

**Development of a real-time RT-PCR assay specific for
the Winterfield 2512 strain of infectious bursal
disease virus**

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Declaration

I, Juliet Ndubu Kabajani, hereby declare that all the work that has been carried out in this study was done by me as a student under the supervision and support of Professor Melvyn Quan at the Department of Veterinary Tropical Diseases and that there is no falsification of results. All sources of information used have been acknowledged.

Signed.....

Place.....

Date.....

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Finally, I would like to give my appreciation to Ceva South Africa as the sponsor of the study, for such a remarkable opportunity, allowing me to work with their vaccine and other resources.

Dedication

I would like to dedicate this thesis to my family (my husband Bernard Sililo, our two boys Joshua and Jordan Sililo).

Table of Contents

1. INTRODUCTION	1
1.1. Background	1
1.2. Aim of the study.....	1
1.3. Objectives	1
2. LITERATURE REVIEW	2
2.1. IBDV genome	3
2.2. Pathogenesis and transmission of IBDV	4
2.3. Clinical signs and lesions of IBDV	5
2.4. Economic impact of IBDV.....	7
2.5. Control of IBDV	8
2.6. Cevac® Transmune® IBD vaccine	10
2.7. Diagnosis of IBDV	12
3. Materials and Methods.....	16
3.1. Viral strain	16
3.2. Nucleic acid purification.....	16
3.3. Sequencing.....	17
3.3.1. Sequencing primer design	17
3.3.2. RT-PCR	17
3.3.3. Gel electrophoresis.....	17
3.3.4. PCR purification	18
3.3.5. Sequencing analysis.....	18
3.4. Assay design	18
3.5. Assay optimisation	19
3.6. Laboratory validation.....	19
3.6.1. Assay efficiency	19
3.6.2. Analytical sensitivity	20
3.6.3. Analytical specificity	20
3.6.4. Inter and intra-run variation.....	20
3.7. Analysis of field samples	20
4. RESULTS.....	22
4.1. Sequencing.....	22
4.1.1. Sequencing primer design	22
4.1.2. Gel electrophoresis.....	23
4.1.3. Sequence assembly	25
4.2. Assay design	25
4.3. Assay optimisation	27
4.4. Laboratory validation.....	28
4.4.1. Assay efficiency	28
4.4.2. Assay sensitivity.....	29
4.4.3. Assay specificity.....	30

4.4.4. Assay variation.....30

4.5. Analysis of field samples31

5. DISCUSSION33

5.1. Benefits arising from the study.....39

6. REFERENCES41

APPENDIX A: Customized protocol for the MagMax™ Express Particle Processor (Life Technologies)47

APPENDIX B: IBDV Segment A GenBank accession numbers.....50

APPENDIX C: IBDV Segment B GenBank accession numbers51

APPENDIX D: IBDV Segment A - W2512 sequence.....52

APPENDIX E: Animal ethics committee approval certificate.....53

List of Figures

Figure 2.1. Structure of <i>Avibirnavirus</i> , showing VP2, VP1 covalently attached to virus protein genomic end (Vpg) and T=13 which is a total number of icosahedral capsid proteins of the virus. (ViralZone:www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics).....	4
Figure 2.2. Genomic characteristics of <i>Avibirnavirus</i> showing the open reading frames (ORF) of segment A and segment B, RNA-directed RNA polymerase VP1 (RdRpVP1). (ViralZone:www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics).....	4
Figure 2.3. Normal (right) and atrophied IBDV-infected bursa (left) three days post infection (Intervet, 1999).	5
Figure 2.4. IBDV infected chicken (right) (Intervet, 2013) appears to be depressed and has ruffled feathers. The healthy, uninfected chicken (left) seems to be alert, with smooth feathers and good body condition.	6
Figure 2.5. Swollen and hyperaemic bursa of Fabricius, due to IBDV infection (Mohammed <i>et al.</i> , 2013).	7
Figure 2.6. Chicken carcass affected with IBD, showing haemorrhages in the thigh muscles (Mohammed <i>et al.</i> , 2013).	7
Figure 2.7. Transmune® IBD vaccine packaging and dosage (Ceva, 1999 – 2014).	10
Figure 2.8. Transmune® IBD vaccine strain (W2512) binding to specific antibodies and virus protecting immunoglobulins (van Oort <i>et al.</i> , 2016).	11
Figure 2.9. Structure and mechanism of action of TaqMan probe. Before PCR begins, the TaqMan® probe is intact and the reporter and quencher are in close proximity to each other. The quencher absorbs the energy of the reporter dye due to Förster resonance energy transfer (FRET). During PCR, the primers and probe anneal to the target. DNA polymerase extends the primer upstream of the probe. If the probe is bound to the correct target sequence, then the polymerase's nuclease activity cleaves the probe, releasing a fragment containing the reporter dye. Once cleavage takes place, the reporter and quencher dyes are no longer in close proximity to each other. The emission of fluorescence is due to the degradation of the bound hydrolysis probe by the 5'-3'exonuclease activity of the polymerase, fluorescence is measured during the extension phase (Navarro <i>et al.</i> , 2015).	14
Figure 3.1. Transmune® IBD vaccine containing Winterfied 2512 IBDV strain, 2000 doses.....	16
Figure 3.2. Impression smears of bursa samples collected on Whatman® FTA cards, samples 1 to 12 were W2512 negative (collected from chickens that were vaccinated with TABic® M.B. (Phibro) vaccine), samples 13 to 24 were W2512 positive (collected from chickens that were vaccinated with Cevac® Transmune® IBD vaccine).	21
Figure 3.3. QIAamp DNA mini kit Qiagen extraction reagents, used in the protocol of extracting RNA from FTA cards.	21
Figure 4.1. Amplification of Transmune® IBD using conventional RT-PCR. DNA ladder: 100 bp (SN0243, ThermoFisher Scientific) loaded in outside lanes. Migration was from top to bottom, Lane 1: fragment B of IBDV W2512, size 301 bp. Lane 2 – 9 are Segment A of IBDV: 2 and 3 were not detected, 4 – the fragment amplified is from position 812 -1372 = 560 bp, 5 - the fragment amplified is from position 1239 – 1798 = 559 bp, 6 - the fragment amplified is from position 1622-	

2189 = 567 bp, 7 - the fragment amplified is from position 2000-2554 = 554, 8 was not detected, 9 - the fragment amplified is from position 2611-3255 = 644 bp.....	23
Figure 4.2. Conventional RT-PCR gel electrophoresis (100 bp DNA marker (SN0243, ThermoFisher Scientific) loaded in outside lanes). Migration was from top to bottom. A band in a corresponding lane is the detected cDNA. Lane 1 is Segment B of IBDV W2512, size 301 bp. Lane 2 – 9 are Segment A of IBDV W2512, 3 - the fragment amplified is from position 416-982 = 566 bp, sizes (bp) for fragments 4 – 7 & 9 are as shown in Figure 4.1, fragments 2 and 8 were not detected. .	24
Figure 4.3. Repeat of conventional RT-PCR gel electrophoresis (100 bp DNA marker (M) SM 0313, ThermoFisher Scientific). Migration was from top to bottom. Fragment 2 of segment A detected = 591 bp and segment B detected.	24
Figure 4.4. Sequence variation in the primers and probe region of a TaqMan® MGB assay to detect SNP2451 IBDV W2512, using NC004178 for numbering. The dotted lines represent identical nucleotides to the first sequence. Sequences are identified by Genbank accession numbers, followed by the number of sequences that have identical sequences. *Not able to confirm if this isolate is a field or vaccine virus sequence. The arrows and the block in between represent the primers and probe respectively.	26
Figure 4.5. Primer optimisation of a TaqMan® MGB assay targeting SNP2451 IBDV W2512. Threshold baseline of 0.1 (blue horizontal line). The vaccine was detected at all concentrations of primer.	27
Figure 4.6. Probe optimisation of a TaqMan® MGB assay targeting SNP2451 IBDV W2512. Threshold baseline of 0.1 (green horizontal line). The vaccine was detected at all concentrations of probe.	28
Figure 4.7. A TaqMan® MGB assay targeting SNP2451 IBDV W2512 standard curve, generated from a ten-fold serial dilution. Assay efficiency is 87%, $R^2 = 0.9924$	29
Figure 4.8. A TaqMan® MGB assay targeting SNP2451 IBDV W2512 limit of detection curve.	29
Figure 4.9. Specificity of a TaqMan® MGB assay targeting SNP2451 IBDV W2512. Other IBDV isolates (Isolate IBD, IBD #6 vero and isolate 1327108) were not detected.....	30

List of Tables

Table 2.1. IBDV isolates adapted for production of attenuated vaccines (Lasher and Shane, 1994).....	9
Table 3.1. Real-time RT-PCR thermal cycling conditions.	19
Table 4.1. Sequencing primers for IBDV Segment A and B, designed using PrimerQuest online tool (Integrated DNA Technologies). AY029166 was used as a reference sequence. * This primer did not work and was replaced by ^.	22
Table 4.2. Assay SNP 2451 primers and probe sequences, designed by using Primer Express V3.0.1, (Applied Biosystems).....	25
Table 4.3: Variation of a TaqMan™ MGB assay specific for Transmune® IBD vaccine. Log CID ₅₀ – logarithm of chicken infection dose ₅₀ , SD - standard deviation, CV - coefficient of variation.	31
Table 4.4. C _T values obtained from analysis of field samples tested with a TaqMan™ MGB assay specific for Transmune® IBD vaccine, using a C _T threshold of 0.1. Neg - negative, indicates samples collected from chickens that were vaccinated with Tabic M.B vaccine. Pos - positive indicates samples collected from chickens that were vaccinated with Transmune® IBD vaccine.	31
Table 5.1. Recommended practices to consider under various conditions in order to get good RT-PCR results (Applied Biosystems guide to relative quantitation RT-PCR).	38

Glossary of terms and abbreviations

AAC	Antigen antibody complex
AC-ELISA	Antigen capture enzyme-linked immunosorbent assay
AGID	Agar gel immunodiffusion
Asp	Analytical specificity
BF	Bursa of Fabricius
cDNA	Complimentary deoxyribonucleic acid
CI	Confidence interval
CID ₅₀	Chicken infection dose ₅₀ (viral load that can infect 50% of chickens)
C _T	Cycle threshold
CV	Coefficient of variation
ddNTPs	Dideoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme linked immunosorbent assay
EtBr	Ethidium bromide
HVR	Hypervariable region
IBDV	Infectious bursal disease virus
ICx	Immune complex
LOD	Limit of detection
NA	Nucleic acid
NGS	Next generation sequencing
NFQ	Non-fluorescent quencher
MDA	Maternally derived antibodies
MGB	Minor groove binding
OIE	World Organisation for Animal Health
R ²	Coefficient of determination
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RT	Reverse transcriptase
SD	Standard deviation
SNP	Single nucleotide polymorphism
VPI	Virus protecting immunoglobulin
VNT	Virus neutralisation test
W2512	Winterfield 2512 strain

Abstract

Cevac® Transmune® IBD was developed by CEVA Santé Animale for vaccination of poultry against infectious bursal disease (IBD, or Gumboro). The vaccine contains the Winterfield 2512 (W2512) strain of infectious bursal disease virus (IBDV). There is currently no rapid method to detect this strain specifically in fowl vaccinated with the Transmune® vaccine.

This study describes the development and optimisation of a real-time RT-PCR assay that is sensitive and specific for the W2512 strain in Cevac® Transmune® IBD vaccine. IBDV sequences available publically on Genbank were downloaded and aligned. No sequence unique to IBDV W2512 Segment B was identified in the downloaded sequences, so sequencing of the IBDV W2512 Segment A was undertaken.

A single nucleotide polymorphism (SNP) at nucleotide position 2451 of segment A of IBDV W2512 was identified. An assay targeting this SNP was designed and made use of a TaqMan™ minor groove binding (MGB) probe. The optimum primer and probe concentrations of the assay were 200 nM and 100 nM respectively. The efficiency of the assay was 87%, with an R² of 0.9924. The 95% limit of detection (LOD) was determined through probit analysis (SPSS) of 25 replicates of a two-fold dilution series and determined to be of 4.7x10⁻⁵ chicken infection dose (CID₅₀) (95% confidence interval of 1.7x10⁻⁵ to 3.4x10⁻⁵). The assay was found to be potentially specific after testing other IBDV strains (IBD#6 vero, isolate 1327108, isolate IBDV). However, there is a need to investigate the specificity further by validating the assay with more IBDV field and vaccine strains. The assay was also tested on 12 W2512 positive and 12 W2512 negative impression smears of bursa samples stored on Whatman® FTA cards. All the 12-W2512 negative samples tested negative while 11 out of 12-W2512 positive samples tested positive with this assay.

Based on these performance characteristics and findings, the assay is sensitive and could be specific for Cevac® Transmune® IBD's W2512 vaccine strain. It is rapid, efficient, easy to perform and may be suitable for its intended use, which is to detect W2512 IBDV strain in poultry flock vaccinated with Cevac® Transmune® IBD vaccine.

Key words: Winterfield 2512, Ceva, Transmune®, Infectious Bursal Disease Virus, Gumboro, reverse transcriptase polymerase chain reaction (RT-PCR), RNA.

CHAPTER

1. INTRODUCTION

1.1. Background

Cevac[®] Transmune[®] IBD is an immune–complex infectious bursal disease (IBD) vaccine developed by Ceva Santé Animale for active immunization of poultry against IBD. Cevac[®] Transmune[®] IBD vaccine contains the live IBDV Winterfield 2512 (W2512) G-61 strain, in complex with infectious bursal disease virus (IBDV) immunoglobulins, in a freeze-dried form. The W2512 strain is an intermediate plus strain of IBDV. Live vaccines are referred to as mild, intermediate, or ‘intermediate plus’ (‘hot’ or ‘invasive’), based on their increasing ability to i) replicate and cause lymphocytic depletion in the bursa, and to ii) overcome residual maternally derived antibodies (MDA) (OIE Terrestrial manual, 2016). This means that it can stimulate an immune response in the presence of MDA. The vaccine can be administered via the *in ovo* route to 18 days old embryonated eggs or via the subcutaneous route to day old chicks.

There is no rapid method to specifically detect fowl vaccinated with the W2512 strain of IBDV used in the Cevac[®] Transmune[®] IBD vaccine. The current technique of confirming the presence of IBDV is by using PCR, followed by restriction fragment length polymorphism (RFLP) and genetic sequencing, which is laborious and time consuming. In this study, we investigated whether a more rapid and simple assay could be developed to detect this vaccine strain in fowl.

1.2. Aim of the study

Develop a real-time reverse transcription polymerase chain reaction (RT-PCR) assay specific for W2512 IBDV strain in Cevac[®] Transmune[®] IBD vaccine.

1.3. Objectives

- Sequence the W2512 vaccine strain of IBDV, using primers developed for conventional PCR.
- Identify sequences unique to W2512 of Cevac[®] Transmune[®] IBD vaccine.
- Develop and optimise a real-time RT-PCR assay specific to W2512 IBDV strain in Cevac[®] Transmune[®] IBD vaccine.

