive real-time PCR equipment that many laboratories do not have. The developed assay will contribute to the poultry industry, as it can be used as a monitoring tool for poultry vaccinated with Vectormune® HVT NDV. It will largely assist in differentiating chickens that are vaccinated with Vectormune® HVT NDV vaccine from AA1 field infections and other NDV vaccines on the market. The test can also be used during outbreaks and for monitoring vaccine efficiency.

5. REFERENCES

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6. APPENDIX

6.1. Customized protocol for purification of nucleic acid using a MagMax™ Express Particle Processor.

[PLATE LAYOUT]
Plate type=PCR plate 200ul, Microstrips
A:
Volume 50, name=Vectomune NDV HVT vaccine
Volume 65 Isopropanol, 65 lysis binding solution
Volume 20, name=bead mix (10ul RNA binding beads, 10ul lysis/binding enhancer)
B:
Volume 150, name=wash solution 1
C:
Volume 150, name=wash solution 1
D:
Volume 150, name=wash solution 2
E:
Volume 150, name=wash solution 2
F:
Volume 50, name=Elution buffer
G:
Empty
H:
Empty

[STEPS]

BIND
Step parameters
Name=lysis binding 5 min
Well A, Default
Beginning of step:
No action=Yes
Bind parameters:
Bind time=5min 0s, speed=fast dual mix
End of step:
Collect beads=Yes, count =5

WASH
Step parameters
Name=1st Wash 1 1min
Well Default
Beginning of step:
Release=Yes, time=0s, speed=fast
Wash parameters
Wash time: 1 min 0s, speed=fast
End of step:
Collect beads=Yes, count=3

WASH
Step parameters
Name=2nd Wash 1 1min
Well Default
Beginning of step:
Release=Yes, time=0s, speed=fast
Wash parameters
Wash time: 1 min 0s, speed=fast
End of step:
Collect beads=Yes, count=3

WASH
Step parameters
Name=1st Wash 1 1min
Well Default
Beginning of step:
Release=Yes, time=0s, speed=fast
Wash parameters
Wash time: 1 min 0s, speed=fast
End of step:
Collect beads=Yes, count=2

WASH
Step parameters
Name=1st Wash 1 1min
Well Default
Beginning of step:
Release=Yes, time=0s, speed=fast
Wash parameters
Wash time: 1 min 0s, speed=fast
End of step:
Collect beads=Yes, count=2

DRY
Step parameters
Name=Dry 1 min
Well: Default
Dry time=1min 0s
Tip position=outside well

ELUTION
Step parameters
Name=Elution 3 min
Well=Default
Beginning of steps:
Release=Yes, time=Os, speed=fast
Elution Parameters
Elution time=3min 0s, speed=bottom medium
Pause parameter
Pause for manual handling=No

