

# **The Isolation and Genetic Characterization of Canine Distemper Viruses from Domestic Dogs (*Canis familiaris*) in South Africa**

BY

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## DEDICATION

To my Lord and Saviour, Jesus Christ. In whom I live and move and have my being.

Acts17:28.

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## TABLE OF CONTENTS

**DEDICATION**

**ACKNOWLEDGEMENTS**

**TABLE OF CONTENTS**

**LIST OF FIGURES**

**LIST OF TABLES**

**LIST OF ANNEXURES**

**ABSTRACT** **1**

**BRIEF OVERVIEW** **3**

**CHAPTER 1**

**GENERAL INTRODUCTION** **4**

**CHAPTER 2**

**LITERATURE REVIEW** **11**

2.1 Classification and characterization of canine distemper viruses 11

2.2 Epidemiology 16

2.2.1 Geographical distribution 16

2.2.2 Transmission and sources of infection 17

2.2.3 Host susceptibility 18

2.3 Pathogenesis 19

2.4 Clinical signs 20

2.5 Diagnosis 21

2.5.1 Haematology 22

2.5.2 Serum chemistries 22



2.5.3 Antibody detection	22
2.5.3.1 Virus neutralization test	23
2.5.3.2 Indirect immunofluorescence	23
2.5.3.3 Enzyme-linked immunosorbent assay (ELISA)	23
2.5.4 Antigen detection	23
2.5.4.1 Direct immunofluorescence	23
2.5.4.2 Immunochromatography	24
2.5.4.3 Enzyme-linked immunosorbent assay (ELISA)	24
2.5.4.4 Immunohistochemistry	24
2.5.5 CSF analysis	25
2.5.6 Viral isolation	26
2.5.7 Nucleic acid detection	27
2.6 Treatment	28
2.7 Prevention and control	29
2.8 Public health considerations	30
<b>CHAPTER 3</b>	
<b>ISOLATION OF WILD-TYPE CANINE DISTEMPER VIRUSES FROM DOMESTIC DOGS IN SOUTH AFRICA</b>	<b>32</b>
3.1 Introduction	32
3.2 Materials and Methods	34
3.2.1 Clinical specimens	34
3.2.2 Vero.DogSLAM cells	35
3.2.3 Virus isolation	36
3.2.4 Direct fluorescent antibody test	36



3.2.5 Electronmicroscopy	37
3.3 Results	37
3.4 Discussion	42
<b>CHAPTER 4</b>	
<b>GENETIC COMPARISON BETWEEN FIELD AND VACCINE STRAINS OF CDV AND DETERMINATION OF THE LINEAGE CIRCULATING IN SOUTH AFRICA</b>	<b>49</b>
4.1 Introduction	49
4.2 Materials and Methods	52
4.2.1 Specimens	52
4.2.2 RNA extraction	52
4.2.3 Complementary DNA (cDNA) synthesis	53
4.2.4 Polymerase chain reaction (PCR)	53
4.2.5 Sequencing	54
4.2.6 Phylogenetic analyses	55
4.2.7 Nucleotide sequence accession numbers	55
4.3 Results	56
4.4 Discussion	67
<b>CHAPTER 5</b>	
<b>GENERAL DISCUSSION</b>	<b>73</b>
5.1 Conclusions and recommendations	76
<b>REFERENCES</b>	<b>78</b>
<b>ANNEXURES</b>	<b>94</b>

## LIST OF FIGURES

Figure 1: Vero.DogSLAM cells infected by CDV from clinical specimens	41
Figure 2: Vero.DogSLAM cells uninoculated negative control	41
Figure 3: CDV nucleocapsid as revealed by electronmicroscopy	42
Figure 4: Agarose gel photo of the RT-PCR amplification of the 2100 bp of the H gene of CDV vaccines used in South Africa	59
Figure 5: Agarose gel photo of the RT-PCR amplification of the 2100 bp of the H gene of CDV isolates from South Africa	59
Figure 6: Neighbour-joining phylogenetic tree of complete CDV H gene Sequences	63
Figure 7: Bayesian analysis phylogenetic tree of complete CDV H gene Sequences	64
Figure 8: Maximum likelihood phylogenetic tree of complete CDV H gene sequences	65



## LIST OF TABLES

Table 1: Samples analysed by viral isolation in Vero.DogSLAM cells	39
Table 2: Description of the animals and samples positive for CDV by viral isolation in Vero.DogSLAM cells	40
Table 3: Oligonucleotide primers used for RT-PCR and sequencing	60
Table 4: GenBank accession numbers of CDV isolates obtained from this study (South Africa)	61
Table 5: Mean, standard deviation and number of pairwise measurements of the genetic distances between CDV H gene sequences	62

## LIST OF ANNEXURES

Appendix 1: Nucleotide sequence alignments of the sequences used in this study and reference strains	94
Appendix 2: Protein sequence alignments of the sequences used in this study and reference strains	110

## ABSTRACT

Studies comparing canine distemper virus (CDV) strains from Africa with that of previously described lineages have been hampered due to a lack of field strains for *in vitro* experiments. There are no reports of CDV isolations in southern Africa, and although CDV is said to have geographically distinct lineages, molecular information of African strains has not yet been documented. Clinical specimens consisting of whole blood, spleen, lungs, brain and cerebrospinal fluid from dogs with clinical signs suggestive of distemper were obtained from private veterinary practices and diagnostic laboratories in Gauteng province, South Africa. One hundred and sixty two specimens from 124 dogs were inoculated into Vero cells expressing canine signalling lymphocyte activation molecule (Vero.DogSLAM). Cytopathic effects (CPE) in the form of syncytia formation and cell necrosis were observed in 33 (20.4%) specimens within 24 hours of inoculation and the presence of CDV was confirmed with the aid of the direct fluorescent antibody test and electronmicroscopy (EM). Viruses isolated in cell cultures were subjected to reverse transcription – polymerase chain reaction (RT-PCR), and the complete H gene was sequenced and phylogenetically analysed with other strains from GenBank. The animals all displayed the catarrhal, systemic, or nervous signs of the disease. Four of the dogs positive for CDV by viral isolation had a history of prior vaccinations to canine distemper. Seventy three percent of all positive dogs were less than 12 months old. There was no breed or sex predisposition to CDV infection in domestic dogs. Phylogenetic comparisons of the complete H gene of CDV isolates from different parts of the world (available in GenBank) with wild-type South African isolates revealed nine clades. All South African isolates form a

separate African clade of their own and thus are clearly separated from the American, European, Asian, Arctic and vaccine virus clades. It is likely that only the 'African lineage' of CDV may be circulating in South Africa currently, and the viruses isolated from dogs vaccinated against CDV are not the result of reversion to virulence of vaccine strains, but infection with wild-type strains.

## BRIEF OVERVIEW

This thesis consists of five chapters. Chapter one provides a background on canine distemper virus (CDV) isolation and genetic characterization. The chapter is concluded with a list of the aims of the study. Chapter two consists of a review of the documented literature on the subject of canine distemper with special reference to South Africa. The chapter also notes the diagnostic tests presently available, the controversy about vaccinated dogs becoming infected; the proposition that CDV causes some human diseases, and publications on CDV genetic characterization.

Chapter three presents the first report of the isolation of wild-type CDV from domestic dogs in South Africa using Vero cells expressing canine signalling lymphocyte activation molecule (Vero.DogSLAM). Chapter three also attempts to correlate isolates with clinical disease. The confirmation of the isolation procedures with immunofluorescence (IF) and electronmicroscopy (EM) is described. Chapter four represents the first study on the genetic characterization of CDV in South Africa. Phylogenetic analysis performed with South African field strains, vaccines used in the country, and CDV sequences from all over the world deposited by researchers in GenBank is reported. The epilogue of this work is chapter five which summarises the discussions of chapters three and four. Chapter five also include conclusions reached and recommendations.

The main limitation of this work was the lack of clinical specimens from other provinces in the country. Another limitation was the inability to also characterize the fusion (F) gene, which, though not as variable as the haemagglutinin (H) gene, also plays a role in immunogenicity. These limitations were due to financial and logistical reasons.

## CHAPTER 1

### GENERAL INTRODUCTION

Canine distemper was the most lethal disease of dogs during the first half of the 19<sup>th</sup> century. It was first reported in Europe in 1760 (Appel & Gillespie, 1972a). The disease was initially thought to be caused by *Bordetella bronchiseptica* but Laidlaw and Dunkin reproduced both visceral and neurological forms of distemper in ferrets and dogs with bacteria-free filtrates of infectious secretions and tissue homogenates (Castro & Heuschele, 1992). Edward Jenner first described the course and clinical signs of the disease in 1809 (Appel & Gillespie, 1972b; Shell, 1990) and the virus was isolated by Carré in 1905 (Carré, 1905).

Canine distemper virus (CDV), the causative agent of canine distemper is a member of the genus *Morbillivirus*, family *Paramyxoviridae*. Other members of the genus includes measles virus (MV) of man, rinderpest virus (RPV) of cattle, peste des petits ruminants virus (PPRV) of small ruminants and emerging viruses of aquatic mammals (phocine, dolphin, seal and porpoise distemper viruses) (Griffin, 2001; Murphy *et al.*, 1999). Measles virus has decimated human populations for centuries and remains a significant cause of childhood mortality in developing countries. Rinderpest was responsible for great cattle plagues in Europe and Africa where it caused massive die-offs around the turn of the 19<sup>th</sup> century (Anderson, 1995). Emerging distemper viruses of aquatic mammals have been reported to cause epizootics in seals in northern Europe during the autumn and summer of 1988 and in freshwater seals in Lake Baikal, Siberia in December 1987 (Mamaev *et al.*, 1996). Peste des petits ruminants virus is responsible for major economic losses in sheep and goats in West Africa, the Middle East and India.

Canine distemper virus has the widest host range among morbilliviruses and has long been recognized to cause potentially lethal disease among 8 families of the order Carnivora, namely *Ailuridae*, *Canidae*, *Mustellidae*, *Procyonidae*, *Ursidae*, *Viverridae*, *Hyaenidae* and *Felidae* (Appel *et al.*, 1994; Harder & Osterhaus, 1997; Mamaev *et al.*, 1995). Wildlife species susceptible to CDV infection and that are present in South Africa include lions, leopards, wild dogs, hyenas, foxes and otters (Leisewitz *et al.*, 2001). Of these species, exposure to CDV has been demonstrated serologically in lions and leopards. However, no catastrophic mortality due to CDV infection has been reported in South African wildlife (Van Vuuren *et al.*, 1997).

Although small antigenic differences have been demonstrated serologically between different CDV strains, it is generally accepted that there is only one serotype. However, there are considerable differences in the pathogenicity of different virus isolates and geographically distinct lineages have been described. Established CDV lineages include: lineage Asia 1 (Japan, China), Asia 2 (only in Japan), Arctic, Europe wildlife, Europe, lineage America 1 and 2, old CDV strains (Onderstepoort, Convac, Rockborne and Snyder Hill) (Haas *et al.*, 1997; 1999; Harder & Osterhaus, 1997; Martella *et al.*, 2006; 2007; Yoshida *et al.*, 1998). Sequence data of field isolates from Africa available for comparison are non-existent. This study was an attempt to determine if African strains form a distinct clade of their own, or group with one of the published lineages. Geographically distinct lineages have been described for other morbilliviruses such as rinderpest (Chamberlain *et al.*, 1993; Wamwayi *et al.*, 1995) and measles viruses (Taylor *et al.*, 1991).