CHAPTER

2. LITERATURE REVIEW

Infectious bursal disease virus (IBDV) is the aetiological agent for a highly contagious disease of young chickens, causing severe inflammation of the bursa of Fabricius (BF) and immunodeficiency (Müller *et al.*, 1979). It was described initially by Cosgrove in 1957 in Gumboro – southern Delaware, hence it's commonly known as Gumboro disease. It was also referred to as avian nephrosis, due to its cause of tubular degenerative lesions in the kidney (Cosgrove, 1962). Due to these characteristic kidney lesions, the initial description was that the disease was caused by a variant infectious bronchitis virus (Gray strain) Winterfield and Hitchner, 1962). This misunderstanding occurred because the two infections occurred concurrently on a frequent basis, and it was a challenge to isolate or identify the actual aetiological agent, due to limited capacity of diagnostic tools at the time (Lasher and Davis, 1997). The disease appeared to be highly infectious and contagious and affected chickens showed signs of ruffled feathers, watery diarrhoea, trembling and prostration (Cosgrove, 1962). Cosgrove, (1962) further reported that the disease affected young chicks up to 5 weeks of age severely, older birds were also affected, but with mild or no clinical signs. Edgar (1966), as quoted by Lasher and Davis (1997), was the first to name the syndrome infectious bursal disease in his paper presented at the 10th annual poultry health and management short course at Clemson University in 1966. One of the outcomes from this session was the official adoption of Edgar's naming of the syndrome as infectious bursal disease and not avian nephrosis (Lasher and Davis, 1997).

Nagarajan and Kibenge (1997) proposed that IBDV should be regarded as one of the most important viral pathogens affecting the commercial poultry industry. IBD is a World Organisation for Animal Health (OIE) listed disease, due to its economic impact and potential to affect trade. The most likely sources of contamination during commercial trade of poultry products are live animals and poultry meat.

IBDV is classified as a birnavirus. The “bi” prefix signifies the double stranded ribonucleic acid (RNA) and the bi-segmented nature of the virus genome, while “rna” refers to the nucleic acid of the genome. It belongs to the family *Birnaviridae* and the genus *Avibirnavirus* (King *et al.*, 2011). Other members of the family *Birnaviridae* are the aquatic viruses such as infectious pancreatic necrosis virus (IPNV), Tellina virus (TV), yellowtail ascites virus (Aquabirnavirus), blotched snakehead virus (Blosnavirus) and Drosophila X virus (DXV) (Entomobirnavirus). Members of this family contain a double stranded (ds)RNA genome consisting of two segments A and B, of molecular weights 2.2×10^6 daltons and 2.5×10^6 daltons respectively,

within a non-enveloped single-shelled icosahedral capsid of 60 nm diameter (Dobos *et al.*, 1979; King *et al.*, 2011; Müller *et al.*, 1979).

IBDV invades and replicates in the BF. It targets and infects actively dividing and differentiating lymphocytes of the B-cell lineage (Burkhardt and Müller, 1987). This leads to B-cell immunodeficiency (Kibenge *et al.*, 1988), making chickens more susceptible to other infections and attributing to their poor immune response to vaccines against diseases such as Marek's disease and infectious bronchitis (Lasher and Shane, 1994).

2.1. IBDV genome

The genome of IBDV consists of two segments designated A and B (Müller *et al.*, 1979). The larger segment A is approximately 3400 base pairs (bp) long, while the smaller genome segment B is approximately 2800 bp long (van den Berg, 2000; King *et al.*, 2011). Segment A encodes for viral structural proteins VP2 and VP3, the protease VP4 and the non-structural protein VP5. Segment A has two open reading frames (ORF) of which the smaller ORF partially overlaps the larger ORF and encodes for VP5 - a non-structural protein not essential for viral replication, but considered to play a role in releasing virus particles from the cell. The larger ORF encodes for structural proteins VP4, VP3 and VP2, of which VP4 is a protease that shares common features with the bacterial ion protease and cleaves the polyprotein to generate the three polypeptides (pre-VP2, VP3 and VP4). Pre-VP2 is processed by a slow maturation cleavage to produce VP2 (King *et al.*, 2011). VP3 forms the inner surface of the capsid and is a group specific antigen (Müller *et al.*, 2003). The outer viral capsid is comprised of trimeric VP2 (Xu *et al.*, 2014) (Figure 2.1), a virulence protein where the major neutralising epitopes are located (Michael and Jackwood, 2005). The VP2 gene contains a hyper variable region (HVR) that is used as an indicator for different IBDV strains in some diagnostic protocols, such as immunohistochemistry and genomic sequencing (Kusk *et al.*, 2005). In reviewing various investigations addressing the molecular basis for antigenic and virulence variations of IBDV, it appeared that there are amino acid residues located in the central variable regions of VP2 and they are a cause for antigenic variation in IBDV (Nagarajan and Kibenge, 1997).

The smaller segment B encodes VP1 (Kusk *et al.*, 2005), which has RNA-dependent RNA polymerase (RdRP) enzyme activities (Müller *et al.*, 2003) (Figure 2.1 and Figure 2.2).

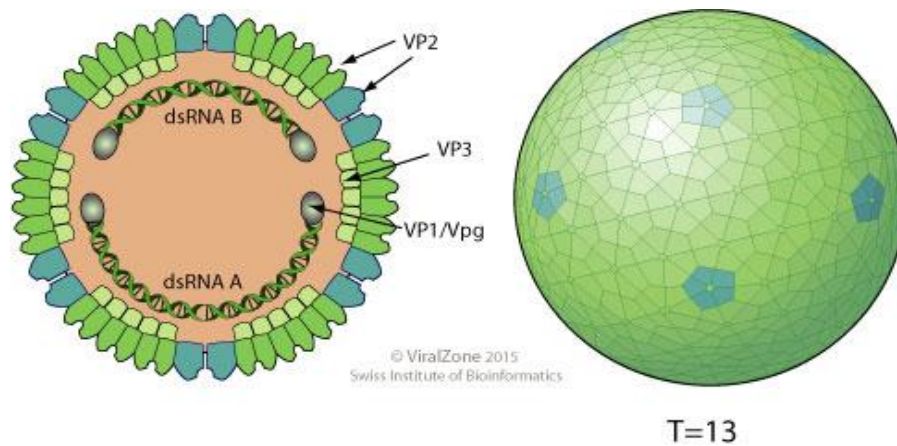


Figure 2.1. Structure of *Avibirnavirus*, showing VP2, VP1 covalently attached to virus protein genomic end (Vpg) and T=13 which is a total number of icosahedral capsid proteins of the virus. (ViralZone:www.expassy.org/viralzone, SIB Swiss Institute of Bioinformatics).

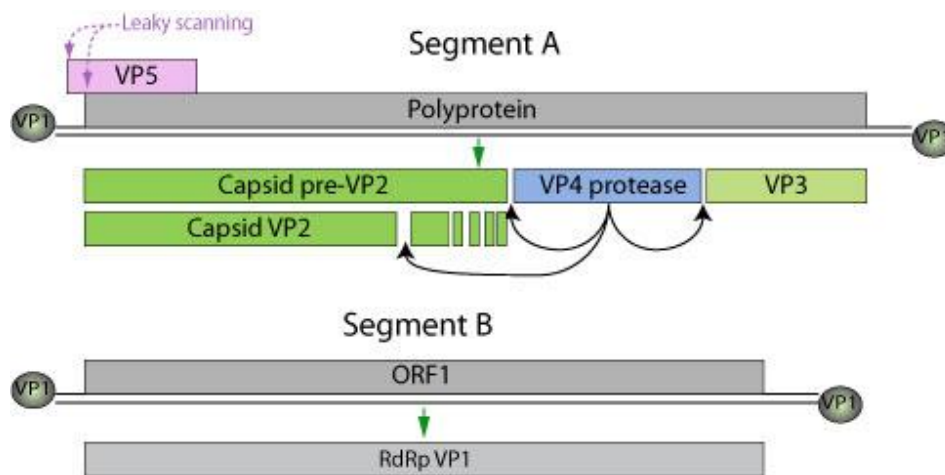


Figure 2.2. Genomic characteristics of *Avibirnavirus* showing the open reading frames (ORF) of segment A and segment B, RNA-directed RNA polymerase VP1 (RdRpVP1). (ViralZone:www.expassy.org/viralzone, SIB Swiss Institute of Bioinformatics).

2.2. Pathogenesis and transmission of IBDV

IBDV replicates in differentiating lymphocytes of the BF, causing immunosuppression (Jenberie *et al.*, 2014; Li *et al.*, 2007). The virus is extremely lymphocidal, with an affinity for immature B cells, resulting in bursal atrophy which becomes evident after four days of infection (Lasher and Shane, 1994) (Figure 2.3). From embryonic development up to approximately 10 weeks of age, lymphocytes migrate to the BF to become programmed into antibody-producing cells. If the BF is damaged due to IBDV infection, it will not be capable to produce sufficient lymphocytes and thus the chickens will be immunosuppressed (Butcher and Miles, 1995).

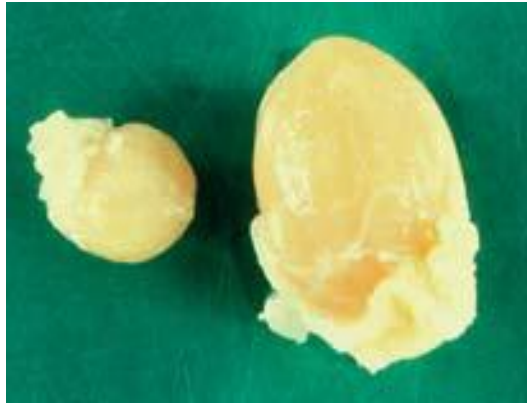


Figure 2.3. Normal (right) and atrophied IBDV-infected bursa (left) three days post infection (Intervet, 1999).

Benton *et al.*, (1967) conducted studies on the transmission of IBDV and demonstrated that it is highly contagious and can easily spread by direct contact with infected fowl, ingestion of contaminated feed and water. The disease can also be transmitted by indirect contact with fomites (van den Berg *et al.*, 2000), or via the adult lesser worm (*Alphitobus diaperinus*), which is capable of serving as a reservoir for IBDV, rather than a fomite (McAllister *et al.*, 1995). Mechanical transmission can also occur through poor biosecurity measures such as, uncontrolled movement of farm workers, equipment and vehicles among farms (Butcher and Miles, 1995).

IBDV is extremely resistant in the environment and even after strict cleaning and disinfecting procedures is able to persist in houses (Lasher and Shane, 1994). Materials from contaminated environments spread the disease in susceptible flocks and contaminated buildings still contained infective virus 122 days after removal of infected chickens, indicating how long the virus may persist and remain viable in the environment (Benton *et al.*, 1967). This supports the reason why the disease was characterised by spreading from one pen to another in a poultry house and the tendency to recur in successive broods (Cosgrove, 1962).

2.3. Clinical signs and lesions of IBDV

The natural hosts of IBDV are chickens, although ducks, turkeys, ostriches and other domestic fowls may also be infected (King *et al.*, 2011; Aiyeduni, 2014). However, the clinical disease occurs solely in chickens and particularly those younger than 10 weeks (Cosgrove, 1962; OIE Terrestrial Manual, 2016).

Although the clinical signs of IBD vary considerably from one farm, region, country or even continent to another (van den Berg *et al.*, 2000), it can be classified into three forms: the classical form caused by classical virulent strains and characterised by low mortalities, the

immunosuppressive or subclinical form which is caused by low pathogenicity strains, and the acute form caused by very virulent strains, which cause clinical disease and high mortality. The primary feature of very virulent IBDV is the ability to induce higher mortality than classic IBDV strains in susceptible chickens (Stoute *et al.*, 2009).

In severe acute cases, the incubation period is short: two to three days (van den Berg, 2000), chickens of 3 - 6 weeks old are usually affected, and the disease is characterised by high mortality of 20 - 30% (Teshome *et al.*, 2015). Clinical signs include anorexia, dehydration, trembling, ruffled feathers, vent pecking, depression and death (Cosgrove, 1962, Butcher and Miles, 1995; van den Berg, 2000; OIE Terrestrial Manual, 2016) (Figure 2.4).



Figure 2.4. IBDV infected chicken (right) (Intervet, 2013) appears to be depressed and has ruffled feathers. The healthy, uninfected chicken (left) seems to be alert, with smooth feathers and good body condition.

On necropsy, the major lesions are found in the BF, which is often swollen, oedematous and hyperaemic. In some cases, the BF can be completely haemorrhagic, resembling a black berry grossly (Figure 2.5). There is often a yellowish transudate covering the serosa within the first four days, followed by bursal atrophy later during the course of the disease. Muscular and proventricular haemorrhages or nephritis and areas of necrosis may be present in more severe cases (Figure 2.6) (Butcher and Miles, 1995; van den Berg, 2000; OIE Terrestrial Manual, 2016).



Figure 2.5. Swollen and hyperaemic bursa of Fabricius, due to IBDV infection (Mohammed *et al.*, 2013).

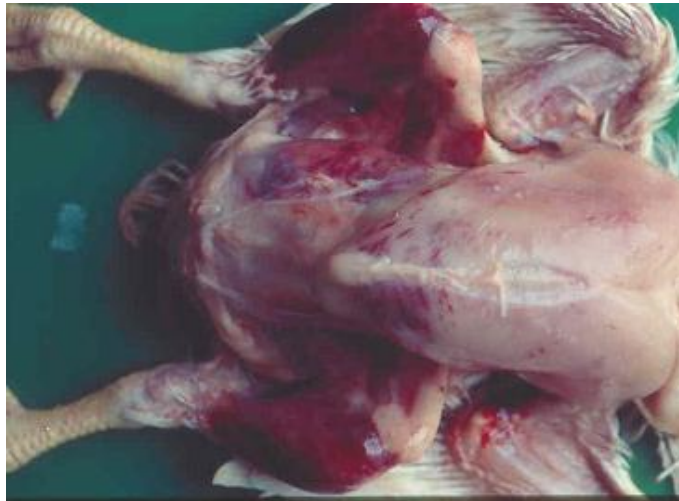


Figure 2.6. Chicken carcass affected with IBD, showing haemorrhages in the thigh muscles (Mohammed *et al.*, 2013).

On the other hand, the subclinical form occurs in chickens less than three weeks of age and presents no clinical signs of disease, but the chicks experience permanent and severe immunosuppression (Butcher and Miles, 1995). The only significant lesion associated with the subclinical form of IBD is bursal atrophy which can be observed microscopically when doing histopathology (OIE Terrestrial Manual, 2016).

2.4. Economic impact of IBDV

IBD is of economic importance as it affects both small and large-scale poultry farmers. Direct losses are specifically linked to mortality and depend on the dose and virulence of the strain

of the IBDV, age, breed of poultry, presence or absence of passive immunity (Aiyeduni, 2014; van den Berg *et al.*, 2000; Müller *et al.*, 2003).

Indirect losses occur from immunosuppression, growth rate and productivity may be adversely affected by subsequent exposure to a wide range of viral, bacterial, and protozoal agents. IBDV is therefore a major health and production constraint of young chickens (Lasher and Shane, 1994).

2.5. Control of IBDV

Vaccination of hens and the subsequent maternal immunity imparted to chicks is the primary means of controlling IBDV (Wu *et al.*, 2007). Despite advances in vaccination programmes, outbreaks of IBD still occur. One cause may be related to serological variants (Liu *et al.*, 1994). Therefore, differentiation and identification of local IBDV strains is crucial for selection of the appropriate vaccine strain (Jenberie *et al.*, 2014; Giambrone and Closser, 1990).