Remove beads
Remove beads=Yes, collect count 5, disposal well=B

6.2. Genbank accession numbers of F gene sequences used to design an assay specific for the D26 strain of AA1.

M24692	DQ485274	EU330230	FJ969393	GQ245814	HQ398797
AB465605	EF128053	EU346659	FJ969394	GQ245815	HQ398798
AB465606	EF128054	EU477188	FJ969395	GQ245816	HQ398799
AB465607	EF128055	EU477189	GQ245765	GQ245817	HQ398800
AB853329	EF464163	EU477190	GQ245766	GQ245818	HQ398801
AB853929	EF540729	EU477191	GQ245767	GQ245819	HQ398802
AB853930	EF564813	EU477192	GQ245768	GQ281085	HQ398803
AB853931	EF564814	EU493451	GQ245769	GQ281086	HQ398804
AB853932	EF564815	EU493454	GQ245770	GQ281087	HQ398805
AB853933	EF564816	EU583503	GQ245771	GQ281088	HQ398806
AB858995	EF564817	FJ011441	GQ245772	GU166154	HQ398807
AB858996	EF564818	FJ011442	GQ245773	GU227738	HQ398808
AB858997	EF564819	FJ011443	GQ245774	GU227739	HQ398809
AB858998	EF564820	FJ011448	GQ245775	GU332645	HQ398810
AB858999	EF564821	FJ426563	GQ245776	GU332646	HQ398811
AB859000	EF564822	FJ480774	GQ245777	GU332647	HQ398812
AB859001	EF564823	FJ480775	GQ245778	HM188393	HQ398813
AB859002	EF564824	FJ480776	GQ245779	HM188394	HQ398814
AB871655	EF564825	FJ480777	GQ245780	HM188395	HQ445943
AB871656	EF564826	FJ480778	GQ245781	HM188396	HQ445944
AB871657	EF564827	FJ480779	GQ245782	HM188397	HQ445945
AF048763	EF564828	FJ480780	GQ245783	HM188398	HQ445946
AF079172	EF564829	FJ480781	GQ245784	HM188400	HQ445947
AF079322	EF564830	FJ480782	GQ245785	HM188401	HQ445948
AF079323	EF564831	FJ480783	GQ245786	HM188402	HQ445949
AF079324	EF564832	FJ480784	GQ245787	HM625835	HQ445950
AF109885	EF564833	FJ480785	GQ245788	HM748944	HQ917080
AF456438	EF589131	FJ480786	GQ245789	HM748945	HQ917083
AF456442	EF589132	FJ480787	GQ245790	HM748946	HQ997378
AY028995	EF589133	FJ480788	GQ245791	HM748947	HQ997379
AY034801	EF589134	FJ487637	GQ245792	HM748948	HQ997380
AY325799	EF589135	FJ600539	GQ245793	HM776583	HQ997381
AY325909	EF589136	FJ600542	GQ245794	HQ398777	HQ997382
AY338284	EF589137	FJ665433	GQ245795	HQ398778	HQ997383
AY341061	EF589138	FJ772446	GQ245796	HQ398779	HQ997384
AY427817	EF592500	FJ772449	GQ245797	HQ398780	HQ997385
AY508514	EF592501	FJ772452	GQ245798	HQ398781	HQ997386
AY727881	EF592502	FJ772455	GQ245799	HQ398782	HQ997387
AY727882	EF592503	FJ772458	GQ245800	HQ398783	HQ997388
AY727883	EF592504	FJ772463	GQ245801	HQ398784	HQ997389
AY734534	EF592505	FJ772466	GQ245802	HQ398785	HQ997390
AY734535	EF592506	FJ772469	GQ245803	HQ398786	HQ997391
AY734536	EF592507	FJ772472	GQ245804	HQ398787	HQ997392
DQ195265	EF592508	FJ772475	GQ245805	HQ398788	HQ997393
DQ485256	EF592509	FJ772478	GQ245806	HQ398789	HQ997394
DQ485257	EF592510	FJ772481	GQ245807	HQ398790	HQ997395
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DQ485261	EU140950	FJ772494	GQ245811	HQ398794	JF824013
DQ485269	EU239663	FJ882014	GQ245812	HQ398795	JF824032
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JN255774	JN942038	JQ966080	JX901315	JX901372	KC503453
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JN255776	JN942040	JQ966082	JX901317	JX901374	KC503455
JN255777	JN942041	JQ966083	JX901318	JX901375	KC503456
JN255778	JN942042	JQ966084	JX901319	JX901376	KC503457
JN255779	JN942043	JQ966085	JX901320	JX901377	KC503458
JN255780	JN942044	JQ966086	JX901321	JX915243	KC503459
JN255781	JN942045	JX094510	JX901322	KC020114	KC503460
JN255782	JN967789	JX186997	JX901323	KC424427	KC503461
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JN682188	JQ013864	JX244798	JX901333	KC503414	KC503471
JN682189	JQ013865	JX244799	JX901334	KC503415	KC503472
JN682190	JQ013866	JX244800	JX901335	KC503416	KC503473
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JN941993	JQ013874	JX436340	JX901343	KC503424	KC503481
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JN942021	JQ039393	JX840454	JX901357	KC503438	KC750154
JN942023	JQ039394	JX840455	JX901358	KC503439	KC750155
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KF767467	KJ525687	KJ525718	KR869091	KT892752	KU295453
KF767468	KJ525688	KJ600777	KR869092	KT892753	KU295454
KF767469	KJ525689	KJ600778	KT380032	KT892754	KU295455
KF767470	KJ525690	KJ600779	KT381585	KT892755	KU594613
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KJ136261	KJ525695	KJ865706	KT381590	KT965730	KU594618
KJ174522	KJ525696	KJ865709	KT381591	KT965731	KU594619
KJ450977	KJ525697	KJ865711	KT381592	KT965732	
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KJ525672	KJ525703	KJ958914	KT381599	KU200242	
KJ525673	KJ525704	KM016457	KT381600	KU200243	

6.3. Ethical clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Development of PCR specific for vectormune-Newcastle disease vaccine
PROJECT NUMBER	V019-16
RESEARCHER/PRINCIPAL INVESTIGATOR	MBN Phala

STUDENT NUMBER (where applicable)	UP_23376598
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SPECIES	n/a	
NUMBER OF ANIMALS	n/a	
Approval period to use animals for research/testing purposes		February 2016-February 2017
SUPERVISOR	Dr. M Quan	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	9 February 2016
CHAIRMAN: UP Animal Ethics Committee	Signature	