Virulent (wild-type) CDV replicates readily in activated canine lymphocytes and in canine macrophages *in vitro* but only after adaptation in monolayers of epithelial cells or fibroblasts. In contrast, attenuated vaccine virus replicates in lymphocytes/macrophages as well as in epithelial and fibroblastic cells *in vitro* (Appel & Summers, 1999). Recently, Vero cells expressing canine signalling lymphocyte activation molecules (Vero.DogSLAM) were developed to efficiently isolate CDV from clinical samples (Seki *et al.*, 2003). Virus isolation is important to confirm a diagnosis, facilitate direct sequence analysis, and investigate pathogenesis in animal experiments and future vaccine development in the event that unusual viral variants are detected for which current vaccines are ineffective (Lednicky *et al.*, 2004a). In this study, Vero.DogSLAM cells were used to isolate CDV from dogs with natural infection in South Africa to provide a basis for further molecular characterization.

In a developing peri-urban community of southern Africa, canine distemper incidence was highest in the spring and early summer months of August to November (Eckersley *et al.*, 1992). In some urban areas however, the incidence is higher during winter (Moritz van Vuuren, University of Pretoria, unpublished results). Canine distemper virus infection rates in South Africa are higher than the disease rates with between 25 and 75 % of susceptible dogs clearing the infection without showing any clinical disease (Leisewitz *et al.*, 2001).

In a cross-sectional study of the canine population in a rural town in southern Africa, 5.5% of the dogs examined were diagnosed with active CDV infection (Eckersley *et al.*, 1992). In a second study that compared the disease status of



canine hospital patients from developed communities with those from developing communities over a 3-year period, a remarkable difference was found between the 2 groups. While 43.6 % of the dogs from the developing community had infectious disease, only 8.2 % of dogs from the developed community were similarly diagnosed. Canine distemper was diagnosed in 4.1 % of the infectious disease cases in the developing community dogs, but infection with CDV was rarely diagnosed in the dogs from the developed community (Eckersley *et al.*, 1992). These data indicate that despite vaccinations, CDV is still an important disease among dogs in South Africa. This study focused on the isolation and genetic characterization of South African CDV strains to provide information that may be used in the evaluation of the protection afforded by the current generation of vaccines.

Canine distemper virus is enveloped with an unsegmented negative-strand RNA genome and an RNA polymerase. The lipoprotein envelope is readily destroyed by lipid solvents which renders the virus non-infectious. It contains the H and F glycoproteins that induce a neutralizing antibody response (Appel & Summers, 1999). The H glycoprotein is responsible for viral attachment to the host cell and may also play a role in induction of protective immunity (Pardo *et al.*, 2005). It also shows the greatest genetic variation that allows for the distinction of various lineages according to a geographical pattern of distribution irrespective of the species of identification (Martella *et al.*, 2006). Immunological and sequence data have clearly shown that the H protein is the most variable of the morbillivirus proteins and reverse transcription- polymerase chain reaction (RT-PCR) of H genes in combination with fast sequencing methods is a sensitive tool to precisely

characterize current CDV field viruses (Haas *et al.*, 1999). Phylogenetic analyses of the CDV H gene nucleotide and amino acid sequences have been performed to study evolutionary relationships between CDV isolates and to find genetic variation among wild-type and CDV vaccine strains. This study therefore characterised the H gene (nucleotides) and H proteins (amino acids) of South African CDV isolates in comparison with other published strains.

The control of canine distemper is only achieved by the use of vaccination (Chappuis, 1995). However, many outbreaks of the disease in vaccinated dogs have been documented (Calderon *et al.*, 2007; Jozwik & Frymus, 2002; Lan *et al.*, 2006). Studies showed that present CDV strains may differ from known vaccine strains, and it has also been reported that wild-type CDV isolates detected in naturally occurring cases clustered according to geographical distribution, rather than to host species origin following phylogenetic analysis (Bolt *et al.*, 1997; Carpenter *et al.*, 1998; Mochizuki *et al.*, 1999). There may be genetic variation between recent CDV strains and the old CDV isolates used in vaccines, and cases of canine distemper in vaccinated animals suggest the emergence of CDV with different antigenic properties from the vaccine strains. Following the attenuation of the Onderstepoort strain of CDV, there have not been any reports of CDV isolation in South Africa and there are no sequence data of field CDV isolates from Africa available for comparison with established lineages.

Domestic dogs (*Canis familiaris*) are probably the most numerous carnivores in the world today (Daniels & Bekoff, 1989). Dogs have been introduced wherever man has settled; hence the exponential growth of the human population in the past

century has led to an equally rapid increase in dog numbers. Dogs have been implicated in the transmission of canine distemper to lions (Roelke-Parker *et al.*, 1996). Dogs are also said to be partly responsible for the extinction of the African wild dog (*Lycaon pictus*) in areas of the Serengeti ecosystem (Woodroffe, 1999). Butler *et al.*, (2004) postulated predation of diseased dogs as a route of infection to large carnivores in Zimbabwe. The dog population in South Africa was conservatively estimated to be around 4 million in 1999/2000 and it was reported that approximately 1 million dogs visit a veterinarian at least once a year (Leisewitz *et al.*, 2001). Assuming an annual dog population growth rate of 5 % as postulated for Tanzania (Cleaveland *et al.*, 2000) and 6.5 % for Zimbabwe (Butler & Bingham, 2000), the South African dog population can be put at 6 million (2007/2008) with approximately 1.5 million said to visit a veterinarian at least once a year. This implies that the greater proportion of the South African dog population is unvaccinated and probably also reproductively active. The conditions for persistent large-scale CDV disease therefore exist, as it has been estimated that at least 300 000 individuals are required to maintain a morbillivirus in circulation (Black, 1991). This situation poses a threat to the domestic dog population and the wildlife resource of the country. This study therefore genetically characterized current canine distemper viruses from dogs in South Africa to lay a foundation for further molecular epidemiological surveillance in wild and domestic members of the family Canidae.

Enlarging the collection of CDV-specific sequences obtained globally from a growing number of host species would provide the basis not only for understanding the molecular epidemiology of CDV but also for the improvement of

current CDV vaccines. Considering the implications of the geographically distinct lineages in the diagnosis, epidemiology and control of CDV infections, it is deemed important to characterise the canine distemper viruses occurring in a region.

The aims of this research project were:

1. To isolate canine distemper viruses and provide a pool of local strains for genetic characterization.
2. To identify the lineage(s) of CDV circulating in South Africa and obtain phylogenetic data of local strains, and thus determine whether contemporary strains had undergone significant genetic changes relative to the currently used vaccine strains.
3. To evaluate South African canine distemper viruses in terms of the global classification and determine if a unique African lineage exists.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Classification and characterization of canine distemper viruses

Canine distemper virus (CDV) is classified in the genus *Morbillivirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, in the order *Mononegavirales*. Genera of the family include *Paramyxovirus*, *Morbillivirus* and *Pneumovirus* (Pringle, 1997). Paramyxoviruses and orthomyxoviruses were originally grouped together as the “myxoviruses,” because of the morphological similarity of the virions and the fact that the prototype viruses, newcastle disease virus and influenza virus, each carry a haemagglutinin and a neuraminidase. However, it was later realized that the viruses of each group differ in such basic properties as genome structure and mode of replication; hence they were separated into two families (Fenner, 1987).

There is only one serotype of CDV, but strains vary in virulence. It was believed that growth of CDV in cell cultures from dogs, ferrets, monkeys, and humans, or in embryonated chicken eggs, was only possible after adaptation (Fenner, 1987). However, CDV has been most easily and efficiently isolated in Vero.DogSLAM cells from clinical specimens (Seki *et al.*, 2003). Biochemical analysis of CDV was hampered for a long time by the poor growth of the virus *in vitro*, the difficulty in eliminating cellular contaminants, and the high susceptibility of some of its proteins to proteolysis. But, since the beginning of the 1980s, sequencing and cloning technologies have made it possible to produce substantial new data on the biology of CDV (Diallo, 1990).

Canine distemper virus has an unsegmented, negative-sense, single-stranded, approximately 15.7 Kb RNA genome and an enveloped virus particle that is 150 to 300 nm in diameter (Murphy *et al.*, 1999). It is a labile virus and is sensitive to heat, UV irradiation, lipid solvents, detergents and oxidising agents (Grone *et al.*, 1998). It can survive at room temperature in tissues and exudates for between 20 minutes and 3 hours. In environmental temperatures below zero it will survive several days if protected by organic material (Greene & Appel, 2006). Canine distemper virus has one known non-structural protein (C) and six structural proteins: large protein (L), haemagglutinin (H), phosphoprotein (P), nucleocapsid protein (N), fusion protein (F) and matrix protein (M) (Diallo, 1990). The non-structural protein (C) is produced by an alternative open reading frame in the P gene (Lamb & Kolakofsky, 2001). The actual function of C protein in the biology of the virus is not clear. The most commonly reported mutations are in the H, F and N proteins. The H and F proteins are the major target antigens for the host immune response, where the highest genetic/antigenic variation in the CDV has been found (Mochizuki *et al.*, 1999).

The RNA polymerase and transcriptase-associated phosphoprotein P gene is highly conserved among members of CDV strains and has been used for phylogenetic analysis by some workers (Carpenter *et al.*, 1998; Pardo *et al.*, 2005; Rockborn, 1958; Rzezutka & Mizak, 2003). The P protein is susceptible to proteolysis and has a molecular weight of between 54.9 to 66 kDa. It has a polymerase entry site and is expected to play a function in RNA transcription, RNA replication or both (Sidhu *et al.*, 1993).

The fusion protein F is one of the viral surface glycoproteins. The gene is conserved within morbillivirus species (Barrett *et al.*, 1991; Liermann *et al.*, 1998) and has also been used to determine phylogenetic relationships among these viruses (Visser *et al.*, 1993). The F protein is synthesized as a precursor, F<sub>0</sub>, which undergoes glycosylation and endoproteolytic cleavage into two disulfide-linked fragments (F<sub>1</sub>-F<sub>2</sub>) which is critical for functional activation. The molecular weight of F<sub>1</sub> is 40 kDa, while the molecular weight of F<sub>2</sub> is approximately 20 kDa (Diallo, 1990). Both F<sub>1</sub> and F<sub>2</sub> have structural function (penetration). It induces neutralizing antibodies and is one of the protective viral proteins. The F protein enables CDV to fuse cells together to cause syncytia. It mediates fusion between the virus and the infected cell or between the infected cell and an adjacent cell, giving it an essential role in spreading the virus within the host. The transformation of F<sub>0</sub> to F<sub>1</sub> and F<sub>2</sub> is necessary for the biological activity of the protein, and any event which could impede cleavage leads to a less virulent virus (Barrett *et al.*, 1987).

The nucleocapsid protein N is highly conserved in all morbilliviruses (Blixenkrone-Moller *et al.*, 1992a; Kamata *et al.*, 1991) and it plays an important role in the development of persistence. It is the most abundant structural virus protein. It influences virus assembly and has some regulatory functions in virus transcription and replication (Stettler & Zurbriggen, 1995). The N gene contains 1683 nucleotides which express 523 amino acids (Yoshida *et al.*, 1998). The protein has a molecular weight of 58 kDa and its function is structural (protects the genome). It folds and protects the genome and is very sensitive to intracellular proteolysis. The N protein of CDV is phosphorylated and its mobility in SDS-polyacrylamide gels varies slightly from one strain to another (Diallo, 1990).