According to Zierenberg *et al.*, (2000), there are two distinct serotypes of the IBDV: serotype 1 viruses are pathogenic to chickens while serotype 2 viruses are non-pathogenic. Serotype 1, which causes naturally occurring disease in chickens, has been divided into several groups on the basis of antigenic variation and virulence: classical strains, variant strains and virulent strains. High genetic variability among different strains of IBDV results in different antigenic and pathogenic types of IBDV (Banda *et al.*, 2004).

Thus detection and strain identification of IBDV is important, because antigenic subtypes within serotype 1 make it necessary to design vaccination programmes specific to the antigenic type found in the bird's environment (Jackwood, 2004; Michael and Jackwood, 2005). Due to the low degree of protection offered by vaccination with heterologous antigenic subtypes, it would be helpful to match the vaccine subtype to the antigenic subtype present in the flock's environment (Michael and Jackwood, 2005). Furthermore, if multiple IBDV subtypes are present in the field, two or more IBDV sub-type vaccines may need to be combined for improved coverage, as is commonly done with inactivated IBDV vaccine products (Giambrone and Closser, 1990).

Vaccines made by different manufacturers are available to address the challenge of controlling IBD. A good vaccine should not cause disease or bursal lesions, must not be immunosuppressive nor be excreted, and must confer long lasting immunity, even in birds with high level of immunity (van den Berg *et al.*, 2000). Subtype 6 viruses are called variant because vaccines produced from viruses in the first 5 subtypes produce only minimal protection against subtype 6 viruses (Liu *et al.*, 1994). It should also be taken into

consideration that very virulent IBDV will break through immunity provided by highly attenuated vaccine strains. Alternatively, it is well known that less attenuated strains (“hot vaccines”) may cause lesions in the bursa follicles and immunosuppression even in vaccinated birds (Müller *et al.*, 2003). In the study conducted by Kelemen *et al.*, (2000), one of the observations was that the higher the virulence of the vaccine virus strain, the more severe the damage to the lymphocytes of the BF.

Selection of vaccines from the ‘mild,’ ‘intermediate,’ and low attenuation or ‘hot’ classification depends on management and stock-related factors, level and uniformity of maternal antibody transfer, virulence of field virus strains, and risk of challenge. Table 2.1 lists the significant and documented IBDV isolates adapted for production of attenuated vaccines from 1965 onwards (Lasher and Shane, 1994).

Table 2.1. IBDV isolates adapted for production of attenuated vaccines (Lasher and Shane, 1994).

Designation	Country	Vaccine type	Reference
Edgar	USA	No attenuation	Edgar and Cho (1965)
Moulthrop	USA	Intermediate	Moulthrop (1966)
2512 low passage	USA	Intermediate	Winterfield (1969)
244/1968	German	Intermediate	Bengsdorf and Bernhardt (1971)
I-65 PV	Italy	Mild	Rinaldi <i>et al.</i> , (1972)
Edgar-Lukert	USA	Mild to intermediate	Leonard (1974)
WB (PBG-98)	UK	Very mild	Baxendale (1976)
IZ	Italy	Mild	Winterfield and Thacker (1978)
MS	USA	Intermediate	Winterfield and Thacker, (1978)
N1/77 (PM 877)	Australia	Mild to intermediate	Chubb and Jackson (1979)
Cu-1M	German	Mild	Cursifien <i>et al.</i> , (1979)
Moulthrop-Edson	USA	Intermediate	Thayer (1980)
2512 intermediated passage	USA	Mild	Haffer (1982)
DL (clone D-78)	Holland	Mild to intermediate	Lütticken and Cornelissen (1985)
Delaware variant E	USA	Mild	Rosenberger <i>et al.</i> , (1987)
GLS-5	USA	Mild	Snyder <i>et al.</i> , (1988)
002-73	Australia	Mild to intermediate	Wood <i>et al.</i> , (1988)
Delaware variant A (TC)	USA	Mild	Cruz-Coy (1992)
V877	Australia	Intermediate	Claxton and McGavin (1993)
89/03	USA	Very mild	Hein <i>et al.</i> , (1993)

It is important to note that IBDV is a very persistent virus and can easily survive in the environment. Complete IBDV control is only possible with a strong focus on cleaning and disinfection, in addition to a breeder vaccination programme (van Oort *et al.*, 2016).

2.6. Cevac® Transmune® IBD vaccine

Cevac® Transmune® IBD is a vaccine manufactured by CEVA Santé Animale. It is a live virus vaccine that contains the Winterfield 2512 strain of IBDV, in complex with IBDV antibodies (an antigen-antibody complex [AAC] or immune-complex vaccine) in a freeze-dried form (CEVAC, 2000-2014) (Figure 2.7) The vaccine has been tested under different types of conditions, both in the laboratory and in field trials, and in all situations the vaccine demonstrated very good efficacy and excellent safety (Gardin *et al.*, 2008).

The name of the strain Winterfield 2512 is derived from its history, whereby the virus isolate that was modified for vaccine production was isolated initially from tissue samples recovered from case accession number 2512 at the Georgetown substation. Through various levels of embryo passage, Winterfield successfully demonstrated in 1969, that 2512 could protect against virulent field strains of IBDV (Winterfield, 1969), and thus this strain was named after Winterfield and became commonly known as Winterfield 2512.

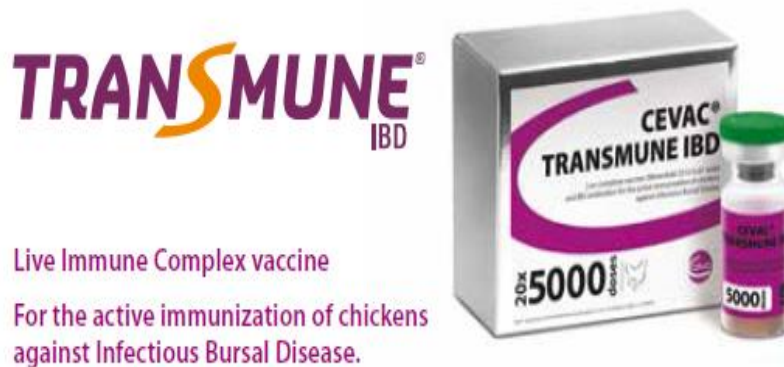


Figure 2.7. Transmune® IBD vaccine packaging and dosage (Ceva, 1999 – 2014).

Kelemen *et al.*, (2000) demonstrated the effects of three IBD vaccine strains: G87 (mild), IBDL (intermediate), IBDV 2512 (intermediate-plus) on specific pathogen free (SPF) broiler chicken embryos immunised *in ovo*. One of the findings was that only strain IBDV 2512 induced production of antibodies in chickens, which were protected against IBD by three weeks of age. Furthermore, weight gain in the IBDV 2512 vaccinated group was higher compared to controls and those vaccinated with other strains. Al-Mufarrej (2013) showed that the Cevac® vaccine induced significantly higher antibody titres against IBDV in chickens vaccinated at 18 days old

compared to those vaccinated with D78 IBDV (Intervet). Ivan *et al.*, (2001) observed that changes in the BF due to vaccine uptake were evident, although much milder and more transient in commercial broilers compared to specific pathogen free chickens. Furthermore, signs of complete bursa architecture and functionality restoration were evident in less than two weeks. The results obtained in the study by Dren *et al.*, (2005), demonstrated the compatibility of Cevac® Transmune® with HVT frozen vaccine in terms of Marek protection.

The principle behind how this vaccine works is that the immune-complex formulation protects the vaccine virus, out of the reach of MDA. As a consequence, vaccine uptake is ensured, whatever the MDA level is (Gardin *et al.*, 2008). The vaccine virus is covered and protected from recognition by the immune system of chickens by virus protecting immunoglobulins (VPI) which are stored in the same way as MDA in dendritic cells (van Oort *et al.*, 2016) (Figure 2.8). After vaccination, the immune complex is trapped by the follicular dendritic cells of the spleen until the level of MDA drops to a residual level that allows the virus to be released gradually (van Oort *et al.*, 2016). The virus then colonizes the bursa, viral replication takes place and induces an active immunity (Gardin *et al.*, 2008).

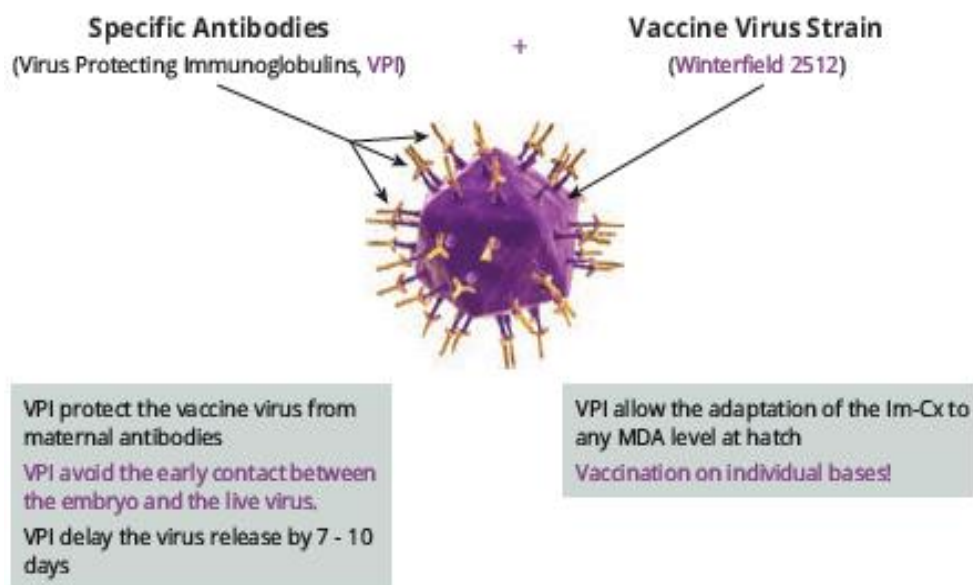


Figure 2.8. Transmune® IBD vaccine strain (W2512) binding to specific antibodies and virus protecting immunoglobulins (van Oort *et al.*, 2016).

The main advantage associated with an AAC vaccine is the capacity to stimulate antibody responses even when MDA are present, making vaccination much simpler and more effective for the producers: one shot, at one age, with one vaccine. Another advantage is the possibility to follow replication of the vaccine virus and the induction of the immune response at defined times using simple (histology and serology) or sophisticated (RT-PCR with RFLP or sequencing) techniques (Gardin *et al.*, 2008).

2.7. Diagnosis of IBDV

The current techniques to diagnose IBD are based on clinical signs, pathology, histopathology, virus isolation and serological tests. These techniques are all laborious, time consuming and in some cases, expensive (Kusk *et al.*, 2005). Early diagnosis of IBD is necessary for the control of the disease, and rapid detection and differentiation of IBDV serotypes is warranted (Lin *et al.*, 1994).

In chicken flocks, the clinical picture and the course of the disease are usually indicative of an IBDV infection. Pathological changes observed at the BF are characteristic, and histopathological investigations combined with the demonstration of viral antigens by immunohistochemistry confirm an IBDV infection (Müller *et al.*, 2003). IBDV can also be isolated by inoculation of antibody-free embryonated chicken eggs. Viral antigens can be demonstrated by the agar-gel precipitation assay or by the antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) (Müller *et al.*, 2003).

The OIE Terrestrial Manual (2016) recommends methods such as the use of histopathological examination of bursae, virus detection in the bursae by immunoassays (agar gel immunodiffusion (AGID) test, AC-ELISA, immunostaining), RT-PCR and virus characterisation (typing, antigenicity and nucleotide sequencing) for the purpose of confirming clinical cases. The Manual further recommends ELISA (for antibody detection) for purposes of establishing individual or population freedom from infection, monitoring of post-vaccination immune response in vaccinated chickens and developing eradication strategies.

Diagnostic approaches that are able to infer the presence of new antigenic subtypes are necessary to assist in the development of strategies for prevention and control of IBDV (Michael and Jackwood, 2005). Lukert and Saif (1991), as quoted by Lin *et al.*, (1994), stated that polyclonal and monoclonal antibodies have been developed and used to differentiate IBDV serotypes by the virus neutralization test (VNT). However, VNT requires that the amounts of IBDV or its replication products should be in a sufficient quantity to enable an antigen-antibody reaction (Lin *et al.*, 1994).

Rapid identification of viral strain is a primary focus to poultry flock health programs to ensure administered vaccines will protect against current strains of virus circulating in the flock (Peters *et al.*, 2005). Strain identification has been accomplished using a variety of post-RT-PCR assays, including restriction enzyme digestion of the RT-PCR products. The resulting restriction fragment length polymorphisms (RFLP) are used to differentiate viruses into molecular groups that correlate with antigenic and pathogenic types (Jackwood, 2004). The

RFLP discrimination system depends on the existence of specific restriction enzymes for single nucleotide polymorphism (SNP) identification (Tomás *et al.*, 2012).

When developing assays, it is important to consider and address the current challenges in diagnostics. As such, simpler and more rapid assays for detection and classification of IBDV strains are necessary for monitoring strain spread and improving the control of disease (Tomás *et al.*, 2012).

Conventional PCR for instance, requires a thermal cycler machine, to amplify target DNA fragments into thousands or millions of copies, following three steps of PCR (denaturation, annealing and extension). In the first step, DNA is denatured at high temperatures (90 - 97°C), breaking the hydrogen bonds in between and detaching the two DNA strands into single strands. In the second step, temperature is lowered (50-56°C) to allow primers to anneal to the DNA template strands, and in the third step extension by DNA polymerases occurs at 72°C, using nucleotides (A), thymine (T), cytosine (C) and guanine (G) as building blocks, a primer pair (small fragment of ssDNA) to which the nucleotides attach, and a DNA template for constructing the new complimentary DNA strand. These three steps are repeated in 20-40 cycles, the amount of DNA doubles with each subsequent cycle (Joshi and Deshpande, 2010).

Conventional PCR assays have disadvantages compared to real-time PCR. They rely on visualization of PCR products using intercalation of ethidium bromide (EtBr) (or other intercalating dyes), at an empirically determined PCR cycle number. These products migrate on agarose gels in an electric field and then quantified with radio-imaging or other densitometric means (Ginzinger, 2002).

Real-time PCR on the other hand, permits the analysis of the products while the reaction is in progress (Joshi and Deshpande, 2010). Real-time PCR, which does not require post-PCR processing on gels, has the advantages over conventional PCR of being rapid, sensitive and accurate (Wu *et al.*, 2007, Peters *et al.*, (2005). A limitation however of DNA polymerases (and real-time PCR itself) is that they only utilise DNA as their template, and cannot amplify RNA in a similar manner. This problem can be overcome by other enzymes called reverse transcriptases. They are enzymes generally derived from RNA-containing retroviruses, which generates complementary DNA (cDNA) from an RNA template and hence the name reverse transcription PCR (RT-PCR). Like PCR, RT-PCR can either be conventional or Real-time RT-PCR with advantages and disadvantages (Valasek and Repa, 2005).

In terms of IBDV diagnostics, real-time RT-PCR can rapidly detect and differentiate IBDV subtypes by subtype-specific primers and/or melting temperature measurement. It can provide additional information, such as quantification of virus for measurement of viral load

(Wu *et al.*, 2007). Its ability to quantify virus concurrently is necessary for identifying the progress of disease outbreaks within the flock (Peters *et al.*, (2005).

Real-time RT-PCR can be a one, or two-step procedure (Qiagen QPCR guide, 2010). With two-step RT-PCR, the RNA is first reverse transcribed into cDNA using oligo-dT primers, random oligomers, or gene-specific primer and then an aliquot of the reverse-transcription reaction is added to real-time PCR reagents, while in one-step both reverse transcription and real-time PCR take place in the same tube. The use of reverse transcriptase to evaluate RNA levels and the extension of PCR technology to quantify DNA amplification in real time has brought major advances to the application of PCR (Joshi and Deshpande, 2010).

Current real-time methods generally involve fluorogenic probes that fluoresce when excited, to show the quantity of amplicon at each cycle of PCR (Valasek and Repa, 2005). Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA-binding dyes (SYBR green I, Evagreen or Resolight) and real-time PCR instruments that measure fluorescence while performing the thermal cycling needed for the PCR (Life Technologies, real-time PCR handbook).

The qPCR instrument consists of a thermal cycler with an integrated excitation light source (a lamp, a laser or light emitting diode), a fluorescence detection system or fluorimeter and software that displays the recorded fluorescence data as a DNA amplification curve. It is necessary to add a dsDNA intercalating dye or fluorophore-labelled probe to the reaction mixture (Navarro *et al.*, 2015). Several types of probes exist, including hydrolysis probes (5-nuclease probes), hybridization probes, molecular beacons, sunrise and scorpion primers, and peptide nucleic acid (PNA) probes. Different probes have different mechanisms. However, they generally create a relation between fluorescence detected and quantity of target amplified (Valasek and Repa, 2005). An example of how this is achieved is shown in Figure 2.9, using a hydrolysis probe as an example.

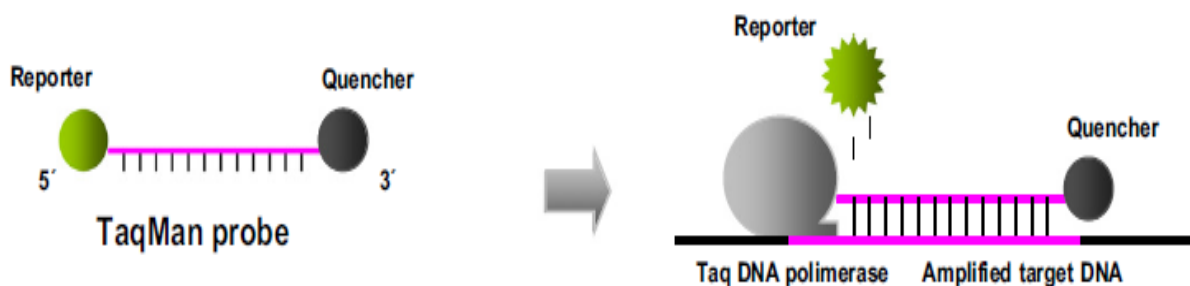


Figure 2.9. Structure and mechanism of action of TaqMan probe. Before PCR begins, the TaqMan® probe is intact and the reporter and quencher are in close proximity to each other. The quencher

absorbs the energy of the reporter dye due to Förster resonance energy transfer (FRET). During PCR, the primers and probe anneal to the target. DNA polymerase extends the primer upstream of the probe. If the probe is bound to the correct target sequence, then the polymerase's nuclease activity cleaves the probe, releasing a fragment containing the reporter dye. Once cleavage takes place, the reporter and quencher dyes are no longer in close proximity to each other. The emission of fluorescence is due to the degradation of the bound hydrolysis probe by the 5'-3' exonuclease activity of the polymerase, fluorescence is measured during the extension phase (Navarro *et al.*, 2015).

Several research and diagnostic assays have been developed for IBDV using RT-PCR. Tomás *et al.*, (2012) described the development and validation of a TaqMan minor groove binding (MGB) real-time RT-PCR assay to detect and discriminate virulent from non-virulent (classic and variant) IBDV strains using allelic discrimination probes and targeting a SNP contained in a highly conserved genomic region. This assay improved diagnostic capacity in conducting general and genotype specific detection and quantification. Ashraf *et al.*, (2007) developed a RT-PCR assay for differentiating various types of IBDV. Two sets of primers specific for serotype 2 and virulent IBDV were designed based on the sequences of segment A and B of IBDVs. Furthermore, Peters *et al.*, (2005) developed a real-time RT-PCR assay utilizing dual-labelled fluorescent probes which bind to VP4 specific sequences of the classical (CI), variant (V) and virulent strains of IBDV. In another study, virulent IBDV RNA in the BF and spleen from experimentally infected chickens or field samples was detected by *in situ* hybridisation (ISH) with subsequent RT-PCR and sequence analysis. Five IBDV isolates (V97/TW, T1/ TW, 2512, T2/CH and Luckert) were used in the study, of which the 2512 strain of IBDV was a vaccine strain used in Taiwan (Liu *et al.*, 2001). Jackwood, (2004) used a two-probe assay to identify single-nucleotide mutations among IBDV strains. A mutation probe was used in this assay to detect substitution mutations in a region of the viral genome that encodes a neutralizing epitope of the virus.

Although different types of real-time RT-PCR assays have been developed, there is none yet that has been developed to specifically detect the W2512 IBD vaccine strain. As such there is no rapid method to specifically detect fowl vaccinated with the Winterfield 2512 strain of IBDV used in the Cevac® Transmune® IBD vaccine. The current test uses PCR to confirm the presence of IBDV in the BF of vaccinated adult chicken flocks, but is not specific. To characterise the strain of IBDV, either sequencing or RFLP is used. In this study, the focus was to develop a real-time RT-PCR that would detect the W2512 IBD vaccine strain in vaccinated poultry flocks. This will provide an effective and efficient way to specifically assess the vaccine status of chicken flocks vaccinated with Cevac® Transmune® IBD vaccine. It will also reduce costs for both poultry farmers and Ceva SantéAnimale, by eliminating the use of multiple, costly and lengthy tests. A real-time RT-PCR assay specific for the Cevac® vaccine will significantly reduce the turnaround time needed to get results and will be specific.

CHAPTER

3. Materials and Methods

3.1. Viral strain

Transmune® IBD vaccine (Ceva South Africa) was used in this project. It contained a freeze-dried IBDV (Winterfield 2512 G 61 strain in complex with IBD antibodies), with 2000 doses of virus/vial. The registration number was G3457, Act 36/1947 and batch/lot number 0201E4LYKHF (Figure 3.1). The vaccine was reconstituted by adding 10 ml of diluent (Ceva), aliquoted into volumes of 2 ml and kept at -80°C.

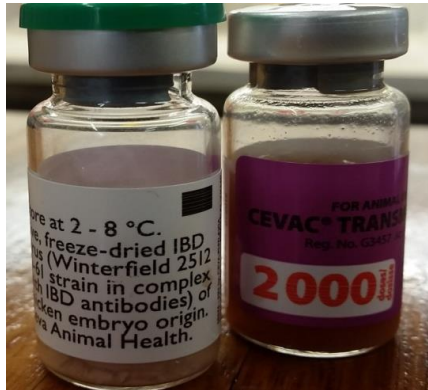


Figure 3.1. Transmune® IBD vaccine containing Winterfield 2512 IBDV strain, 2000 doses.

3.2. Nucleic acid purification

Virus RNA extraction was performed using the MagMax™ Pathogen RNA/DNA kit and the MagMax™ Express Magnetic Particle Processor (Life Technologies). It is based on the protocol of RNA isolation by Chirgwin *et al.*, (1979). The protocol utilise guanidium thiocyanate as a protein denaturant for rapid release of nucleic acid and protein denaturation so that the isolated nucleic acid is free of proteins. Other reagents include: alcohol (100% isopropanol) as a precipitant of nucleic acid, microspherical and paramagnetic beads create a larger surface area for nucleic acid molecules to be dispersed in solution, allowing efficient nucleic acid binding. The washing buffers 1 and 2 are for eliminating proteins and contaminants and excess binding solutions respectively. In the last step, nucleic acid is eluted with elution buffer. Reagents were prepared according to the manufacturer's guidelines as follows: 125 ml of 100% isopropanol was added to the bottle of Wash Solution 1 Concentrate and mixed. Similarly, 232 ml of 100% ethanol was added to the bottle of Wash Solution 2 Concentrate and

mixed. The bead mix was prepared by mixing 10 µl binding beads and 10 µl lysis enhancer per sample.

The sample and reagents were loaded onto an extraction plate, the plate was placed into a MagMax™ Express Magnetic Particle Processor and a custom protocol run (Appendix A) that took 15 min. The purified nucleic acid was transferred into Eppendorf tubes and stored at -80°C.

3.3. Sequencing

3.3.1. Sequencing primer design

IBDV sequences available on Genbank® (<https://www.ncbi.nlm.nih.gov>) were downloaded (see tables of accession numbers in Appendix B and C) and aligned online with MAFFT version 7.299b.1 (Standley, 2013). Sequences were edited with BioEdit v7.2.5 (Hall, 2013) and Dambe5 (Xia, 2013) was used to remove duplicate sequences. Sequencing primers were designed with the PrimerQuest Tool (Integrated DNA Technologies, Inc.), using AY029166 as a reference sequence. Sequencing primers were designed to amplify overlapping fragments of approximately 600 - 700 nucleotides in length.

3.3.2. RT-PCR

Purified nucleic acid (RNA) was amplified through a conventional RT-PCR using designed sequencing primers. RNA was transcribed into cDNA and amplified using a Veriti Thermal Cycler machine (Applied Biosystems). SuperScript™ III One-Step RT-PCR System (Invitrogen) was used as follows: 25 µl of 2X reaction mix, 2 µl of RNA template, 2 µl of Superscript™ III/*Taq* mix, 5 µl of sense and anti-sense primer (at 0.2 µM final concentration) and 20 µl of RNase free water, to make up a total volume of 50 µl. The thermal cycler was setup to run a protocol, as recommended by the manufacturer: reverse transcription at 60°C for 30 min, 94°C for 2 min, 40 cycles of denaturation at 94°C for 15 sec, annealing at 60 °C for 30 sec and extension at 68°C for 1 min, before a final extension at 68°C for 5 min.

3.3.3. Gel electrophoresis

Gel electrophoresis confirmed the presence of amplified cDNA visually. A 2% agarose gel was prepared in tris-acetate ethylenediaminetetraacetic (TAE) buffer (10 mM, pH 8.0). Five µl of a 100 bp DNA ladder/marker (SN 0243, ThermoFisher Scientific) were loaded into the outer wells, then 5 µl of each PCR products was mixed with 1 µl of loading dye, before loading onto

the gel. When samples had migrated half way, the gel was imaged with the Gel Doc (BioRad) imaging system to visualise the cDNA.

3.3.4. PCR purification

Amplicons were purified using CleanSweep™ (ThermoFisher Scientific) PCR Purification reagents, by adding 4 µl Cleansweep™ to 10 µl of PCR product. Using a Veriti Thermal Cycler machine (Applied Biosystems), samples were incubated at 37°C for 15 min, then 80°C for 15 min. Purified PCR products, together with respective forward and reverse primers at 6.4 pmol/µl, were submitted for Sanger sequencing (Inqaba Biotec).

3.3.5. Sequencing analysis

Sequences in an ab1 file format were quality trimmed and assembled using Pregap4 and Gap4 in the Staden 2.0.0b11-2016 package (Staden *et al.*, 2003).

This process involved investigating the electropherographs to evaluate the sequence quality, assembling of various fragment sequences, and editing through cut-offs of low quality sequences, fixing any discrepancies and creating consensus files and using different functions in the programme. The aim was to minimise errors, produce high quality sequences for further use.

Furthermore, sequences were aligned with ClustalX-2.1 (Larkin *et al.*, 2007), and used for developing an assay by determining a unique SNP.

3.4. Assay design

Consensus sequences of both segments A and B obtained from sequencing of IBDV W2512 vaccine strain were assembled with W2512 sequences that were downloaded from Genbank (see tables of accession numbers in Appendix B and C) and aligned using Clustal X V2.0 (Larkin *et al.*, 2007). Single nucleotide polymorphisms (SNPs) were identified, using sequence KJ198844 as a reference sequence. Primer express V3.0.1 (Applied Biosystems) was used to design a TaqMan™ minor groove binder (MGB) RT-PCR assay (Applied Biosystems, United Kingdom) to target and amplify the region with a specific SNP.

TaqMan® Fast Virus 1-Step Master Mix (ThermoFisher Scientific) was used to perform the assay on a StepOnePlus™, real-time PCR system (Applied Biosystems), with the manufacturer recommended cycling conditions indicated in Table 3.1. Two µl of RNA was added to 18 µl of

master mix, which was prepared as follows (per sample): 5 µl of 4x master mix, forward and reverse primers at 200 nM final concentration, probe at 100 nM final concentration and 12.5 µl RNase free water.

Table 3.1. Real-time RT-PCR thermal cycling conditions.

Thermal cycling step	Stage	Number of cycles	Temperature	Time
Reverse transcription (RT)	1	1	50°C	5 min
RT inactivation/initial denaturation	2	1	95°C	20 sec
Amplification and extension	3	40	95°C	3 sec
			60°C	30 sec

3.5. Assay optimisation

The primer concentration was optimised by keeping the probe concentration constant at 250 nM, while varying primer concentrations: 50 nM, 100 nM, 200 nM, 400 nM and 800 nM. Each concentration was tested in triplicate. Amplification curves were inspected visually and the lowest concentration of primer that yielded the lowest cycle threshold (C_T) curve was selected.

The probe concentration was optimised by using the optimised primer concentration determined in the previous experiment and varying the probe concentration: 50 nM, 100 nM, 150 nM, 200 nM and 400 nM. Similarly, each concentration was tested in triplicate and the lowest concentration of probe that yielded the lowest cycle threshold (C_T) curve was selected.

3.6. Laboratory validation

3.6.1. Assay efficiency

A vial of Transmune® IBD vaccine (lot number: 0201E4LYKHF) was used and a ten-fold dilution series from 10^0 to 10^{-8} was prepared. The nucleic acid was purified as described in 3.2 and real-time RT-PCR performed in triplicate for each dilution, using the thermal cycler temperature conditions described in Table 3.1. The resulting C_T values for each input amount of template were plotted as a function of the logarithm of chicken infection dose₅₀ (Log CID₅₀). A linear regression was calculated in Microsoft Excel and the slope of the regression line was used to calculate the efficiency of the assay using the following equation: PCR efficiency (%) = $100 \times (10^{1/\text{slope}} - 1)$.

3.6.2. Analytical sensitivity

A two-fold dilution series of the Transmune® IBD vaccine (lot number: 0201E4LYKHF), from $10^{-3.3}$ to $10^{-5.7}$, was made to cover the range from 100% to 0% detection. Five separate nucleic acid purifications were made from each dilution. Each purified dilution was tested five times in five separate runs. The 95% limit of detection (LOD) was determined by probit analysis using SPSS Statistics 24 (IBM Corp.).