The envelope-associated matrix protein M forms a structure surrounding the nucleocapsid (Peeples, 1991). The attachment of the matrix protein to the nucleocapsid is essential for the budding process. An internal layer of the matrix protein (M) stabilizes the membrane. It has a molecular weight of 34 kDa and its function is structural (penetration). The matrix protein is readily translated *in vitro*. It consists of 335 amino acids and lies under the lipid bilayer. It is thought to play an important function during the maturation of the virus and also serves as a link between the nucleocapsid and the two surface glycoproteins, H and F (Diallo, 1990).

The transcriptase-associated large protein L is a multifunctional RNA-dependent RNA virion polymerase. It is a minor viral nuclear protein with a molecular weight of 180 to 200 kDa; it plays a functional role (polymerase complex). This gene is the last transcribed by the virus. In association with N, P proteins and the genomic RNA it forms the nucleocapsid. Because of its large size, it is expected to exhibit the majority of the RNA polymerase activity (Diallo, 1990).

The haemagglutinin/attachment gene H is an enveloped glycoprotein. The H gene consists of a fragment of 1824 base pairs and a single reading frame encoding 607 amino acids (Lan *et al.*, 2005a). It is the CDV receptor binding protein with a molecular weight of 76 kDa and its function is structural (viral attachment). The H protein serves to attach the virus to the host cell. The division of the Paramyxoviridae family reflects the activity of this attachment protein. The paramyxovirus genus has an attachment protein with both haemagglutinating and neuraminidase activities; while the attachment protein of the morbilliviruses



exhibits only haemagglutinating activity and that of the pneumoviruses has neither haemagglutinating activity nor neuraminidase activity (Diallo, 1990). The H protein is the major determinant of tropism and cytopathogenicity (Von Messling *et al.*, 2001). The protein induces neutralizing antibodies and is a protective viral protein. H is more variable among CDV isolates perhaps due to the role the protein plays in the host immune reaction, and thus has been widely used for phylogenetic analyses (Bolte *t al.*, 1997; Haas *et al.*, 1997; Hashimoto *et al.*, 2001; Mochizuki *et al.*, 1999).

The geographical classification of CDV on the basis of the nucleotide alignment of the H protein (Harder & Osterhaus, 1997; Martella *et al.*, 2006) includes 7 major lineages:

1. Arctic-like: This group consist of strains from seals in Siberia and from dogs in Greenland, China and Italy.
2. America 1: This group has vaccine strains (Convac, Lederle, Onderstepoort and Snyder-Hill) and strains from raccoons in the United States.
3. America 2: These are isolates from a Javelina, a Black leopard, leopards, raccoons and dogs in the United States.
4. Asia 1: This group consists of isolates from dogs and raccoons in Japan, Taiwan and China.
5. Asia 2: These are isolates from domestic dogs in Japan.
6. Europe: Isolates from dogs in Italy, Turkey, Hungary, Germany and Denmark.
7. Europe-wildlife: These are from a panda (China), ferret (Germany), mink (Denmark) and fox (Italy).

Due to the few epidemiological surveys and the different genes targeted in the various studies, the distribution of the major CDV lineages throughout the world is not clear. This work on field strains from South Africa was an attempt to compare and see if African field strains group with the Onderstepoort vaccine strain in lineage America 1 or if they form a separate African lineage.

## **2.2 Epidemiology**

### **2.2.1 Geographical distribution**

Canine distemper virus has a worldwide distribution. Serological surveys and molecular epidemiological studies in many parts of the world have shown that CDV infection is endemic globally. In Africa, CDV infections have been documented among domestic dogs in South Africa and Nigeria as in other parts of the continent (Ezeibe, 2005; Leisewitz *et al.*, 2001). Evidence of CDV infection has also been reported among wildlife species in Botswana, Zimbabwe, South Africa, Tanzania and other parts of the African continent (Butler *et al.*, 2004; Van Vuuren *et al.*, 1997; Woodroffe, 1999).

Canine distemper is widespread amongst domestic and wildlife species in America (Pardo *et al.*, 2005). Over 300 dogs were reported dead during an outbreak of canine distemper in Alaska (Maes *et al.*, 2003) and evidence of CDV infection has been reported amongst vaccinated dogs in Mexico (Simon-Martinez *et al.*, 2007). The presence of the disease in Brazil has been documented (Headley & Graça, 2000; Saito *et al.*, 2006). Canine distemper has been detected in both vaccinated and unvaccinated dogs in Argentina (Calderon *et al.*, 2007), while an epidemic of the disease among raccoons in Chicago occurred in 1998 (Lednicky *et al.*, 2004b).

In Europe, evidence of CDV infections has been reported in Italy (Martella *et al.*, 2006), Germany (Frisk *et al.*, 1999), Hungary (Demeter *et al.*, 2007) and Northern Ireland (Harder & Osterhaus, 1997). An epidemic in vaccinated dogs has occurred in Finland and canine distemper is still an important infection in Poland where 71% of dogs are unvaccinated (Jozwik & Frymus, 2002). Recently, canine distemper outbreaks have been reported on dog breeding farms in Australia (Norris *et al.*, 2006).

Canine distemper is endemic in Asia as in other parts of the world. Its endemicity in different part of that continent has been documented most recently in Japan (Lan *et al.*, 2006), Thailand (Keawcharoen *et al.*, 2005), the Republic of Korea (An *et al.*, 2008) and India (Latha *et al.*, 2007b). Canine distemper infections occur on all continents and it has remained an important worldwide disease. Analysis of CDV strains detected globally and from a variety of host species will give a more in-depth understanding of the global ecology of CDV and will provide the basis for improvement of current vaccines.

### **2.2.2 Transmission and sources of infection**

Canine distemper virus is most abundant in respiratory exudates and is commonly spread by aerosol or droplet exposure. However, the virus can be isolated from most body tissues and secretions, including urine. Transplacental infection can occur from viraemic bitches. Virus can be excreted up to 60 to 90 days after infection although in the majority of dogs shedding stops within 2 weeks. Contact between recently infected and susceptible animals maintain the virus in a

population, and a constant supply of puppies helps provide a susceptible population for infection (Greene & Appel, 2006).

### **2.2.3 Host susceptibility**

Canine distemper infects a broad range of animals, such as *Canidae* (domestic dog, coyote, wolf, jackal, fox, dingo), *Mustelidae* (ferrets, minks, otter, skunks, weasels, badgers), *Procyonidae* (coatimundi, raccoons, pandas), *Ursidae* (bears), *Viverridae* (civets, genets, linsangs), *Hyaenidae* (hyenas), *Felidae* (lions, tigers) and *Ailuridae* (lesser and giant pandas) (Appel *et al.*, 1974; Deem *et al.*, 2000; Evermann *et al.*, 2001; Haas *et al.*, 1996).

A major outbreak of CDV occurred in large cats in 1992 in North America and mortalities were recorded in lions (*Panthera leo*), tigers (*Panthera tigris*), leopards (*Panthera pardus*) and jaguar (*Panthera onca*) (Appel *et al.*, 1994). In 1994, CDV outbreaks killed 30% of the free-ranging lion population and an unknown number of hyenas in the Tanzanian Serengeti National Park and adjacent areas (Roelke-Parker *et al.*, 1996). Six years later, CDV outbreaks with mortalities amongst African wild dogs (*Lycaon pictus*) in Tanzania were documented (Bildt *et al.*, 2002).

Spontaneous, clinically overt infections with CDV have been described in captive Japanese primates (*Macaca fuscata*) (Yoshikawa *et al.*, 1989).

Mamaev *et al.*, (1995) characterised isolates from Lake Baikal seals and (Bergman *et al.*, 1990) reported pathological findings of CDV in European seals. Van Moll *et al.*, (1995) reported evidence of CDV in mustelids and foxes from southwest Germany.

Stanton *et al.*, (2002) reported the prevalence of antibodies against CDV in foxes and wolves from Spain and the prevalence of antibodies to CDV in free-ranging jackals (*Canis adustus* and *Canis mesomelas*) in Zimbabwe has been reported (Spencer *et al.*, 1999) . Serological surveys also revealed evidence of CDV infections in foxes and martens in different parts of Germany (Frolich *et al.*, 2000). In South Africa, exposure to CDV has been demonstrated serologically in lions and leopards. However, no catastrophic mortality due to CDV infection has been reported in South African wildlife (Van Vuuren *et al.*, 1997).

### **2.3 Pathogenesis**

Inhalation of airborne virus leads to infection of macrophages in the respiratory tract. The virus spreads within 24 hours to local lymph nodes and within seven days to all lymphatic tissues. During this period, usually between three and six days post-infection, the first temperature elevation occurs along with lymphopenia (Appel, 1969). During the second and third weeks post-infection, dogs either initiate vigorous humoral and cellular immune responses and they recover without further clinical signs, or have weak immune responses and develop acute or subacute disease. In dogs that fail to recover early, infected lymphocytes and macrophages carry the virus to the surface epithelium of the alimentary,

respiratory, and urogenital tracts and to the central nervous system. Clinical signs follow the local infection (Greene & Appel, 2006; Shell, 1990).

Neurological manifestations of distemper usually begin 1 to 3 weeks after recovery from systemic illness. Neurological signs vary according to the area of the CNS involved. The virus strains that affect the grey matter induce acute fatal infection and neuronal damage. A more chronic disease is seen when the virus affects mainly the white matter of the CNS causing demyelination. Neurological signs (acute or chronic) are typically progressive and old dog encephalitis (ODE) is characterised by this type of progressive history (Andrea, 1995). The ODE is due to an inflammatory reaction associated with persistent CDV infection in the CNS and defective viral particles may play a role in its pathogenesis (Merck, 1998).

## **2.4 Clinical signs**

The wide variety of clinical signs observed during CDV infections demonstrates the multisystemic nature of the disease. The clinical signs of distemper relate essentially to the respiratory, gastrointestinal and central nervous systems. Canine distemper is often characterized by a biphasic fever, accompanied by serous oculo-nasal discharges, later mucopurulent discharges, anorexia, conjunctivitis, bronchitis, pneumonia, gastroenteritis, and nervous signs (Summers & Appel, 1994; Thulin *et al.*, 1992).

Skin lesions such as vesicular and pustular dermatitis may occur in dogs. Hyperkeratosis and parakeratosis with vesicles and pustule formation may also be

noticed. However, skin lesions are rarely associated with neurological complications and this is usually a favourable prognostic sign (Maeda *et al.*, 1994).

Dental abnormalities may be seen in young puppies infected with CDV (Bittegeko *et al.*, 1995). Young, growing dogs may show metaphyseal osteosclerosis of long bones (Baumgartner *et al.*, 1995; Frame *et al.*, 1999)

Neurological signs may include localized involuntary twitching of a muscle or group of muscles in the leg or facial muscles (Andrea, 1995). Paresis or paralysis, convulsions characterized by salivation and chewing movements of the jaw, the dog falling on its side and paddling its legs; involuntary urination as well as defecation are among the many neurological manifestations of CDV infections (Greene & Appel, 2006).

Clinically, the most common form of canine distemper seen at the Onderstepoort Veterinary Academic Hospital (OVAH) is the neurological form in older dogs (Leisewitz *et al.*, 2001), but at a satellite clinic of the Medical University of Southern Africa that served a more resource-deprived community than the OVAH, catarrhal signs were the most common (Eckersley *et al.*, 1992; Rautenbach *et al.*, 1991).

## **2.5 Diagnosis**

For practitioners, the diagnosis of canine distemper is primarily based on clinical signs and history. A characteristic history of 3 to 6-months-old unvaccinated puppy with clinical signs compatible with the disease supports the diagnosis. However,

some laboratory techniques have been useful to confirm the diagnosis of CDV infection under various conditions.

### **2.5.1 Haematology**

A typical haematological profile of a dog with acute CDV infection includes anaemia, thrombocytopenia, absolute lymphopenia, neutropenia and monocytopenia (Shell, 1990). Canine distemper virus inclusion bodies within erythrocytes are usually singular, oval, or irregular in shape and appear red or blue on Wright's stain (Gossett *et al.*, 1982). Leukocyte inclusion bodies have been described as homogenous, round, oval, or irregular bodies that measures 1 to 4 microns in diameter. They appear red with Schorr's stain (Cello *et al.*, 1959) and gray with Wright's stain (Gossett *et al.*, 1982).

### **2.5.2 Serum chemistries**

Hypoalbuminaemia, hyperglobulinaemia, or hypocalcaemia have been occasionally reported in CDV infections (Appel, 1969). Gnotobiotic pups infected with virulent CDV at 6 to 10 days of age were found to have significantly reduced serum calcium concentrations (Weisbrode & Krakowka, 1979).

### **2.5.3 Antibody detection**

Canine distemper virus titers in serum may help support the diagnosis of CDV infection. The detection of anti-CDV IgM antibody indicates recent infection or recent vaccination (Guy, 1986). Acutely infected dogs may die without neutralizing antibody in their serum and subacutely or chronically infected dogs may have antibody levels comparable to vaccinated dogs (Appel & Summers, 1999).



### **2.5.3.1 Virus neutralization test**

The neutralization test is still considered the gold standard for measuring protection against infection, and serum titers correlate well with the level of protection. Neutralizing antibodies directed against the membrane proteins (H and F) of the virus, appear beginning 10 to 20 days post infection, and may persist for the life of a recovered animal (Appel & Robson, 1973).

### **2.5.3.2 Indirect immunofluorescence**

The indirect fluorescent antibody test detects anti-CDV IgG in the serum and CSF, or anti-CDV IgM in the serum. However, the presence of IgG alone may be due to subacute or chronic infection or vaccination, while the presence of IgM is an indication of a recent infection, if vaccination during the preceding 3 weeks can be ruled out (Maes *et al.*, 2003).

### **2.5.3.3 Enzyme-linked immunosorbent assay (ELISA)**

Whole-virus ELISA has been used to detect serum IgG and IgM antibodies to CDV (Greene & Appel, 1984). Recently, an ELISA test which uses a recombinant protein N, has been recommended for the serodiagnosis of CDV infection in dogs in India (Latha *et al.*, 2007a).

## **2.5.4 Antigen detection**

### **2.5.4.1 Direct immunofluorescence**

Blood, CSF, urine sediment, bone marrow, and cells from conjunctival, genital and respiratory epithelium are good samples for the diagnosis of CDV by direct immunofluorescence (Fairchild *et al.*, 1967). The direct fluorescent antibody test is

used for antigen detection. The cells from test samples are smeared on clean slides and stained with a fluorescein dye conjugated with CDV antibody. A negative response does not prove the absence of CDV; antibody produced by the dog may coat the viral antigen and produce a false-negative result. False-negative results may also occur if samples are taken late in the course of infection (Guy, 1986).

#### **2.5.4.2 Immunochromatography**

Recently, an immunochromatographic assay which employs two monoclonal anti-CDV antibodies has been developed for rapid antemortem diagnosis of dogs with CDV infection. This assay does not require special instruments and may be considered simple enough for dog owners to use (An *et al.*, 2008).

#### **2.5.4.3 Enzyme-linked immunosorbent assay (ELISA)**

ELISA has been used to detect viral antigen in whole blood and CSF of natural and experimentally infected dogs (Gemma *et al.*, 1996; Soma *et al.*, 2003). In one study, modified-live virus vaccination produced a false-positive result in testing for viral antigen by ELISA (Soma *et al.*, 2003).

#### **2.5.4.4 Immunohistochemistry**

Monoclonal antibodies are commercially available and used to detect CDV by immunohistochemical methods. The detection of CDV antigen in the nasal mucosa, footpad epithelium, and haired skin of the dorsal neck has been used consistently for the antemortem diagnosis of CDV by immunohistochemistry

(Haines *et al.*, 1999). Immunohistochemistry has been used to study CDV antigen among wild carnivores in southwest Germany (Van Moll *et al.*, 1995).

Some immunohistochemical techniques have been developed for histological detection of distemper antigen in formalin-fixed and paraffin-embedded tissues and cell culture (Gathumbi, 1993). Immunohistochemical demonstration of CDV antigen is superior to reliance on inclusion bodies in brain tissue to confirm distemper encephalitis. Results are more likely to be positive in acute than chronic infections in which viral antigens may not be expressed (Palmer *et al.*, 1990).

### **2.5.5 CSF analysis**

Dogs with subacute to chronic inflammatory forms of CDV encephalomyelitis show increases in protein levels (>25 mg/dl) and cell counts (>10 cells/ $\mu$ l with a predominance of lymphocytes) in CSF. Intracytoplasmic inclusions may also be found in the CSF cells of dogs with CDV infection. The levels of interferon (IFN) are also increased in the CSF of dogs with acute and chronic distemper encephalitis (Tsai *et al.*, 1982). Differences in the humoral immune response in CSF and sera to the H and F envelope proteins have been noted between some dogs with chronic progressive encephalitis and those with other forms of distemper encephalitis (Rima *et al.*, 1987).

The presence of anti-CDV IgG antibody in CSF offers definitive evidence of distemper encephalitis because antibody is locally produced; these increases have not been found in vaccinated dogs or those with systemic distemper without CNS disease. However, CSF antibody may be present from traumatic collection

procedures causing contamination by whole blood. Although the test for CSF antibodies is sensitive and specific for CDV, the sample collection require experienced personnel (Greene & Appel, 2006).

### **2.5.6 Viral isolation**

Various cell culture systems have been used for the isolation of CDV. Virulent CDV can be readily cultured in macrophages or activated lymphocytes, but it grows only with adaptation in epithelial or fibroblast cell lines. In alveolar macrophage cultures, giant cell (syncytia) formation, a characteristic cytopathic effect of CDV in many other tissue cultures, is detected within 2 to 5 days, at which time the virus can be isolated by overlays made on other cells. Macrophage cultures have been replaced by dog lymphocyte cultures for the isolation of CDV. Buffy coat cells or tissues from infected animals can be cultivated with mitogen-stimulated canine blood lymphocytes, and cultures are examined 72 to 144 hours later by immunofluorescence (Appel *et al.*, 1992). A marmoset lymphoid cell line (B95a) has also been used for CDV isolation (Kai *et al.*, 1993). Ferret peritoneal macrophages has been used for the isolation of CDV (Poste, 1971; Whetstone *et al.*, 1981). Less commonly, CDV isolation on the chorioallantoic membrane of embryonated chicken eggs has been used (Ezeibe, 2005; Haig, 1956). Canine distemper virus has been isolated effectively from naturally infected free-ranging raccoons using cell lines such as MDCK (canine epithelial kidney cells), MV 1 LU (mink lung), and Vero (African green monkey kidney cells) (Lednicky *et al.*, 2004a). Vero cells expressing canine signalling lymphocyte activation molecule (Vero.DogSLAM) has been established to aid the isolation of CDV from clinical material within 24 hours of inoculation (Seki *et al.*, 2003).

### 2.5.7 Nucleic acid detection

Reverse transcription has been used to detect CDV RNA in buffy coat cells from dogs with acute CDV infection, and CDV nucleoprotein RNA has been detected by reverse transcription- polymerase chain reaction (RT-PCR) using serum, whole blood and CSF from dogs with distemper (Frisk *et al.*, 1999). Regardless of the duration and form of distemper, a positive result was highly specific for diagnosis. Viral mRNA has been detected in footpad specimens from infected dogs (Grone *et al.*, 2003). Polymerase chain reaction (PCR) and nucleic acid hybridization studies using single stranded RNA probes have been performed to detect virulent virus in tissue culture and histologic sections (Zurbriggen *et al.*, 1993). A real-time RT-PCR assay was developed for detection and quantitation of CDV. The assay exhibited high specificity and a quantitative TaqMan was validated on clinical samples, including various tissues and organs collected from dogs naturally infected by CDV (Elia *et al.*, 2006). Reverse transcription-PCR, nested-PCR and southern blot hybridization has been used for the detection of the phosphoprotein gene of CDV in peripheral blood mononuclear cells and internal organs of dogs and fur animals in Poland (Rzezutka & Mizak, 2002). Recently, a hemi-nested multiplex PCR which provides a rapid approach for the investigation of CDV outbreaks has been reported. The system was used to genotype the major CDV lineages and is advocated to be useful for large-scale molecular epidemiological studies of CDV and for the diagnosis of vaccine-related disease (Martella *et al.*, 2007).

Semi-nested PCR has also been used to efficiently detect CDV in paraffin-embedded nervous tissue. Sequence determination using this method may yield molecular epidemiologic information regarding vaccine efficacy (Stanton *et al.*, 2002). During the outbreak of CDV in Alaska, PCR was used to detect the infection and trace the origin of the responsible strain to Siberia (Maes *et al.*, 2003). Various nucleic acid detection methods have been used to diagnose, characterize and trace the origin of CDV in outbreaks. Generally, RT-PCR is very valuable for the antemortem diagnosis of CDV. However, it is not widely available. A positive RT-PCR result is indicative of infection, whereas a negative one can result from many factors including improper sample handling, nucleic acid extraction method and choice of primers.

## **2.6 Treatment**

Lack of an effective antiviral treatment for CDV infection creates the need for supportive care, symptomatic treatment, and a guarded prognosis. Antibiotic therapy is indicated because of the common occurrence of secondary bacterial infections of the respiratory and alimentary tracts. Fluid and electrolyte support is also indicated for dehydrated dogs with CDV infection as a result of diarrhoea. Sedatives and anticonvulsants may ameliorate neurological clinical signs but they do not have a curative effect. Parenterally administered antiemetics may be necessary to control vomiting. Expectorants and coughage may also be useful if the cough is productive. Multicomplex B vitamins administered intramuscularly or added to intravenous fluids may help stimulate appetite in cases of anorexia (Appel & Summers, 1999; Greene & Appel, 2006; Shell, 1990). Controlled studies have documented a decrease in morbidity and mortality in children with measles

who received two 200,000-IU (60-mg) doses of vitamin A within 5 days of the onset of systemic illness (Hussey & Klein, 1990). Although its effectiveness in treating distemper is unproven, a similar regimen could be tried for puppies with acute systemic infection.

## **2.7 Prevention and control**

The amount of maternal antibody transferred to a pup is directly proportional to the amount possessed by the bitch. This maternally transferred antibody causes a period of temporary immunity that varies in duration of only a few days to 3 - 4 months. During this period of passive immunity, CDV antibodies from the colostrum prevent both infection and successful immunization with CDV vaccines (Baker *et al.*, 1968; Robson *et al.*, 1959). In most pups, the concentration of maternal antibody is sufficiently reduced at 6 to 8 weeks of age; this renders the animals susceptible to infection and allows them to respond to CDV vaccines. In some pups, maternal antibody is still protective at 14 weeks of age, thus preventing immunization by CDV vaccines that were previously administered. Because practitioners cannot routinely determine when the concentration of maternal antibody is insufficient in each patient, vaccinations should be done every 2 to 4 weeks beginning at 6 to 8 weeks of age and continuing until 14 to 16 weeks of age to protect the majority of pups (Shell, 1990). Immunity following natural CDV infection is considered life-long.