3.6.3. Analytical specificity

The assay was used to test different IBDV strains from the Department of Veterinary Tropical Diseases, University of Pretoria: IBD#6 vero, isolate 1327108 and Isolate IBD. All the samples were diluted 1:10, before purifying the RNA.

3.6.4. Inter and intra-run variation

Results from 3.6.2 were used to calculate the intra-run and inter-run standard deviations (SD) and coefficient of variation (CV) with Microsoft Excel.

3.7. Analysis of field samples

The developed real-time RT-PCR assay was tested on impression smears of bursa samples collected on Whatman® FTA cards (Sigma-Aldrich), from chickens that were 21 and 28 days old. There were 12 W2512 bursa samples collected from chickens vaccinated with TABic® M.B. (Phibro) vaccine and 12 samples collected from chickens vaccinated with Cevac® Transmune® IBD vaccine (Figure 3.2).

The nucleic acid purification protocol developed for this assay was not suitable for the extraction of nucleic acid from Whatman® FTA cards, so a protocol using a QIAamp DNA mini extraction kit (Qiagen, Germany) was used (Figure 3.3). A 2 mm diameter disk of each FTA card was cut with a clean scalpel blade and placed into a labelled Eppendorf tube. ATL reagent (180 µl) was added to each tube and incubated for 10 min at 85°C, then 20 µl of Proteinase K was added to the sample and vortexed. Samples were incubated at 56°C for one hour. Thereafter, 200 µl of AL buffer was added and vortexed thoroughly, before incubating at 70°C for 10 min. Two hundred µl 96-100% ethanol was added to the samples, followed by vortexing for 15 sec. The mixture was transferred to a QiaAmp mini column, centrifuged at 8000 rpm for 1 min and the filtrate discarded. AW1 reagent (500 µl) was added to the column, centrifuged at 8000 rpm for 1 min, then 500 µl of AW2 added and centrifuged at 1400 rpm for 3 min. The columns

were transferred into new collection tubes and centrifuged at 1400 rpm for 1 min to remove residual AW2. The columns were then placed into labelled Eppendorf tubes, and 100 µl of buffer AE added to the column, incubated at room temperature for 2 min and centrifuged at 8000 rpm for 1 minute to elute the nucleic acid in the Eppendorf tube, and stored at -80°C.



Figure 3.2. Impression smears of bursa samples collected on Whatman® FTA cards, samples 1 to 12 were W2512 negative (collected from chickens that were vaccinated with TAbic® M.B. (Phibro) vaccine), samples 13 to 24 were W2512 positive (collected from chickens that were vaccinated with Cevac® Transmune® IBD vaccine).



Figure 3.3. QIAamp DNA mini kit Qiagen extraction reagents, used in the protocol of extracting RNA from FTA cards.

The real-time RT-PCR was then carried out with primers at a final concentration of 200 nM, probe at a final concentration of 100 nM and thermal cycling conditions as outlined in Table 3.1.

CHAPTER

4. RESULTS

4.1. Sequencing

4.1.1. Sequencing primer design

The segment B sequence of IBDV attenuated W2512 vaccine strain (AF083092) has been published (Yehuda *et al.*, 1999). Other IBDV segment B sequences (n = 558) were downloaded from Genbank and aligned with AF083092. After editing and removal of duplicate sequences, the number of sequences was reduced to 431. A single nucleotide polymorphism (SNP) – SNP1385, unique to W2512, was identified within a seven nucleotide region, between nucleotide positions 1382 and 1388 of segment B (using AF083092 as reference).

No segment A IBDV W2512 strain sequences were available in Genbank. However, six partial W2512 segment A sequences were identified. According to the literature the sequences were published in, they were vaccine strains. After searching IBDV sequence length in the range of 2000 – 4000 nucleotides, 137 near full length segment A IBDV sequences were downloaded from Genbank. The six partial IBDV W2512 segment A sequences were assembled with the 137 sequences of IBDV segment A, making a total of 143 sequences.

Sequencing primers were designed to confirm the presence of SNP1385 in IBDV segment B and sequencing primers were designed to sequence Segment A of Transmune® IBD vaccine (Table 4.1.).

Table 4.1. Sequencing primers for IBDV Segment A and B, designed using PrimerQuest online tool (Integrated DNA Technologies). AY029166 was used as a reference sequence. * This primer did not work and was replaced by ^.

	Start	Stop	Length	T _m	GC%	Fragment number	
>IBDV_2512_SegmentB							
Forward	CAGGATCGTCGAGTGGATATTG	1241	1263	22	62	50	1
Reverse	CTGCCTTGACCATAGGTCTTAAT	1521	1544	23	62	44	
>IBDV_2512_SegmentA							
Forward	GGATACGATCGGTCTGACCC*	1	21	20	63	60	2
Forward	TCTCCTTCTACAACGCTATCA^	91	111	22	63	46	
Reverse	TGTCGTTGATGTTGGCTGTT	571	591	20	63	45	3
Forward	TACAACACTGCAGGCTAGTGA	416	438	22	63	46	
Reverse	GCCGTCAGCCCATTGTC	965	982	17	63	65	

Forward	ACACTGTTCTCAGCCAACAT	812	832	20	62	45	4
Reverse	CAGACGGTCTTGATGCCAA	1353	1372	19	62	53	
Forward	ACTTCGAGCTGATCCCAAATC	1239	1260	21	62	48	5
Reverse	GCACCTCTCTTAACACGCAGTC	1775	1798	23	60	57	
Forward	AAAGCAAGAGCTGCCTCA	1622	1640	18	62	50	6
Reverse	TACTTTCTCAATCTCGCCACAA	2167	2189	22	62	41	
Forward	GACTACACCGTTGTCCCAATAG	2000	2022	22	62	50	7
Reverse	TCGCTGAGTGCGAAGTTG	2536	2554	18	62	56	
Forward	ATGGAAGCAGCAGCCAA	2444	2461	17	62	53	8
Reverse	CACGTCCATGGTTGATTTTCATAG	2974	2997	23	62	43	
Forward	GTCGCAAAGGGCCAAGTA	2611	2629	18	62	56	9
Reverse	CCGCGAACGGATCCAATT	3236	3255	19	63	53	

4.1.2. Gel electrophoresis

The following results were obtained after the first round of conventional RT-PCR of Transmune® IBD, using primers described in Table 4.1. Figure 4.1 shows the different amplicons of IBDV that were detected successfully.

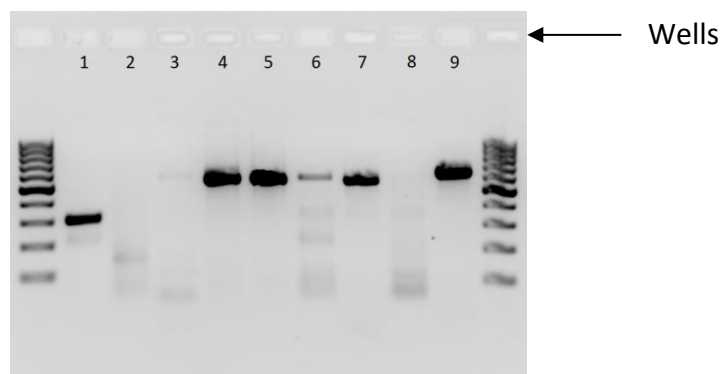


Figure 4.1. Amplification of Transmune® IBD using conventional RT-PCR. DNA ladder: 100 bp (SN0243, ThermoFisher Scientific) loaded in outside lanes. Migration was from top to bottom, Lane 1: fragment B of IBDV W2512, size 301 bp. Lane 2 – 9 are Segment A of IBDV: 2 and 3 were not detected, 4 – the fragment amplified is from position 812 -1372 = 560 bp, 5 - the fragment amplified is from position 1239 – 1798 = 559 bp, 6 - the fragment amplified is from position 1622-2189 = 567 bp, 7 - the fragment amplified is from position 2000-2554 = 554, 8 was not detected, 9 - the fragment amplified is from position 2611-3255 = 644 bp.

From the nine PCR reactions, six fragments were amplified successfully at high concentrations (lanes 1, 4, 5, 6, 7 and 9) while others (lane 2, 3 and 8) were not detected. The expected size of bands ranged from 301 and 644 bp.

The conventional RT-PCR was repeated at a lower temperature of 55°C (previous reactions were done at 60°C) to amplify fragments that were not amplified successfully. Segment A fragment 3 was detected at low concentration (Figure 4.2).

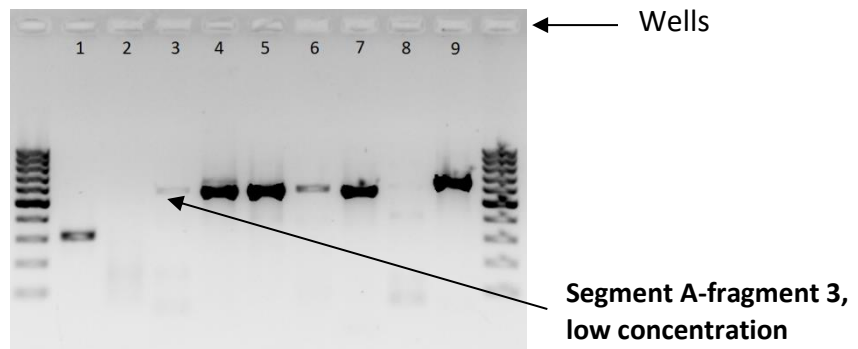


Figure 4.2. Conventional RT-PCR gel electrophoresis (100 bp DNA marker (SN0243, ThermoFisher Scientific) loaded in outside lanes). Migration was from top to bottom. A band in a corresponding lane is the detected cDNA. Lane 1 is Segment B of IBDV W2512, size 301 bp. Lane 2 – 9 are Segment A of IBDV W2512, 3 - the fragment amplified is from position 416-982 = 566 bp, sizes (bp) for fragments 4 – 7 & 9 are as shown in Figure 4.1, fragments 2 and 8 were not detected.

Other attempts to amplify the fragments 2 and 8 (Figure 4.1) included lowering the annealing temperature to 50°C, trying to amplify longer fragment with different primers (e.g. fragment 2 forward and fragment 3 reverse primers; fragment 7 forward and fragment 8 reverse primers). Fragment 4 with its respective primers was used as a positive control. The outcomes of these adjustments were that fragment 8 of segment A was amplified and detected, while there was no amplification for fragment 2 of segment A. Therefore, primers for fragment 2 of segment A were re-designed (Primer Quest online) (Table 4.1), which yielded good results (Figure 4.3) and then sequencing was repeated.

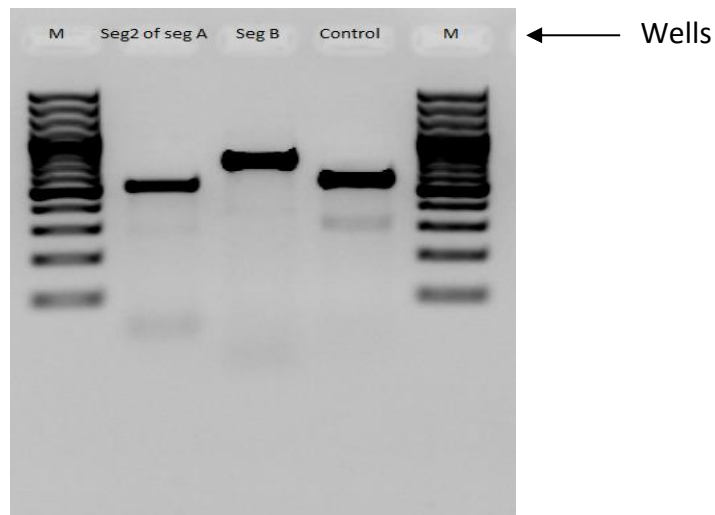


Figure 4.3. Repeat of conventional RT-PCR gel electrophoresis (100 bp DNA marker (M) SM 0313, ThermoFisher Scientific). Migration was from top to bottom. Fragment 2 of segment A detected = 591 bp and segment B detected.

Sequencing results of segment B could not confirm the presence of SNP1385, as indicated on Genbank.

4.1.3. Sequence assembly

IBDV W2512 Segment A from Transmune® IBD vaccine was assembled using the Staden programme package 2.0.0b11-2016 (Staden *et al.*, 2003). The analysis involved the alignment and editing of poor quality sequences

Using NC_004178 as reference sequence, which is a full length sequence 3183 nucleotides in length, a region from nucleotide position 416 – 3241 was sequenced (IBDV segment A W2512 sequence shown in Appendix D).

4.2. Assay design

The SNP1385 that was present in Segment B of AF083092 was not present in the Transmune® IBD vaccine, so this segment could not be used to design an assay unique to the vaccine. A unique SNP at nucleotide position 2451 of Segment A was identified, and was thus used for assay development. Primer Express software (V3.0.1, Applied Biosystems) was used to design a TaqMan® MGB assay (Table 4.2. and Figure 4.4).

Table 4.2. Assay SNP 2451 primers and probe sequences, designed by using Primer Express V3.0.1, (Applied Biosystems).