Current licensed CDV vaccines contain viral strains that have been attenuated by serial passage, either on canine kidney cells (Rockborn), hen eggs (original Onderstepoort) or chicken fibroblast cultures (Lederle strain) (Chappuis, 1995).

Modified-live CDV vaccines produced from either avian or canine cell culture adaptations are very effective in inducing an immunity that lasts at least one year and probably for several years in most dogs. Modified-live CDV vaccines may revert to virulence in certain wildlife and zoo animals. Inactivated virus vaccines must be used in wildlife species. Inactivated CDV vaccines were used some 60 years ago and were unable to control the disease in dogs and are no longer commercially available (Appel & Summers, 1999).

Recombinant CDV and DNA vaccines are emerging with the advancement in biotechnology. Genes coding for the H and F proteins of CDV are being used as inserts in vectors. Immunity to the CDV H and F proteins has been found to be protective. A canarypox recombinant vaccine is now available commercially and its efficacy is comparable to that of the modified-live vaccines (Schultz, 2006).

In addition to vaccination, other control measures include strict isolation of dogs with canine distemper and disinfection of apparatus and the environment with commonly used disinfectants.

## **2.8 Public health considerations**

Canine distemper virus is discussed as a candidate that might play a role in the initiation of multiple sclerosis (Rohowsky-Kochan *et al.*, 1995). Multiple sclerosis (MS) is a neurological affliction of humans. The cause of MS is still uncertain, but no substantial evidence for human measles virus or CDV involvement exists (Hodge & Wolfson, 1997). The evidence for the role of CDV is indirect, and examination of the case control data for reported associations reveals that the



existing evidence is weak. Human measles and paramyxovirus are still likely candidates for MS involvement, and herpes viruses have also been implicated. Furthermore, the incidence of MS has not reduced since before 1960, despite the widespread reduction of measles and distemper through effective vaccines (Greene & Appel, 2006).

A possible link between Paget's disease of bone in humans and CDV infection in dogs has been shown in an epidemiological study and was substantiated by detection of CDV RNA in affected tissues (Gordon *et al.*, 1992; O'Driscoll *et al.*, 1990). Some workers have suggested that Paget's disease, an inflammatory bone disorder in humans, might be related to CDV acquired from exposure to dogs. Paget's disease is a chronic disease that leads to progressive destruction, remodelling, and deformity of bone (Reddy *et al.*, 1995). Using in situ hybridization, CDV genetic sequences have been found in the bone of 63.5% of untreated humans with Paget's disease (Cartwright *et al.*, 1993; Gordon *et al.*, 1991; 1992). In a study using same technique, CDV RNA was detected in 100% of lesions from pagetic patients but in none of the control specimens, including uninvolved sites of pagetic patients, normal bone, and active remodelling bone (Mee *et al.*, 1998). Owning a dog was found to be highly correlated with Paget's disease, but this indirect relationship should not be overstated because a similar correlation was found between Paget's disease and ownership of cats and birds. Other studies have implicated other paramyxoviruses such as measles virus variants (Reddy *et al.*, 1995; 1999). Until such viruses are isolated and completely sequenced, CDV's role if any in such infections is questionable.

## CHAPTER 3

### ISOLATION OF WILD-TYPE CANINE DISTEMPER VIRUSES FROM DOMESTIC DOGS IN SOUTH AFRICA

#### 3.1 Introduction

Canine distemper virus (CDV) is a highly contagious viral pathogen causing lethal disease in both domestic and wild, land and sea living animals. It is classified in the *Morbillivirus* genus of the family *Paramyxoviridae* ((Griffin, 2001; Murphy *et al.*, 1999). Studies on the characteristics of CDV strains prevailing in the field are scanty, perhaps due to difficulties in isolation and titration of the virus (Lan *et al.*, 2005b). Canine distemper virus isolates detected in naturally occurring cases cluster according to geographical distribution (Bolt *et al.*, 1997; Carpenter *et al.*, 1998). Further studies have reported genetic variation between vaccine strains and current circulating wild-types (Lan *et al.*, 2006; Martella *et al.*, 2006). Since the attenuation of the Onderstepoort vaccine strain in the 1940s (Haig, 1956), there has not been any reports of CDV isolations in southern Africa, justifying the need to isolate field strains from clinical specimens to provide a pool of local strains for characterization.

Canine distemper virus occurs as virulent (wild-type) and laboratory-modified strains that have different target cell tropisms (Appel *et al.*, 1992). Attenuated virus grows readily in monolayers of a variety of epithelial and fibroblast cell lines and in primary cell cultures from different species. However, isolation of virulent CDV in these cells is difficult (Appel & Gillespie, 1972a; Appel, 1978). Canine distemper virus has been isolated from animals by co-cultivation of infected tissues with mitogen-stimulated lymphocytes derived from healthy dogs (Appel *et al.*, 1992), or

canine alveolar macrophages (Appel, 1978; Appel & Jones, 1967). The virus has also been isolated with the aid of ferret peritoneal macrophages obtained from specific pathogen free (SPF) ferrets (Poste, 1971; Whetstone *et al.*, 1981). However, isolation using these methods tends to be prolonged and requires multiple blind cell-passages before cytopathic effects (CPE) are noticed. Moreover, many laboratories no longer keep SPF dogs or ferrets due to animal welfare concerns. These methods are therefore not user-friendly for diagnostic laboratories processing clinical specimens. Less commonly, CDV isolation on the chorioallantoic membrane of embryonated chicken eggs has been used (Ezeibe, 2005; Haig, 1956). This technique requires multiple weekly passages in fresh eggs before viral CPE becomes evident and is relatively expensive. Lednicky *et al.*, (2004a) reported effective primary isolation of CDV from naturally infected free-ranging raccoons using cell lines such as MDCK (canine epithelial kidney cells), MV1 LU (mink lung), and Vero (African green monkey kidney cells). However, a minimum of 10 days was required for isolations in addition to the multiple passages. Cells of the B95a type have also been reported to be highly effective for the isolation of CDV (Kai *et al.*, 1993). Unfortunately, B95a cells are derived from marmosets, which are endangered animals and the purchase and possession of B95a cells in some countries, e.g. the U.S.A, requires a government permit.

Signalling lymphocyte activation molecule (SLAM) has been reported to be the principal cellular receptor for morbilliviruses *in vivo* (Tatsuo *et al.*, 2001), and Vero.DogSLAM cells has been shown to aid the isolation of CDV from clinical material within 24 hours of inoculation, with a significant reduction in the costs associated with the isolation of wild-type CDV (Seki *et al.*, 2003). This study

therefore, explored this faster and cheaper alternative to isolate field strains of CDV from dogs in South Africa, and to obtain a pool of viruses that will enable molecular comparisons with vaccine and other published strains.

## **3.2 Materials and Methods**

### **3.2.1 Clinical specimens**

Clinical specimens were obtained from veterinary practices and diagnostic laboratories in South Africa. Specimens obtained from living dogs were limited to sick animals showing clinical signs suggestive of canine distemper and included blood in heparin-containing tubes and cerebrospinal fluid (CSF).

Necropsy specimens were collected from dogs that were euthanized or died with signs of canine distemper and included spleen, lung, and brain. All specimens were chilled during transport to the laboratory. One hundred and sixty two clinical specimens from 124 living and dead animals were received for virus isolation. A litter of 10 puppies was euthanized and the spleen and whole blood from all 10 puppies were submitted. For economic reasons, three blood samples were selected randomly, and the 10 spleen samples were pooled as 2 samples.

Peripheral blood mononuclear cells (PBMCs) were extracted from blood samples as described (Appel *et al.*, 1992). Briefly, heparinized blood collected from dogs tentatively diagnosed with canine distemper by clinical examination was diluted with an equal part of RPMI 1640 medium with L- glutamine. The diluted blood was overlaid on an equal volume of Histo-Paque® and centrifuged for 30 minutes at 500 *g*. The cell band was removed and washed 3 times in RPMI 1640 with L-

glutamine plus 2% foetal bovine serum in polypropylene tubes. Cells were resuspended at a concentration of  $5 \times 10^6$  cells/ml in RPMI 1640 with L-glutamine plus 2% foetal bovine serum and 50  $\mu$ l gentamycin. Cerebrospinal fluid was used directly to inoculate Vero.DogSLAM cells since they were collected in a sterile manner by clinicians and thus suitable for viral isolation in cell cultures. Necropsy tissues were processed on receipt as described by (Seki *et al.*, 2003) with some minor modifications. Sterile sand was used to grind 0.5 grams of the organ in a mortar and resuspended in 10 ml phosphate buffered saline with calcium and magnesium (PBS plus). The suspension was centrifuged at 1800 *g* for 5 minutes and the supernatant filtered through a 0.22  $\mu$ m filter to remove bacterial and fungal contaminants. The filtrate was used for inoculation into cell cultures and the remaining fluid poured into 2ml freezing tubes (Nunc) for storage. The remaining unprocessed tissues were stored at -80 °C for future use.

### **3.2.2 Vero.DogSLAM cells**

Vero.DogSLAM cells were obtained from Dr Yusuke Yanagi, Kyushu University, Fukuoka, Japan and were grown in 25 cm<sup>2</sup> tissue culture flasks with minimum essential medium supplemented with 7% foetal bovine serum and incubated in a 37 °C incubator. The preparation of Vero.DogSLAM cells was described by Tatsuo *et al.*, (2001) and Seki *et al.*, (2003). Briefly, total RNA from canine PBMCs stimulated with 2.5  $\mu$ g of phytohaemmagglutinin per ml was extracted and amplified using various combinations of the primers for human and marmoset SLAMs to yield dog SLAM cDNA. The dog SLAM cDNA was then subcloned into the eukaryotic expression vector pCAGGS, and the resulting construct was named pCAGDogSLAM. Vero.DogSLAM cells were then generated by transfecting Vero

cells with the eukaryotic plasmid called pCXN2 and pCAGDogSLAM. The Vero.DogSLAM cells was then grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum with 0.5 % sodium carbonate, 0.5 mg/ml gentamycin, 100 unit/ml penicillin and 100 µg streptomycin.

### **3.2.3 Viral isolation**

Each specimen was individually inoculated at a volume of 0.5 ml into a 25 cm<sup>2</sup> plastic tissue culture flask containing a sub-confluent monolayer of Vero.DogSLAM cells. Each sample was inoculated in duplicate and uninoculated flasks used as negative controls were included in each run. The flasks were incubated in a 37 °C humidified incubator and examined daily for cytopathic effects (CPE). CPE was observed as giant multinucleated syncytium formation and detachment of cells. Blind passages were done for those not showing CPE after 4 days. The presence of CDV was confirmed with a direct fluorescent antibody test and electronmicroscopy (EM) as described below.

### **3.2.4 Direct fluorescent antibody test**

A direct fluorescent antibody test as described by (Maes *et al.*, 2003) was used for canine distemper virus antigen detection. Ten µl of scraped cells from inoculated flaks showing CPE was placed on a blank 12-well Teflon slide and allowed to air dry. It was fixed in an acetone-alcohol mixture (75:25) for 10 minutes at room temperature and 10 µl CDV-specific FITC antibody conjugate (VMRD, Inc) was added to each well and incubated in a humid chamber for 30 minutes at 37 °C. A 0.16 M sodium carbonate solution was used as wash buffer for 10 minutes in a

magnetic stirrer. Mounting fluid consisting of a buffer-glycerol mixture (50:50) was added to the slide and viewed with an epi-fluorescence microscope.