Type	Name	Nucleotide start	Nucleotide end	T _m
Forward Primer	TCGAGAGCGCCGTCAGA	2423	2439	59
Reverse Primer	ACACTGAGTGCAGATTGGAATAGTG	2492	2468	58
Probe	ATGGAAGCTGCGGC	2443	2456	67

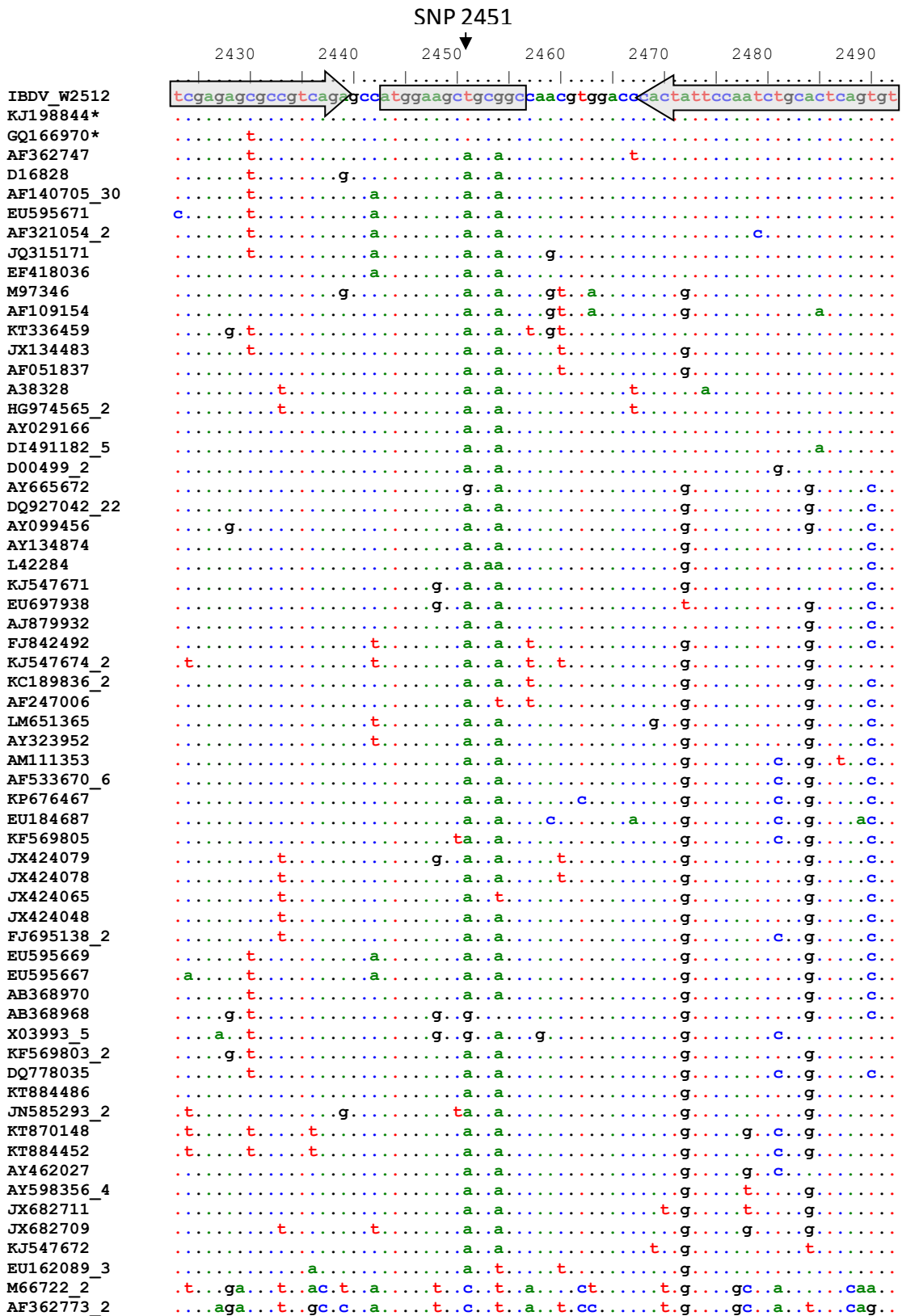


Figure 4.4. Sequence variation in the primers and probe region of a TaqMan® MGB assay to detect SNP2451 IBDV W2512, using NC004178 for numbering. The dotted lines represent identical nucleotides to the first sequence. Sequences are identified by Genbank accession numbers, followed by the number of sequences that have identical sequences. *Not able to confirm if this isolate is a field or vaccine virus sequence. The arrows and the block in between represent the primers and probe respectively.

4.3. Assay optimisation

The primers were optimised by testing various concentrations: 50 nM, 100 nM, 200 nM, 400 nM and 800 nM, while keeping the probe concentration constant at 200nM (Figure 4.5). The optimum concentration of primer was the lowest one that produced a low C_T value with a steep increase in fluorescence, determined to be 200 nM.

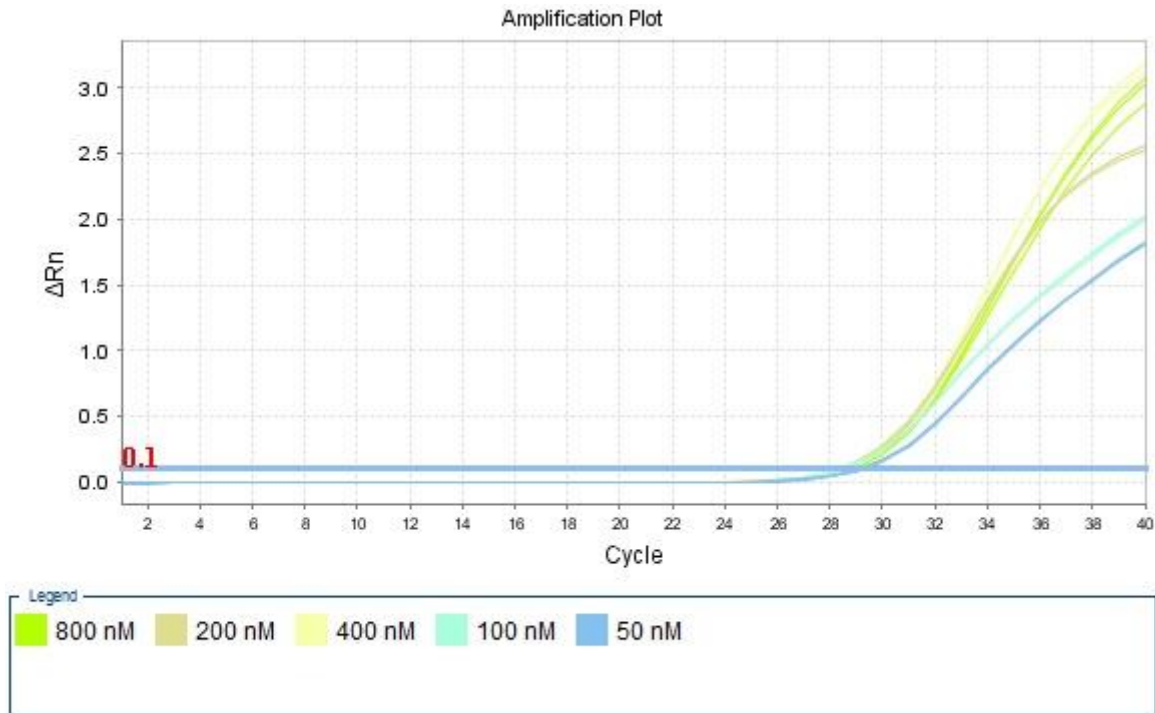


Figure 4.5. Primer optimisation of a TaqMan® MGB assay targeting SNP2451 IBDV W2512. Threshold baseline of 0.1 (blue horizontal line). The vaccine was detected at all concentrations of primer.

The probe concentration was optimised by testing various probe concentrations: 50 nM, 100 nM, 150 nM, 200 nM and 400 nM. The optimum concentration of probe of 100 nM was selected (Figure 4.6).

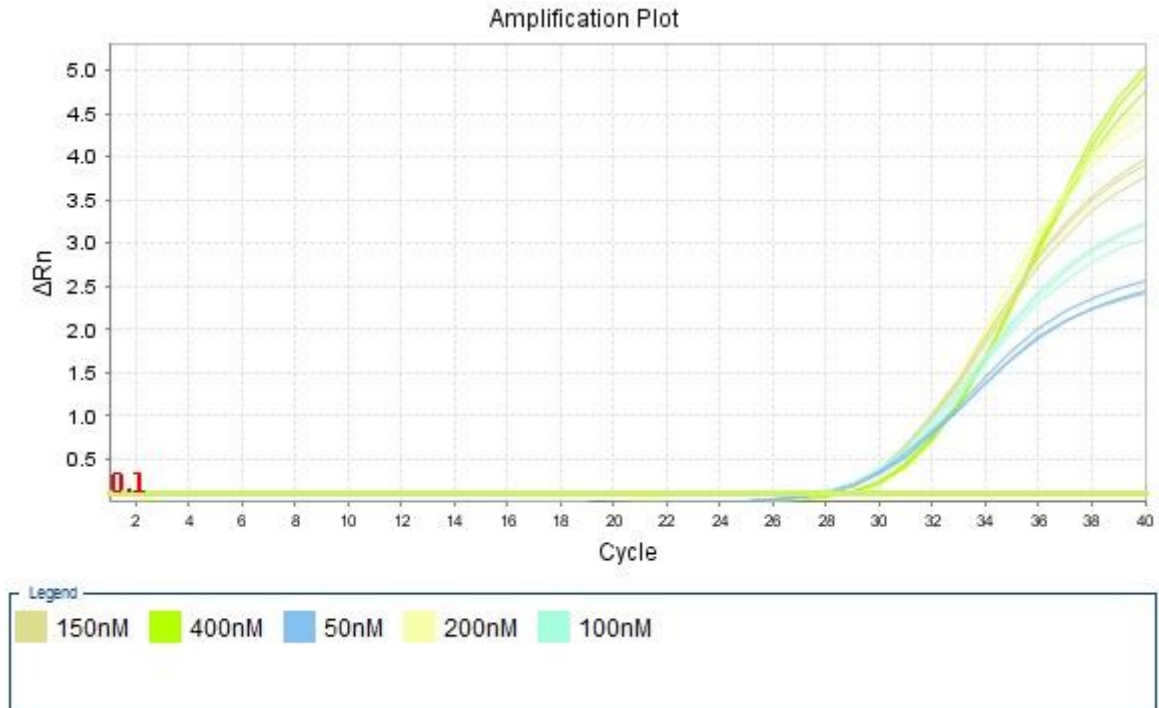


Figure 4.6. Probe optimisation of a TaqMan® MGB assay targeting SNP2451 IBDV W2512. Threshold baseline of 0.1 (green horizontal line). The vaccine was detected at all concentrations of probe.

4.4. Laboratory validation

4.4.1. Assay efficiency

A ten-fold dilution series of Transmune® IBD vaccine, from 10^0 to 10^{-8} , was used to generate a standard curve (Figure 4.7). IBDV RNA was extracted from each dilution, and five replicates tested in one run. The logarithm of chicken infection dose 50 (CID50) (x-axis) was plotted against the C_T values of the assay. The efficiency of the assay was 87%, while the correlation coefficient (R^2) was 0.9924. The correlation coefficient is a measure of how well the data fit the standard curve and reflects the linearity of the standard curve.

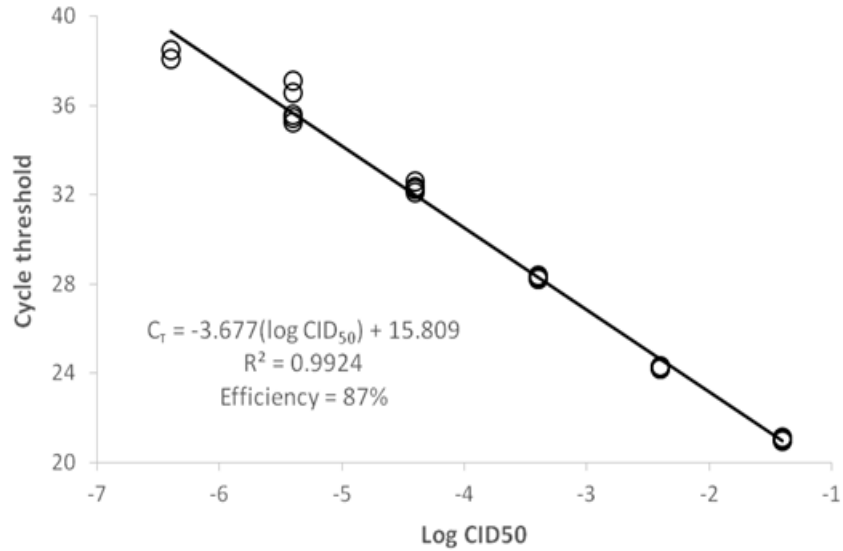


Figure 4.7. A TaqMan® MGB assay targeting SNP2451 IBDV W2512 standard curve, generated from a ten-fold serial dilution. Assay efficiency is 87%, $R^2 = 0.9924$.

4.4.2. Assay sensitivity

The 95% LOD is the input concentration giving a positive result in 95% of replicates and was calculated at 4.7×10^5 (95% confidence interval range of 1×10^5 to 3.4×10^5) CID₅₀ (Figure 4.10).

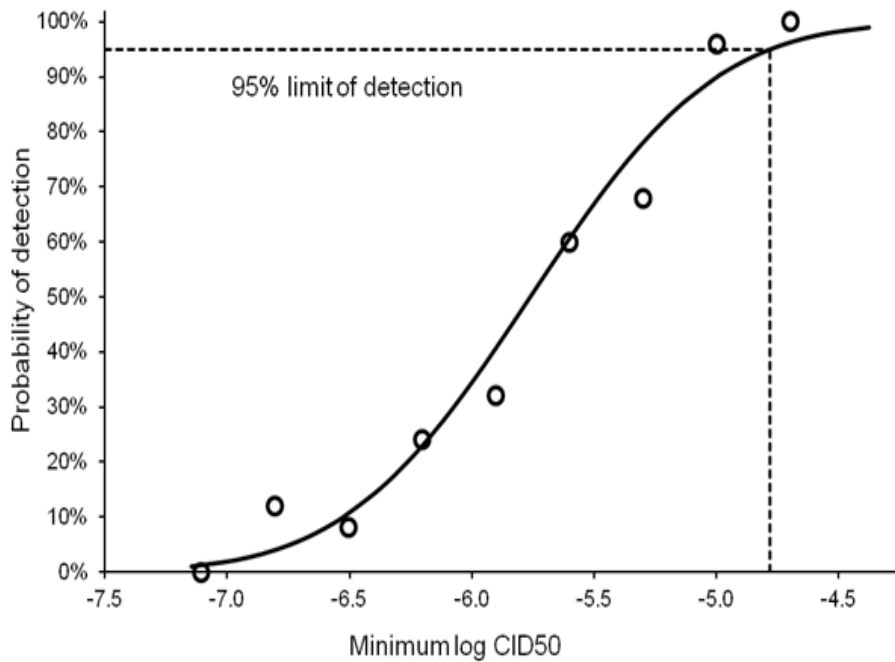


Figure 4.8. A TaqMan® MGB assay targeting SNP2451 IBDV W2512 limit of detection curve.

4.4.3. Assay specificity

This was determined by using the Transmune W2512 - specific real-time PCR with other IBDV strains (IBD#6 vero, isolate 1327108 and isolate IBD from the Department of Veterinary Tropical Diseases, University of Pretoria), as template for PCR. Only the W2512 vaccine strain was detected (Figure 4.9).

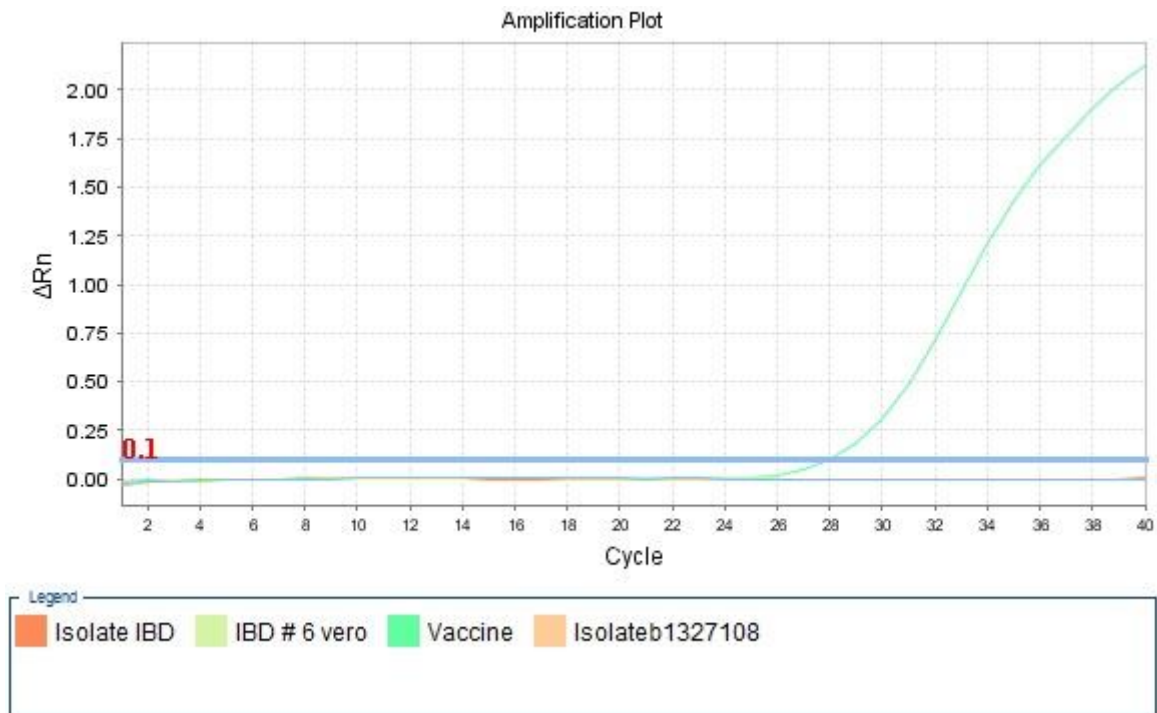


Figure 4.9. Specificity of a TaqMan® MGB assay targeting SNP2451 IBDV W2512. Other IBDV isolates (Isolate IBD, IBD #6 vero and isolate 1327108) were not detected.