### **3.2.5 Electronmicroscopy**

Two ml of tissue culture fluid from each flask with CPE was centrifuged at 2000 *g* for 15 minutes and the supernatant further centrifuged at 14000 *g* for 45 minutes. The supernatant was discarded and the remaining pellet rinsed with deionised water. One drop of deionised water was added to the pellet and mixed vigorously with a pipette. A drop of 3 % phosphotungstic acid and pellet mixture was individually mixed in a tissue culture plate well and left to stain for 15 seconds. A formvar carbon coated grid (coated side under) was floated on top of the mixture and left for 15 seconds. The excess fluid on the grid was blotted onto a paper towel and allowed to dry. The grid was then examined using a Philips CM10 transmission electron microscope operated at 80 kilovolts.

### **3.3 Results**

The number of specimens processed, inoculated in tissue culture and the results are indicated in table 1. Thirty three out of 162 (20.4%) samples were positive for CDV. The 33 specimens were obtained from 26 dogs. Thus virus was isolated from 26 of 124 (21%) dogs studied. Cytopathic effects (CPE) seen as giant multinucleated syncytium formation (Fig. 1), was observed in positive specimens between 24 and 48 hours of inoculation. Uninoculated negative controls included in each run did not show any effects (Fig. 2). The characteristic apple green fluorescence seen with the aid of the direct fluorescent antibody test confirmed the presence of CDV antigen. The herring bone appearance of the viral nucleocapsid

observed with transmission electron microscopy (Fig. 3) provided further confirmation of the presence of CDV.

Of the 26 dogs that were positive in this study, 8 showed systemic signs only (diarrhoea, vomiting, anorexia, emaciation, ocular discharges, depression, dehydration, leukopaenia, fever), 3 showed nervous signs only (jaw clamping, clonic-tonic/ temporal muscle contraction, muscle fasciculations) and 2 showed respiratory signs only (cough, purulent nasal discharges, dyspnoea, increased lung sounds). A combination of systemic and nervous (4 dogs), systemic and respiratory (3 dogs), nervous and respiratory (2 dogs) signs were also noticed. One case of digital hyperkeratosis (so called 'hardpad' disease) was noted in a dog that also presented with bilateral uveitis, blepharospasm and ocular discharge. Three dogs displayed a combination of the catarrhal, systemic and nervous forms of the disease.

The 2 brain samples studied only yielded CPE after 48 hours while spleen samples from the same dogs yielded CPE within 24 hours post inoculation. However, the two CSF samples studied did not yield any CPE despite the detection of CDV IgG in the CSF by indirect fluorescent antibody test. Table 2 shows the vaccination history, clinical presentation, age, sex, and breed distribution of the 26 positive dogs. The spleen sample from dog 7 did not yield CPE but clear CPE was detected in the PBMCs from the same dog. Four (15.4%) of the 26 dogs had a history of CDV vaccination. Others had no known vaccination history, or were never vaccinated. Expectedly, 19 (73%) of the positive dogs in our study were aged 12 months and below. Two (7.7%) were 24 months old, two



were between 25 and 36 months old while 3 (11.5%) were between 60 and 72 months old. Twelve (46.2%) of the positive dogs in our study were males while 14 (53.8%) were females.

**TABLE 1:** Samples analysed by viral isolation in Vero.DogSLAM cells

<b>Specimen</b>	<b>Whole blood</b>	<b>Spleen</b>	<b>Lung</b>	<b>Brain</b>	<b>CSF</b>	<b>Total</b>
Total number tested	102	53	3	2	2	162
Number positive	21	10	0	2	0	33
Number negative	81	43	3	0	2	129

**TABLE 2:** Description of the animals and samples positive for CDV by viral isolation in Vero.DogSLAM cells

Dog No.	Sex	Age (months)	Breed	Sample type	Clinical signs	Vaccination history
1	M	29	Papillon	Spleen, Brain†	muscle fasciculation, jaw clamping	N
2	M	7	Afrkanis	Blood	muscle contraction with ocular discharges	N
3	M	3	Dachshund	Blood	Cough, diarrhoea, vomiting, anorexia	N
4*	F	2	Border Collie	Blood, Spleen	Ocular discharge, cough, twitching	N
5	F	12	Boerboel	Blood	Emaciated, cough, oculonasal discharge, jaw clamping	Y
6	M	5	Cocker spaniel	Blood, Spleen‡	Bilateral uveitis, blepharospasm, hyperkeratosis of right footpad, bilateral mucopurulent ocular discharge	N
7	F	4	Boerboel	Blood	Anorexia, fever, dehydration, vomiting	N
8	F	72	Dachshund	Blood	Nasal discharge, crusty eyes, lung sounds	N
9	M	3	Great Dane	Blood	Muscle contraction, crusty eyes and nose	N
10	F	36	Border Collie	Blood	Diarrhoea, leukopaenia, fever, temporal muscle contraction	N
11	M	6	Boerboel	Blood	Cough, dyspnoea, jaw clamping	Y
12	M	12	German Shepherd	Blood, Spleen	Muscular weakness, ocular discharge, depressed	N
13	F	4	Labrador	Blood, Spleen	Depressed, vomiting, bloody diarrhoea, ocular discharge, increased lung sounds	Y
14	M	7	Afrkanis	Blood, Spleen	Mucopurulent ocular discharge, dry crusty nose	N
15	F	12	Doberman	Blood, Spleen	Oculo-nasal discharge, vomiting, diarrhoea, increased lung sounds	N
16	M	12	Toypom	Spleen	Emaciated, oculo-nasal discharge, depressed	N
17	F	24	Toypom	Spleen	Emaciated, depressed, mucopurulent nasal discharge	N
18	F	24	Dachshund	Blood	Cough, vomiting, haemorrhagic diarrhoea	N
19	F	5	Labrador	Spleen	Emaciated, ocular discharge	N
20	M	60	Jack Russell Terrier cross	Blood	Purulent nasal discharge, cough, dyspnoea	N
21	M	9	Jack Russell	Blood	Dyspnoea, increased lung sounds, muscle contraction	N
22	F	3	Border Collie	Blood, Spleen	Crusty oculo-nasal discharge, depressed, dehydrated	N
23	M	12	Yorkie	Blood	Depressed, fever, dehydration, anorexia	Y
24	F	1.5	Dachshund	Blood, Spleen	Constantly crying, twitching of facial muscles, salivation	N
25	F	72	Dachshund	Spleen‡, Brain†	Vomiting, anorexia, muscle fasciculations, mucopurulent oculo-nasal discharge	N
26	F	12	Chihuahua	Blood	Emaciated, vomiting, anorexia, fever	N

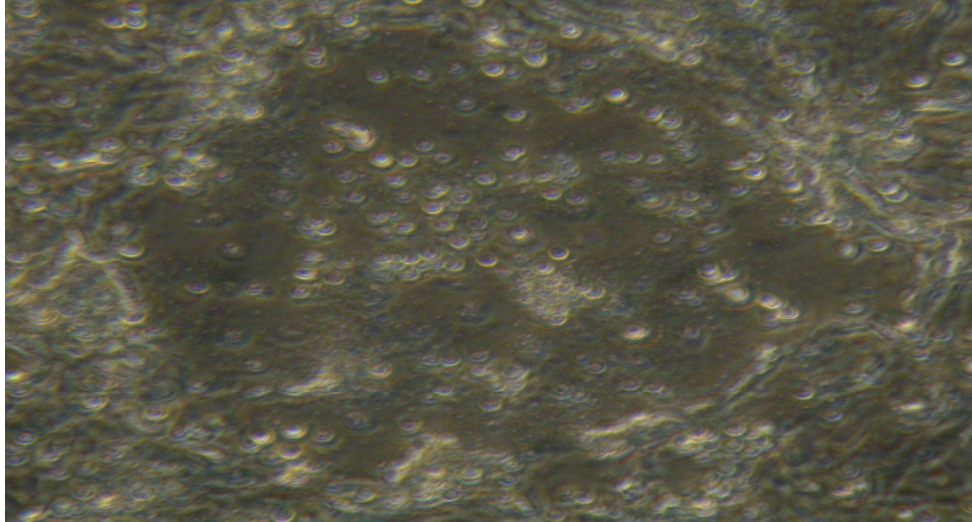
\* A litter of 10 puppies

† Showed CPE after 48 hours

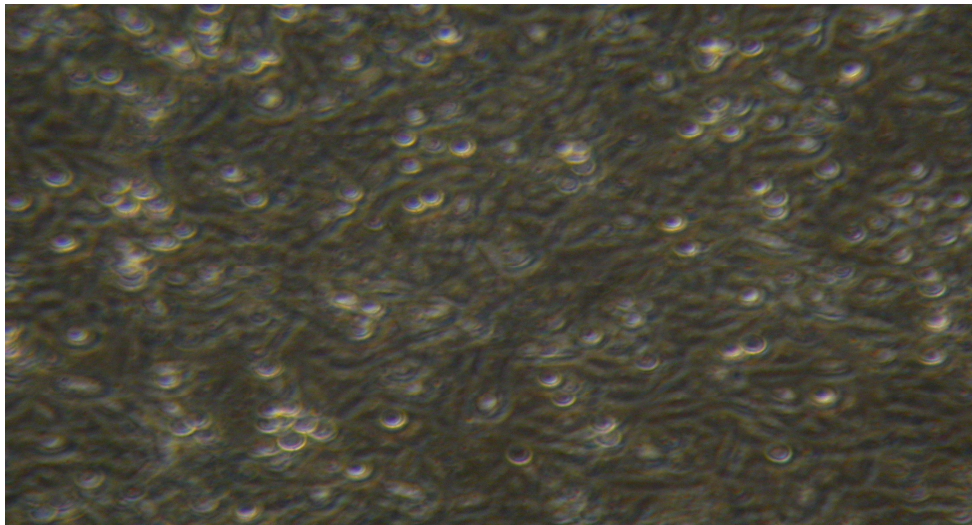
‡ Did not show CPE at all

Y Vaccinated against canine distemper

N Not vaccinated/unknown vaccination history



**Figure 1.** Vero.DogSLAM cells infected by CDV from clinical samples. There is formation of giant cells (syncytia).



**Figure 2.** Vero.DogSLAM cells uninoculated negative control.



**Figure 3.** Herring bone appearance of the CDV nucleocapsid as revealed by electronmicroscopy

### 3.4 DISCUSSION

Canine distemper is endemic in Africa as in other parts of the world. Underreporting is a characteristic of almost every infectious disease in most developing countries. The clinical signs of distemper in some dogs are not typical, emphasising the need for a definitive diagnostic test that is rapid and reliable. Diagnosis of canine distemper in the live animal, although difficult, is important because of the global distribution, broad host range, diverse signs and history of mass mortality events associated with the disease.

Several approaches have been used for the diagnosis of canine distemper. The use of haematology has been reported (Cornwell *et al.*, 1965), but changes in haematological parameters are non-specific and sometimes absent. Canine distemper virus-specific inclusion bodies may be present only during the viraemic stage and only in a small percentage of dogs (Shell, 1990). The use of serum chemistry has also been reported (Weisbrode & Krakowka, 1979), but biochemical

changes are usually non-specific and may reflect other pathological changes such as dehydration or azotaemia (Shell, 1990). Increased protein levels and lymphocytic pleocytosis in CSF may support the diagnosis of CDV infection of the nervous system (Gorham, 1966; Gossett *et al.*, 1982). However, not all dogs infected with CDV will show the latter changes.

The use of direct immunofluorescence for CDV diagnosis is not sufficiently sensitive and specific. It is prone to false negative results (Guy, 1986). Immunohistochemistry for CDV diagnosis may also produce false negative results especially in cases of subacute and chronic infection because the virus is often fleetingly present in certain tissues (Vandeveld *et al.*, 1985; Vandeveld & Zurbriggen, 1995).

The use of techniques such as the reverse transcription polymerase chain reaction (RT-PCR) represent a marked improvement on the classical CDV detection methods (Rima *et al.*, 1985; Rzezutka & Mizak, 2002) but direct RT-PCR of some tissues couldn't detect CDV despite virus isolation from the same tissues in Vero cell cultures (Lednicky *et al.*, 2004a; 2004b). Moreover, some laboratories in resource-poor parts of Africa lack the sophistication and technical expertise of present day molecular diagnostic facilities.

The gold standard for the diagnosis of virus infections has for a long time been virus isolation in cell cultures (Bussell & Karzon, 1965; Cosby *et al.*, 1981; Greene & Appel, 2006; Guy, 1986). The findings of this study confirmed that Vero.DogSLAM cells facilitate the isolation of virulent CDV from infected dogs as

early as 1 day post-inoculation with clinical material, and there is a significant reduction in the costs associated with the isolation of wild-type CDV. The efficiency, ease and cost effectiveness of using Vero.DogSLAM cells for confirmatory diagnosis (by viral isolation) of CDV should encourage wider use especially in third world countries where cost and technical expertise may limit the use of present day molecular diagnostic tools. The ability of laboratories to diagnose, isolate and store current field isolates of CDV has become more important since the host range of CDV appears to have broadened and interspecies transmission occurred, leading to epizootics with high mortality (Barrett *et al.*, 1992; Kennedy *et al.*, 2000; Van Moll *et al.*, 1995). Characterization of wild-type field strains from Africa will assist with studies on the epidemiology of the virus. Virus isolation is important not only to confirm a diagnosis and provide material for direct sequence analysis, but also for investigation of the pathogenesis in animal experiments and vaccine improvements (Lednicky *et al.*, 2004a).

Virus was not isolated from all submitted tissue specimens obtained from virus-positive dogs. The two brain samples studied (table 2) yielded CPE only after 48 hours while spleen samples from the same animals yielded CPE within 24 hours. The spleen sample from dog no. 6 (table 2) did not yield CPE even though CPE was detected from the PBMCs of the same animal. Similarly, the CSF samples studied did not yield CPE despite detection of CDV-specific IgG in the CSF by indirect fluorescent antibody testing. The inability of the CSF to cause CPE in cell cultures was probably due to the neutralizing effect of the IgG produced by the animal in response to the viral antigen present in the brain. Some specimens stored at 4°C for a period of more than 7 days were still positive when tested,

supporting the statement by Greene & Appel, (2006) that CDV survives in the environment for weeks at near-freezing (0°C to 4°C).

Improved vaccination has reduced the frequency and magnitude of canine distemper outbreaks (Chappuis, 1995). However, 4 of the 26 (15.4%) CDV positive dogs in our study had been vaccinated against the virus. There are several reports of distemper outbreaks in which affected dogs had received CDV vaccines (Blixenkron-Moller *et al.*, 1993; Ek-Kommonen *et al.*, 1997; Lan *et al.*, 2006). Speculations to explain these cases are varied but include immunosuppression, insufficient time after vaccination to develop immunity, improper refrigeration of vaccine, excessive exposure of vaccine to sunlight, maternally derived antibody, and overwhelming challenge dose of virus (Blixenkron-Moller, 1989; Blixenkron-Moller *et al.*, 1992b; Iwatsuki *et al.*, 2000). Most of the 124 dogs studied had unknown/unlikely vaccination status against distemper or other canine diseases. Thus the conditions for sporadic large-scale canine distemper outbreaks are evident; and additionally placing a large wildlife resource at risk.

The results of this research project support the contention that the prevalence of CDV in dogs does not follow breed or sex predisposition. (Headley & Graça, 2000) did not find any sex discrimination in an epidemiological study of 250 cases in Brazil. However, (Alex & Dhanapalan, 1994) found that more males than females (60:40%) were infected in Madras city, India, while the contrary (45:55%) was reported in Texas, USA by Gou *et al.*, (1986).

Gorham, (1966) did not find any breed predisposition to CDV infection. However, a Norwegian study found the relative distemper mortality rate low in the boxer, Pekingese, and Scottish terrier and high in the Samoyed, German shepherd, and cocker spaniel (Erno & Moller, 1961). Other workers (Alex & Dhanapalan, 1994; Gou *et al.*, 1986) have also indicated that a difference exists in breed susceptibility to distemper. Brachycephalic dogs have been reported to have a lower prevalence of disease, sequelae and mortality compared with dolichocephalic breeds (Gorham, 1966; Shell 1990).

Rockborn, (1958) reported increased numbers of clinical cases of canine distemper virus infections in the fall and winter months but other studies reported that the incidence of the disease is highest in the spring and early summer (Eckersley *et al.*, 1992; Erno & Moller, 1961; Van Moll *et al.*, 1995). Leisewitz *et al.*, (2001) did not observe any seasonal incidence of CDV infection in a two year retrospective study in South Africa. Thus seasonal variation may or may not affect the incidence of disease.

The severity of canine distemper depends on the virulence of the virus, immune competence and age of the affected dogs. In this study animals displayed a variety of clinical signs characteristic of canine distemper. This agrees with a two year retrospective study of 133 CSF samples in South Africa where 34 (25%) were positive, of which 23, 4, and 2 had only, nervous, systemic and respiratory signs respectively. Four had a combination of nervous and systemic signs, 1 had both respiratory and systemic signs and no dogs were observed to have a combination of nervous and respiratory signs (Leisewitz *et al.*, 2001). In Denmark, a distemper



outbreak was reported in which half of the cases presented with respiratory signs. However, nervous signs and hyperkeratosis were also observed (Blixenkron-Moller *et al.*, 1993). In an outbreak amongst sled dogs in northern Greenland, a 33% mortality was reported with classical respiratory and nervous signs, and there was no digital hyperkeratosis but blindness was observed in a few survivors (Bohm J *et al.*, 1989). In a Finnish study which reported 30% mortality, the majority showed classic respiratory signs and a few cases of digital hyperkeratosis (Ek-Kommonen *et al.*, 1997). Lymphadenopathy has been reported among Nigerian dogs with distemper (Ezeibe, 2005). Since CDV invades lymphoid tissues (Zurbriggen *et al.*, 1995), it was postulated that either a stimulation of the lymph nodes by the virus or attempts by host tissues to produce more lymphocytes to replace lost ones may have lead to the observation of lymphadenopathy in Nigerian dogs. There are many other reports of CDV outbreaks of varying severity among dog populations in several European countries. These reports demonstrate that despite the development of effective vaccines, CDV remains endemic in most parts of the world.

The CDV negative samples in this study were obtained mostly from animals with respiratory and systemic signs. In many cases, animals are incorrectly diagnosed with canine distemper when showing other related clinical signs. These signs are usually due to other viral and/or bacterial agents (Demeter *et al.*, 2007; Leisewitz *et al.*, 2001). The clinical signs of some fungal and parasitic diseases may also be confused with distemper (Merck, 1998). Most early clinical signs of CDV infection can be misleading, necessitating the use of specific diagnostic tests such as PCR

(where available) and isolation in Vero.DogSLAM cells for confirmatory diagnosis of CDV.

In conclusion, the results of this study demonstrated that the availability of Vero.DogSLAM cells makes virus isolation a useful adjunct for diagnosis of canine distemper, whereas previously virus isolation in cell cultures was time-consuming, difficult and expensive.

## CHAPTER 4

### GENETIC COMPARISON BETWEEN FIELD AND VACCINE STRAINS OF CDV AND DETERMINATION OF THE LINEAGE CIRCULATING IN SOUTH AFRICA

#### 4.1 Introduction

Canine distemper virus (CDV) is classified in the genus *Morbillivirus*, family *Paramyxoviridae* along with measles virus of humans, rinderpest virus of cattle, peste des petits ruminants virus of small ruminants and phocine distemper virus of marine animals (Francki *et al.*, 1991). Like other members of the *Morbillivirus* genus, CDV is enveloped and contains a single-stranded, negative-sense RNA genome. The CDV genome is 15,690 nucleotides long and consists of six genes encoding the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin/attachment (H), and large (L) proteins. The sequence organization of the genes coding for these proteins in the genome is 3'-N-P-M-F-H-L-5' (Barrett *et al.*, 1985; 1987; Bellini *et al.*, 1986; Curran *et al.*, 1991; Sidhu *et al.*, 1993). The H protein is important for both CDV itself and its animal hosts as CDV uses this protein for attachment to receptors on the host cell surface in the first step of infection, and an adequate host immune response against the H protein may prevent CDV infection (Martella *et al.*, 2006). The H protein is therefore the major determinant of tropism and cytopathogenicity (Von Messling *et al.*, 2001) and has the largest antigenic variation (Blixenkronne-Moller *et al.*, 1992b).

The disease caused by CDV is transmitted by aerosols or contact with oral, respiratory, and ocular fluids and exudates containing the virus. The clinical progress of CDV infection is characterized by a biphasic fever and gastrointestinal, respiratory or nervous signs. The infection and disease are not age-restricted and

may be prevented by passive or active immunization (Chalmers & Baxendale, 1994). Dogs and many other carnivores and marine mammals are amongst the host spectrum of CDV (Frisk *et al.*, 1999; Grachev *et al.*, 1989; Myers *et al.*, 1997; Van Moll *et al.*, 1995). Domestic dog populations seem to act as reservoirs for other susceptible carnivores (Carpenter *et al.*, 1998; Lednicky *et al.*, 2004b).

Canine distemper virus is highly contagious but the introduction of modified-live vaccines in the 1950s and their extensive application in most countries has greatly helped to keep the disease under control (Chappuis, 1995). Notwithstanding the extensive use of vaccination, CDV infection remains one of the most serious threats to susceptible carnivore populations worldwide and the disease has been reported in vaccinated animals (Calderon *et al.*, 2007; Decaro *et al.*, 2004; Scagliarini *et al.*, 2003).