4.4.4. Assay variation

The inter-run standard deviations was in the range of 0.4 to 0.8 and intra-run standard deviations between 0.0 and 0.9. This was to demonstrate how close the obtained values were within the assay run and in-between assay runs respectively. The coefficient of variation (CV) ranged from 0.1% to 3.2% (Table 4.3).

Table 4.3: Variation of a TaqMan™ MGB assay specific for Transmune® IBD vaccine. Log CID₅₀ – logarithm of chicken infection dose₅₀, SD - standard deviation, CV - coefficient of variation.

Log CID ₅₀	Inter-run SD	Intra-run SD	Mean	Total SD	CV %
-4.7	0.70	0.94	35.4	1.11	3.3
-5.0	0.70	0.69	36.5	0.94	2.6
-5.3	0.38	0.84	37.7	0.81	2.2
-5.6	0.51	0.94	37.9	0.92	2.4
-5.9	0.71	0.58	38.2	0.69	1.8
-6.2	0.77	0.10	38.2	0.69	1.8
-6.5	-	0.02	38.5	0.02	0.1
-6.8	0.59	-	39.0	0.59	1.5

4.5. Analysis of field samples

All samples from birds vaccinated with TABic® M.B. vaccine tested negative, and 11 out of 12 birds that were vaccinated with Transmune® IBD vaccine tested positive (Table 4.4). Overall, the results demonstrated that the assay has potential to correctly identify chickens that were vaccinated with Transmune® IBD vaccine, although there is still a need to investigate the specificity with more field and vaccine strains. Other assessment may also include assessment on impact of sampling time (post vaccination) different age groups involved and possibility of cross reaction with vaccine strains.

Table 4.4. C_T values obtained from analysis of field samples tested with a TaqMan™ MGB assay specific for Transmune® IBD vaccine, using a C_T threshold of 0.1. Neg - negative, indicates samples collected from chickens that were vaccinated with Tabic M.B vaccine. Pos - positive indicates samples collected from chickens that were vaccinated with Transmune® IBD vaccine.

Sample Name	C _T
Neg 01	Undetermined
Neg 02	Undetermined
Neg 03	Undetermined
Neg 04	Undetermined
Neg 05	Undetermined
Neg 06	Undetermined
Neg 07	Undetermined
Neg 08	Undetermined
Neg 09	Undetermined
Neg 10	Undetermined

Neg 11	Undetermined
Neg 12	Undetermined
Negative control	Undetermined
Pos 13	38.31
Pos 14	36.33
Pos 15	37.38
Pos 16	17.38
Pos 17	33.44
Pos 18	37.71
Pos 19	Undetermined
Pos 20	36.80
Pos 21	32.02
Pos 22	35.19
Pos 23	29.31
Pos 24	17.78
Positive control	33.71

CHAPTER

5. DISCUSSION

The aim of this study was to develop and optimise a real-time RT-PCR assay that is specific for W2512 vaccine strain present in Transmune® IBD vaccine. Global surveillance programmes require rapid and reliable assays for diagnosis of IBDV variants. To be applied widely, these methods must be simple to perform, economically feasible, and provide fast results (Tomás *et al.*, 2012). Real-time RT-PCR has been extremely useful for studying viral agents and most of the assays presented in literature, allow an increased frequency of virus detection compared with conventional techniques, which makes its use more attractive in virology (Mackay *et al.*, 2002). This study comprised three stages:

The first stage was the analysis of publically available IBDV sequences on Genbank data base for the purpose of establishing if there were any differences unique to the Winterfield 2512 strain.

In the second phase, sequencing primers (Table 4.1) were designed from the aligned sequences specific for segment A and some for segment B fragments of IBDV. These primers were then used to perform a conventional RT-PCR on the W2512 vaccine strain. The nucleic acid extracted from the vaccine was RNA, which required to be transcribed into cDNA using reverse transcriptase. Some cDNA fragments were not amplified (Figure 4.1) and this could have been due to various reasons. The primers may not have been designed correctly and could not successfully bind to the area of target. Another possibility is that the annealing temperature might not have been optimum, therefore no hybridization to produce homologous complimentary ssDNA occurred and subsequently no amplicons were detected. Annealing temperature plays a major role in RT-PCR. As the PCR annealing temperature is increased, the stringency of primer annealing is also increased, leading to more specific and reproducible amplification (Malhotra *et al.*, 1998).

All the detected and confirmed PCR products were purified using the CleanSweep™ protocol. This was to remove any impurities, excess dNTPs and primers and to have pure PCR products. Having these impurities in the PCR products prior to sequencing can results in low quality sequences. Sanger sequencing was performed at Inqaba Biotec on segment A and B PCR products in order to have complete nucleotide sequences for the W2512 vaccine strain. Sequencing of both segments' PCR products was also necessary to confirm the presence of a SNP that was identified during Genbank sequence analysis, at position 1385 of segment B.

Sequencing refers to methods for determining the order of the nucleotides bases adenine, guanine, cytosine and thymine in a molecule of DNA (Munshi, 2012). There are various methods of sequencing, however, for the purpose of this study, Sanger sequencing was conducted, which utilises dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. This requires a single-stranded DNA template, primer, DNA polymerase, radioactively or fluorescently labelled nucleotides, and modified nucleotides that terminate DNA strand elongation. The dNTPs are not labelled while the ddNTPs are labelled with different coloured fluorophores. Sanger sequencing is different from other type of sequencing such as next generation sequencing (NGS) systems whereby DNA sequencing is determined by addition of nucleotides to the complimentary strand and not by chain termination. In addition, NGS requires that partially segregated, amplified DNA templates are simultaneously sequenced in a massive and parallel manner, with no need for physical separation (Anderson and Schrijver, 2010).

Regions of the W2512 vaccine strain have been sequenced before. Liu *et al.*, (2001) reported that nucleotide sequences of the HVR of VP2 located between nucleotide 618 and 1050 were determined for three IBDV isolates, of which one was a vaccine strain 2512. The segment B sequence of IBDV W2512 vaccine strain (AF083092) has been published before (Yehuda *et al.*, 1999). The presence of a SNP that was identified at nucleotide position 1385 of segment B of AF083092 was not confirmed by our sequencing of the W2512 vaccine strain present in Transmune® IBD vaccine. This meant that SNP1385 could not be used for developing an assay. The reasons for this difference may be that the Winterfield strains in Transmune® IBD and what is published on Genbank may actually differ, or it may be a sequencing error. Sanger sequencing data quality can be affected by some of the reagents used for DNA extraction, PCR product purification and some of the buffers. The presence of contaminants, such as EDTA, phenol:chloroform, salts, and to a lesser extent dNTPs can also affect Sanger sequencing data quality. Despite these factors, Sanger sequencing is reliable, with over 99.99% accuracy.

Genetic drift of IBDV may have altered sequences of viruses being propagated regularly for vaccine production, resulting in sequences obtained on Genbank not to match 100% with sequences of the current W2512 vaccine strain. Patel *et al.*, (2016), conducted a study on evidence of genetic drift and reassortment in IBDV and emergence of outbreaks in poultry farms in India, some of the findings were that VP1 and VP2 region of IBDV field isolates had significant prevalence of genetic reassortants in Indian poultry farms. Furthermore, unique amino acid pattern in the viral RNA polymerase VP1 with significant genetic differences from other strains was identified. This suggested evolution and adaptation of segment B and its reassortment with segment A of virulent or vaccine strains, resulting in emergence of new virus strains Patel *et al.*, (2016).

Good results were obtained with the redesigned forward primer for fragment 2 of segment A, it was detected with high concentration (Figure 4.3) with the expected size of 500 base pairs (bp). This confirmed that the forward primer for fragment 2 of segment A was previously not well designed and therefore no binding and subsequent amplification could occur.

Analysis of segment A sequences of Transmune IBD vaccine strain W2512 revealed that there were potential SNPs that could be used to develop an assay. The first region considered had a SNP at position 660. It was not ideal due to few variations in the region of location, and two of those sequences (reference DQ355819_2512 and DQ355819_2512) were vaccine strains. The other one that had a single variation was sequence KJ198844, it was identical to W2512 vaccine segment A sequence in this study and when checked on Genbank, it was IBDV KK54 strain from Thailand. There were however very few variations in this region, the only sequences in this area that were different are GQ166970 and FJ842492 and when checked on Genbank, they were segment A, B-SD-LY and BDG23 IBDV strains from China and Vietnam respectively. Other SNPs that were identified and evaluated were at nucleotide position 805, 915 and 2513. SNP2451 was of good choice because there was high variation in the probe region, which should make it specific for its target.

Following sequencing and sequence analysis, the third phase of this study was to use an identified SNP for assay development and optimisation. SNP detection has been used extensively for genetic association studies of diseases including cancer (Suhda, *et al.*, 2016). In this study KJ198844 was used as a reference sequence and a suitable SNP was identified at position 2451 of segment A sequence of W2512. SNP2451 was selected because it is found in a variable region, which made it more likely that the probe would be specific for its target, i.e. Transmune® IBD's W2512 vaccine strain sequence. This similar concept of assay development was used by Tomás *et al.* (2012) who analysed sequences of segment A in an overlapping region of VP5/VP2 for different isolates and selected SNP A229G for its consistency to differentiate the strains.

A minor groove binding non-flourescent quencher (MGBNFQ) TaqMan® probe targeting SNP2451 was ordered. The probe is a hydrolysis probe consisting of a donor (reporter) and acceptor (quencher) molecules. Attached to the 5'-end of the TaqMan® probe is the "reporter," which is a fluorescent dye that will report the amplification of the target during, extension phase of the PCR cycle – when cleavage by the 5'-3' nucleolytic activity of the DNA polymerase occurs. On the 3'-end of the probe is a quencher, which quenches fluorescence from the reporter in intact probes (Heidi *et al.*, 1996). MGB ligands improve target DNA-binding specificity and sensitivity (Navarro *et al.*, 2015). Its mechanism of action was explained by Alfonina *et al.*, (2002) who reported that the fluorescence of the single-stranded MGB eclipse probe is efficiently quenched by the interaction of the terminal dye and quencher

groups when in absence of hybridization. Upon hybridization to a complementary target, the MGB molecule folds into the duplex and hyper-stabilizes it, allowing the use of shorter, more specific probe sequences. Furthermore, the 3'-MGB-quencher group also prevents nuclease digestion by *Taq* DNA polymerase during PCR (Heidi *et al.*, 1996; Alfonina *et al.*, 2002).

Analysis of the results revealed that there was successful amplification at the lowest C_T (cycle threshold) value of 28 and 10^{-1} RNA concentration. This was an indication that the assay primers and probe were well designed. Navarro *et al.*, (2015) explains that if a TaqMan™ probe is not well designed, there is a possibility that primer-dimers might be formed during qPCR assay, leading to assay failure or low efficiency. However, there is always the possibility that even well-designed primers may form primer-dimers or amplify a nonspecific product (Life Technologies' real-time PCR handbook.)

The results also demonstrate that the designed primers and probe could successfully bind to the specific region with the target SNP 2451 of the W2512 vaccine strain genome and amplify it to a detectable level at a C_T value of 28. The determination of the C_T depends upon the sensitivity and ability of the instrument to differentiate between specific and non-specific background fluorescence, the concentration and nature of fluorescence-emitting source as well as initial concentration of nucleic acid template (Mackay *et al.*, 2002). C_T is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level) (Jung *et al.*, 2000; Heidi *et al.*, 1996). The C_T levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the C_T level the greater the amount of target nucleic acid in the sample). It is also useful as an indicator of successful target amplification and for predicting the quantity of target input nucleic acid (Heidi *et al.*, 1996). This to a certain extent also demonstrates the good efficiency of this developed assay. However, there is a possibility that the amount of RNA present might have been affected by the easy degradation of RNA, although experiments were done with newly extracted RNA. There is also a possibility of reaction inhibitors as some compounds may have an inhibition effect on the PCR or that reaction conditions might not have been optimum (Mackay *et al.*, 2002). After performing the first test and demonstrating that the assay works, then its performance had to be assessed through a number of parameters (the standard curve to determine linear range and efficiency, 95% LOD, specificity and analysis of field samples).

Optimisation of primers was performed by testing various concentrations (50 nM, 100 nM, 200 nM, 400 nM, 800 nM), while keeping the probe concentration constant at 200 nM. Similarly, optimisation of probe was done by keeping the optimum primer concentration constant (200 nM) and using a range of probe concentrations (50 nM, 100 nM, 150 nM, 200 nM and 400 nM). Optimisation was important for determining the optimum concentration of primers and probe to run the assay with. In the study conducted by Suhda *et al.*, (2016)

optimization system strategies were used such as (1) designing inner and outer primers; (2) determining of their optimum primer concentration ratios; and (3) determining of the optimum PCR annealing temperature. This shows how significant the concept of optimisation is and that there are various parameters that can be used for optimisation depending on the type and purpose of assay. Optimisation parameters are also important for determining the reproducibility of the assay.

Specificity is one of the important assay performance characteristics that was determined by running the developed real-time RT-PCR to detect Transmune® IBD's W2512 vaccine strain, with other strains which were IBD#6 vero, isolate 1327108 and isolate IBD (obtained from the University of Pretoria – DVTD). Analytical specificity for a single genetic sequence that is not known to be present in other organisms or strains of the targeted organism is said to exhibit exclusivity and denotes a confirmatory assay of high analytical specificity (Asp) (OIE Validation Guidelines, 2014). The results (Figure 4.9) were negative for other strains and positive for Transmune® IBD's W2512 vaccine strain, which suggests that the assay is specific for Transmune® IBD's W2512 vaccine strain. The isolates that were tested were what was available from DVTD. With the testing of other isolates, it may occur that the assay cross reacts with other isolates. To improve the specificity of the assay, the developed assay could be converted into an allelic discrimination assay, where a second probe with a different colour fluorophore attached, specific for the cross-reacting isolate, could be added to the assay, to compete with the probe specific for the W2512 strain. The probe that is more specific for the target will anneal preferentially, which may be discriminated by the channel in which the fluorescence increases.

Apart from specificity, another important performance characteristic of the assay was to demonstrate its efficiency through a standard curve. Determining PCR efficiency is especially important for relative quantitation and an ideal slope should be 3.32 for 100% PCR efficiency (Ginzinger, 2002). This however is rarely achieved in most assays due to many factors that influence the PCR (as outlined later on). In this case, the standard curve (Figure 4.10) indicated that this assay was 87% efficient and the coefficient of determination (R^2) as indicated on the graph was 0.9924.

Considering the degree of deviation from 100% in this study, the performance of the assay with efficiency of 87% is acceptable. However, various factors might possibly have contributed to this level of efficiency for this assay. The variables most likely to affect PCR efficiency are, $MgCl_2$ concentration, reaction conditions (i.e., time and temperature), PCR target size and composition, primer sequences and sample purity (Heidi *et al.*, 1996). According to the Applied Biosystems guide to relative quantitation RT-PCR, other conditions that may influence efficiency are the dynamics of the reaction itself, the use of non-optimal reagent

concentrations, and enzyme quality, which can result in efficiencies below 90%. Thus the guide further outlines the following practices that can help to achieve accurate real-time PCR results (Table 5.1).

Table 5.1. Recommended practices to consider under various conditions in order to get good RT-PCR results (Applied Biosystems guide to relative quantitation RT-PCR).

Condition	Recommended practice
Poor quality RNA samples can lead to spurious real-time PCR results	Use high quality RNA
Type of dye	Use reagents that contain the internal reference dye ROXTM, to compensate for minor variations in signal strength, which results in better precision.
PCR master mixes and PCR reagent	The use of PCR master mixes and PCR reagent cocktails will help reduce the potential variability introduced from pipetting multiple reagents during setup. Applied Biosystems real-time PCR Master Mixes contain all of the components of the real time-PCR reaction except primers, probe and nucleic acid template.
Sample and reagent pipetting	Accurate pipetting with regularly calibrated pipettes is critical to obtaining accurate and precise data.
Mix PCR reagents	Different components within a real time PCR reaction may settle down and be distributed unevenly within the testing tube and can have an effect on precision. Always thaw and thoroughly mix all reagents prior to testing.

Finally, the assay was used to test field bursa samples on Whatman® FTA cards (Sigma-Aldrich). There were 12 W2512 negative and 12 W2512 positive samples. The assay performed remarkably well, whereby all the W2512 negative samples tested negative. Eleven out of 12 W2512 positive samples tested positive. One sample (pos19) which was expected to test positive was however not detected despite being repeated twice. This could possibly be that Qiagen extraction kit which is optimised for DNA and not for RNA extraction might have influenced this result. There is also a possibility that this specific sample was lower than the LOD for this developed assay. The important thing is that the assay successfully detected W2512 vaccine strain and thus differentiated most chickens vaccinated with Transmune® IBD's vaccine from those that were vaccinated with TABic® M.B. vaccine.

5.1. Benefits arising from the study

The developed assay will provide an effective and efficient way to specifically assess the vaccine status of chicken flocks that have been vaccinated with Transmune® IBD. This will reduce the costs for both poultry farmers and Ceva Santé Animale, by eliminating the use of multiple, costly and lengthy tests. Currently, characterisation of the W2512 IBDV vaccine strain is done through techniques of RNA sequencing or a combination of PCR and RFLP which is laborious and time consuming. The real-time RT-PCR assay bases its discrimination capacity on the identification of a SNP, while RFLP discrimination systems depend on the existence of a specific restriction enzyme for SNP identification. Thus there are many advantages to using a real-time RT-PCR assays such as accuracy, sensitivity and specificity.

This developed real-time RT-PCR assay is specific for W2512 IBDV vaccine strain although there is a need to investigate the specificity further by subjecting it to many other IBDV field and vaccine strain. The assay it is quick and will significantly reduce the turn-around time to produce results. The assay is also simple to perform and will not require specialised analysts to interpret the results. Being a real-time RT-PCR assay, this assay brings forth most of the advantages that are linked to real-time RT-PCR as a modern diagnostic and research tool in comparison to other type of methods.

Like most of the authors so far, Lin *et al.*, (1994) outlined a number of advantages for using PCR compared to virus neutralisation test (VNT). The first advantage is that there is no need to do pre-isolation of virus from clinical samples prior to PCR amplification, which significantly reduce time and labour required. Secondly, PCR works by exponential amplification of a specific IBDV cDNA sequence. Real-time PCR in particular, has brought about a wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (Heidi *et al.*, 1996). On the other hand, real-time RT-PCR also has some disadvantages, Mackay *et al.*, (2002) provided a number of these, such as the limitation to monitor amplicon size without opening the system, incompatibility of some platforms compared with some fluorogenic chemistries, as well as the high start-up cost which can be a challenge to low-income generating diagnostic and research facilities.

On the other hand, it does not necessarily mean that a combination of methods or other types of methods cannot be used. Selection and usage of methods depends on the purposes, preferences and availability of resources. Various works have been or are still being conducted using the combination of RFLP and PCR. For example, Priyadharsini *et al.*, (2016) used a combination of RT-PCR with restriction fragment length polymorphism (RFLP) for the differentiation of classical virulent (cv), virulent (v) and very virulent (vv) strains of IBDV isolates from chicken bursal tissues in southern states of India. The author concluded that

although RT-PCR combined with RFLP can be used for genotyping, there is a need to conduct further confirmation of serotypes, in order to distinguish vvIBDV from cvIBDV. This demonstrates that the combination of two methods or more has its own limitations. Suhda *et al.*, (2016) did a study on tetra-primer amplification refractory mutation system-PCR (ARMS-PCR) optimization to Detect Single nucleotide polymorphisms of the CYP2E1 gene. The author observed that results were consistent with validation using DNA sequencing and restriction fragment length polymorphisms (RFLP). Thus determination of genomic variations is more sensitive and reproducible than the use of VNT for classification of viruses. Therefore, PCR followed by RFLP analysis offers additional information for classification of IBDV that are closely related (Liu *et al.*, 1994). The author made a conclusion that PCR-RFLP was faster and less complicated option than direct sequence analysis at the nucleic acid or amino acid level.

There are also other tests that have been used in the diagnosis of IBDV such as the virus neutralisation test (VNT). However, Lin *et al.*, (1994) highlighted that this test requires sufficient amounts of replication products in order for antigen-antibody reaction and serotype identification to be done and that it is time consuming.

Despite all the advantages and benefits of real-time RT-PCR as a modern diagnostic and research tool, conventional PCR which is also referred to as traditional PCR is still being used to some extent and is playing a very significant role in various diagnostic and research methods. This study is a good example, whereby conventional PCR had to be done prior to sequencing, it was the only way to assess that the conventional PCR primers works by visualising the cDNA fragment bands. It also allows for approximation of cDNA fragment bands sizes in bp as it is analysed in comparison to the DNA ladder.

Other benefits of using this developed assay will include easy management on farms and good IBDV surveillance – the assay can confirm presence or absence of Cevac® Transmune® IBD vaccine strain – W2512 and thus making it possible to say outbreak is possibly due to other strains.

CHAPTER

6. REFERENCES

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APPENDIX A: Customized protocol for the MagMax™ Express Particle Processor (Life Technologies)

[PROTOCOL PROPERTIES]

Name = AM1830_Mod3
Protocol template version = 2.6.0
Instrument type = KingFisher
Creator = mquan
Created = 30/9/2009 9:31:36
Description = RNA isolation from whole blood.
Kit = MagMAX-96 viral RNA Isolation Kit , AM1836
Plate layouts = Default

[PLATE LAYOUTS]

Default

Plate type = KingFisher plate 200 ul
Plate change message = Change Default

A:

- volume = 20, name = Bead Mix (10 ul RNA binding beads, 10 ul Lysis/Binding Enhancer)
- volume = 50, name = Whole blood
- volume = 130, name = 65 ul Lysis/Binding Solution, 1 ul Carrier RNA, 2 ul Xeno RNA, 65 ul Isopropanol

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 I 1 min
- Well = B, Default

Beginning of step:

- Release = Yes, time = 0s, speed = Fast

Wash parameters:

- Wash time = 1min 0s, speed = Fast

End of step:

- Collect beads = Yes, count = 3
-

WASH

Step parameters

- Name = 2nd Wash I 1 min
- Well = C, Default

Beginning of step:

- Release = Yes, time = 0s, speed = Fast

Wash parameters:

- Wash time = 1min 0s, speed = Fast

End of step:

- Well = E, Default

Beginning of step:

- Release = Yes, time = 0s, speed = Fast

Wash parameters:

- Wash time = 1min 0s, speed = Fast

End of step:

- Collect beads = Yes, count = 2
-

DRY

Step parameters

- Name = Dry 1 min
 - Well = E, Default
 - Dry time = 1min 0s
 - Tip position = Outside well
-

ELUTION

Step parameters

- Name = Elution 3 min
- Well = F, Default

Beginning of step:

- Release = Yes, time = 0s, speed = Fast

Elution parameters:

- Elution time = 3min 0s, speed = Bottom medium

Pause parameters:

- Pause for manual handling = No

Remove beads:

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

APPENDIX B: IBDV Segment A GenBank accession numbers

A12620	D00868	JX424060
A33255	D00869	JX424065
A38328	D16828	JX424076
AB368968	D49706	JX424077
AB368970	D83985	JX424078
AF051837	DI491182	JX424079
AF092171	DQ286035	JX682709
AF092943	DQ355819	JX682711
AF109154	DQ403248	KC109816
AF133904	DQ778035	KC189836
AF140705	DQ906921	KC603937
AF165149	DQ927040	KF241548
AF165150	DQ927042	KF569801
AF165151	EF418033	KF569803
AF194428	EF418034	KF569805
AF240686	EF418035	KJ198843
AF247006	EF418036	KJ198844
AF279288	EF517528	KJ547670
AF321054	EF646853	KJ547671
AF321055	EU162087	KJ547672
AF321056	EU162088	KJ547673
AF322444	EU162089	KJ547674
AF362747	EU184685	KP676467
AF362771	EU184687	KT336459
AF362773	EU184689	KT870148
AF362776	EU595667	KT884452
AF457105	EU595669	KT884486
AF499929	EU595670	KU891986
AF508176	EU595671	L42284
AF527039	EU595672	LM651365
AF533670	EU697938	LM651367
AJ879932	FJ695138	M66722
AM111353	FJ842491	M97346
AM167550	FJ842492	NC004178
AX138257	GQ166970	U30818
AX138258	GQ166972	X03993
AX138259	GQ451330	X16107
AY029166	HG974563	X84034
AY099456	HG974565	X92760
AY134874	HQ224883	Z21971
AY163771	JF811919	
AY319768	JF811920	
AY323952	JF907702	
AY444873	JF907703	
AY462026	JN585293	
AY462027	JQ315158	
AY598356	JQ315159	
AY665672	JQ315171	
AY918948	JQ411012	
AY918950	JX134483	
D00499	JX134485	
D00867	JX424048	

APPENDIX C: IBDV Segment B GenBank accession numbers

AB368952	AJ878649	AY654284	EU595678	JF811921	JN416827
AB368953	AJ878650	AY705393	FJ040159	JF907704	JN416828
AB368954	AJ878651	AY918947	FJ695139	JF907705	JN416829
AB368955	AJ878652	AY918949	FM205024	JN411134	JN416830
AB368956	AJ878653	D12610	GQ166971	JN416779	JN416831
AB368957	AJ878654	D49707	GQ221684	JN416780	JN416832
AB368958	AJ878655	D86860	GQ221685	JN416781	JN416833
AB368959	AJ878656	D86861	GQ449689	JN416782	JN416834
AB368960	AJ878657	D87047	GQ449690	JN416783	JN416835
AB368961	AJ878658	D87048	GQ449691	JN416784	JN416836
AB368969	AJ878659	D87049	GQ449692	JN416785	JN416837
AB368971	AJ878660	D87050	GQ449693	JN416786	JN416838
AF083092	AJ878661	D87051	GQ451331	JN416787	JN416839
AF083093	AJ878662	D87052	GQ452269	JN416788	JN416840
AF083094	AJ878663	DQ118374	HG974564	JN416789	JN416841
AF092944	AJ878664	DQ166818	HG974566	JN416790	JN416842
AF133905	AJ878665	DQ295314	HQ441142	JN416791	JN982245
AF194429	AJ878666	DQ295315	HQ441143	JN416792	JN982246
AF203880	AJ878667	DQ295316	HQ441144	JN416793	JN982247
AF240687	AJ878668	DQ295317	HQ441145	JN416794	JN982248
AF322445	AJ878669	DQ403249	HQ441146	JN416795	JN982249
AF362748	AJ878670	DQ679811	HQ441147	JN416796	JN982250
AF362770	AJ878671	DQ679812	HQ441148	JN416797	JQ241029
AF362772	AJ878672	DQ679813	HQ441149	JN416798	JQ241030
AF362774	AJ878673	DQ679814	HQ441150	JN416799	JQ241031
AF362775	AJ878674	DQ823085	HQ441151	JN416800	JQ241032
AF493979	AJ878675	DQ864983	HQ441152	JN416801	JQ241033
AF499930	AJ878676	DQ906922	HQ441153	JN416802	JQ241034
AF527038	AJ878677	DQ927041	HQ441154	JN416803	JQ241035
AF527040	AJ878678	DQ927043	HQ441155	JN416804	JQ241036
AJ295021	AJ878679	EF517529AF	HQ441156	JN416805	JQ241037
AJ295022	AJ878680	455136	HQ441157	JN416806	JQ241038
AJ295023	AJ878681	EF576658DQ	HQ441158	JN416807	JQ403647
AJ295024	AJ878682	679815	HQ602714	JN416808	JQ411013
AJ295025	AJ878683	EF646854	HQ602715	JN416809	JQ619639
AJ295026	AJ878684	EF688065	HQ602716	JN416810	JQ619640
AJ295027	AJ878685	EU162090	HQ602717	JN416811	JX096983
AJ295028	AJ878686	EU162091	HQ602718	JN416812	JX096984
AJ310186	AJ878687	EU162092	HQ602719	JN416813	JX096985
AJ318897	AJ880090	EU162093	HQ602720	JN416814	JX101468
AJ496637	AM111354	EU162094	HQ602721	JN416815	JX134484
AJ878638	AM167551	EU162095	HQ602722	JN416816	JX134486
AJ878639	AY029165	EU184686	HQ602723	JN416817	JX235358
AJ878640	AY099457	EU184688	HQ602724	JN416818	JX682710
AJ878641	AY103464	EU184690	HQ602725	JN416819	JX682712
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AJ878646	AY459321	EU595675	HQ735292	JN416824	KC968815
AJ878647	AY508720	EU595676	HQ735293	JN416825	KC968816
AJ878648	AY598355	EU595677	HQ735294	JN416826	KC968817

APPENDIX D: IBDV Segment A - W2512 sequence

>IBDV_Winterfield_2512

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APPENDIX E: Animal ethics committee approval certificate



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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Development of a real-time RT-PCR assay specific for Cevac® Transmune ISD
PROJECT NUMBER	V018-16
RESEARCHER/PRINCIPAL INVESTIGATOR	JN Kabajani

STUDENT NUMBER (where applicable)	UP_15399771
DISSERTATION/THESIS SUBMITTED FOR	MSc (DVTD)

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 Quen	

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	29 February 2016
CHAIRMAN: UP Animal Ethics Committee	Signature	