Sequence analysis of CDV strains isolated in diverse geographic areas and from various animal species showed that the H gene undergoes a genetic drift related to the geographic location of the circulating strains (Martella *et al.*, 2007). Geographically distinct lineages have also been described for other morbilliviruses such as rinderpest (Chamberlain *et al.*, 1993; Wamwayi *et al.*, 1995) and measles viruses (Taylor *et al.*, 1991). The H gene of CDV has been shown to be a reliable target to investigate the genetic relationships among CDV strains (Martella *et al.*, 2006; 2007). Phylogenetic analysis of the H gene of CDV shows that the majority of CDV field strains cluster into seven major genetic lineages. These lineages (“genotypes”) are designated America-1 and 2, Asia-1 and 2, Europe, Europe wildlife and Arctic (Bolt *et al.*, 1997; Haas *et al.*, 1997; Hashimoto *et al.*, 2001;

Iwatsuki *et al.*, 1997; Lan *et al.*, 2006; Martella *et al.*, 2006; Mochizuki *et al.*, 1999; Pardo *et al.*, 2005). Current CDV modified-live vaccines have been produced using CDV isolates within the America-1 lineage. America-1 CDVs have not been detected during the last five decades and it is not known whether they are still circulating in the field. Field isolates from European countries, the United States, Japan and China had been reported to show pronounced genetic diversity in the haemagglutinin gene when compared to vaccine strains (Iwatsuki *et al.*, 2000; Mochizuki *et al.*, 1999; 2002). Phylogenetic analyses of CDV H gene nucleotide and amino acid sequences have been performed to study evolutionary relationships between CDV isolates and to find genetic variation among wild-type and CDV vaccine strains. However, molecular information on CDV strains circulating in South Africa is lacking.

The aim of this study was to phylogenetically characterize the H gene of current South African field strains and determine which lineage(s) of the seven established lineages they belong to, or if they form a distinct African lineage. Furthermore, sequence analysis of the H gene of current field viruses was done to determine whether contemporary strains had undergone significant genetic changes relative to currently used vaccine strains and to also determine if current South African field isolates cluster together with the Onderstepoort strain (used worldwide as an attenuated live vaccine) from lineage America-1.

## **4.2 Material and Methods**

### **4.2.1 Specimens**

Specimens ( $n = 32$ ) consisting of 26 viruses obtained by means of virus isolation (Chapter 3) and 5 commercial canine distemper vaccines (A, B, C, D and E) used in South Africa were subjected to reverse transcription–polymerase chain reaction (RT-PCR). Blood specimens in EDTA tubes ( $n = 13$ ) from dogs with clinical signs compatible with canine distemper were obtained from thirteen Nigerian dogs for the purpose of comparison. The Nigerian specimens were also subjected to RT-PCR.

### **4.2.2 RNA extraction**

Total RNA was extracted from 200  $\mu$ l of supernatants of infected cells and 5 commercial CDV vaccines used in South Africa (200  $\mu$ l) using the RNeasy mini kit spin column protocol (QIAGEN®, Southern Cross Biotechnology, South Africa) according to the manufacturer's instructions. The vaccines were initially diluted as prescribed by the manufacturers for injection in dogs. The QIAamp® RNA blood mini kit (QIAGEN®, Southern Cross Biotechnology, South Africa) was used for the extraction of RNA from the blood samples according to the manufacturer's instructions. The extracted RNA was spectrophotometrically quantified (DU® 530 life science UV/Vis spectrophotometer, Beckman Coulter™) and stored at  $-80^{\circ}\text{C}$  until used. Aliquots of ultrapure sterile water were included as negative controls in all of the RNA extraction procedures.

#### **4.2.3 Complementary DNA (cDNA) synthesis**

Reverse transcription (cDNA synthesis) was performed using the GeneAmp® Gold RNA PCR reagent kit (AppliedBiosystems®) according to the manufacturer's instructions. A denaturation mix consisting of 1 µl of ultrapure sterile water, 0.5 µM of random hexamers with 5 – 7 ng of template RNA was initially denatured at 70 °C for 10 minutes and immediately incubated on ice. The RT-MIX solution consisted of 4 µl 5 X RT-PCR buffer, 0.033 mM of magnesium chloride, 0.02 mM of dNTP, 10 units of RNase inhibitor, 0.4 mM of DTT, 16.7 units of Multiscribe reverse transcriptase and 2.2 µl of ultrapure sterile water. The RT-MIX was added to the denaturation mix and reverse transcription was performed in this 20 µl reaction tube at 25 °C for 10 minutes and 42 °C for 60 minutes in an AppliedBiosystems® 2720 thermal cycler. The template cDNA amplified was immediately stored at -20 °C until used for PCR.

#### **4.2.4 Polymerase chain reaction (PCR)**

PCR was performed using a GeneAmp® Gold RNA PCR reagent kit (AppliedBiosystems) according to the manufacturer's instructions. Briefly, a 2100 bp fragment of the H gene was amplified with forward primer CDV-ff1 and reverse primer CDV-HS2 (Lan *et al.*, 2006) with minor modifications. Ten µl of the template cDNA was added to a PCR reaction mix consisting of 9.5 µl ultrapure sterile water, 3 µl of 5 X RT-PCR buffer, 0.017 mM of magnesium chloride, 0.01 mM of dNTP, 12.5 units of AmplitaqGold® enzyme, and 5 µM of each of the forward and reverse primers. The PCR cycling conditions consisted of 40 cycles of denaturation (95 °C x 1 minute), annealing (48 °C x 1 minute), extension (72 °C x 2 minutes) and final extension (72 °C x 7 minutes) in an AppliedBiosystems® 2720 thermal cycler.

Following PCR, 5 µl of the amplicons were electrophoresed in a 1.5% Tris acetate-EDTA-agarose gel (EM Science, Merck KGaA, Germany) at 120 V for 55 minutes. The gel was stained with ethidium bromide and the bands were visualised and photographed with a Kodak DS electrophoresis documentation and analysis system using the Kodak digital science ID software programme. Product sizes were determined with reference to a 100 bp and a 1 kb molecular weight ladder (O'Gene Ruler™ 100 bp DNA ladder Plus, Fermentas Life Sciences).

#### **4.2.5 Sequencing**

The amplified PCR products were purified using a QIA quick® PCR purification kit (Qiagen®, Southern Cross Biotechnology) according to the manufacturer's instructions. Purified products were directly sequenced with a Big Dye® Terminator v.3.1 cycle sequencing kit (AppliedBiosystems) according to the manufacturer's instructions. The internal H gene sequence primers were P2F, P3R, P5R, CDV-HS1, CDV-HforD and CDV-Hr2 (Lan *et al.*, 2006; Pardo *et al.*, 2005)(Table 3). Precipitation of extension products was by the ethanol precipitation protocol of Applied Biosystems ®. Sequence trace files were electrophoresed using the SpectruMedix Genetics analysis system SCE 2410 of Inqaba Biotech (South Africa) and the ABI 3130XL Genetic analyser of the Genetics Laboratory, Equine Research Centre, Faculty of Veterinary Science, University of Pretoria.



#### 4.2.6 Phylogenetic analyses

Sequence data of the full length H gene were assembled and edited to a total length of 1824 bp using GAP 4 of the Staden package (Version 1.6.0 for Windows). The open reading frames of the assembled sequences were aligned with sequences of other H genes collected from different parts of the world and available in GenBank using MAFFT (Kato *et al.*, 2002; 2005). The nucleotide sequences were translated into amino acids using the tranalign programme in EMBOSS (Rice *et al.*, 2000).

A transversional model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (TVM+I+ $\Gamma$ ) substitution model determined by Modeltest v3.7 (Posada & Crandall, 1998), PAUP\* v4b10 (Swofford, 2003) and MrBayes v3.1.1 (Ronquist & Huelsenbeck, 2003; Huelsenbeck & Ronquist, 2001) were used to explore distance (Neighbour-joining) and character (Bayesian, Maximum likelihood) based phylogenetic methods of the nucleotide sequences. The consensus trees were edited in MEGA v4 (Kumar *et al.*, 1994; Tamura *et al.*, 2007). The phocine distemper virus (PDV) with GenBank accession number AF479277 was indicated as outgroup in all 3 of the analysis types.

#### 4.2.7 Nucleotide sequence accession numbers

The nucleotide sequence accession numbers in the GenBank database of H gene sequences of field isolates from dogs in South Africa are listed in Table 4. The nucleotide sequence accession numbers in the GenBank database of H gene sequences of vaccine strains available in South Africa are also listed in Table 4.

The nucleotide sequence accession numbers in the GenBank database of H gene sequences of the reference strains are: Onderstepoort (AF378705), Convac (Z35493), Snyder Hill (AF259552) and A75/17 (AF164967).

### 4.3 Results

The RT-PCRs performed according to conditions described above resulted in amplicons of the expected size of 2100 bp (Figures 5 and 6) using published primer pairs (Table 3) (Lan *et al.*, 2006) with minor modifications. The thirteen blood samples from Nigerian dogs with clinical signs compatible with CDV however, did not yield any amplicons on RT-PCR. The determined nucleotide sequences of the amplicons were deposited into GenBank under the accession numbers shown in Table 4.

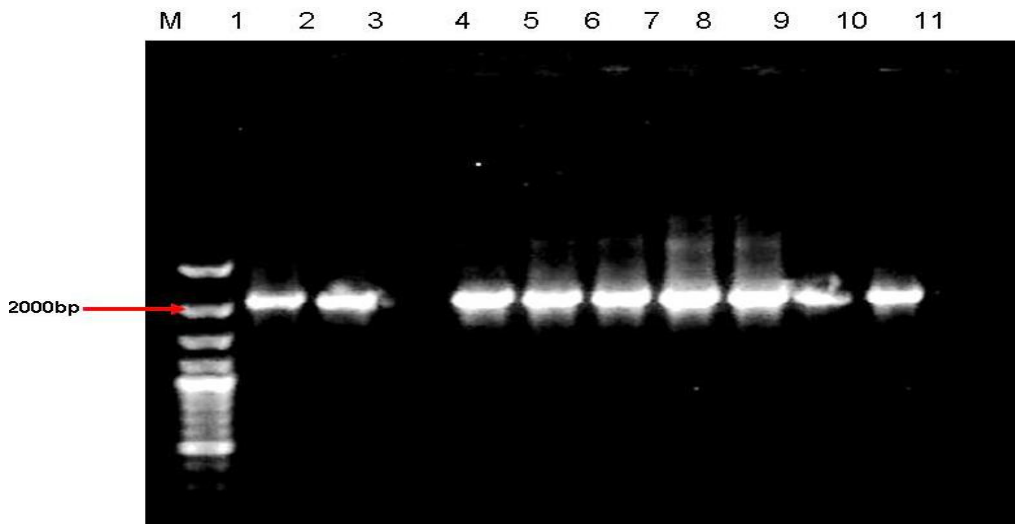
The national center for biotechnology information basic alignment search tool (NCBI BLAST) was optimized for highly similar sequences (megablast) and the South African isolates showed 95% maximum identity to a Hungarian strain (GenBank accession no EF095750), an isolate from a Greenlandic dog (Z47760), an isolate from a Chinese lesser panda (AF178039), a German dog isolate (X85000), the wild-type strain A75/17 (AF164967) and a United States ferret strain 5804 (AY386315). However, sequences from 4 of the commercial vaccines (A, B, C, and E) used in South Africa showed 98 – 99% maximum identity to vaccine (AB212966), the Onderstepoort vaccine (AF378705), the Lederle vaccine (DQ903854) and the Convac vaccine (Z35493) strains. Unexpectedly, one of the commercial vaccines (D) showed 99% maximum identity to the Hungarian strain (EF095750) and the Chinese lesser panda isolate (AF178039).

The multiple sequence alignments of the nucleotides and protein of these wild type isolates and the reference isolates used are presented in appendix 1 and 2 respectively. The sequenced genome fragments from each of the field isolates were translated using the tranalign programme in EMBOSS (Rice *et al.*, 2000), resulting in 607 amino acid (aa) long polypeptides, representing the complete sequence of the H protein. However, only sequences of commercial vaccines D and C yielded 607 amino acids. Vaccines A, B and E had only 604 amino acids similar to the Onderstepoort and some other earlier vaccine strains available in GenBank.

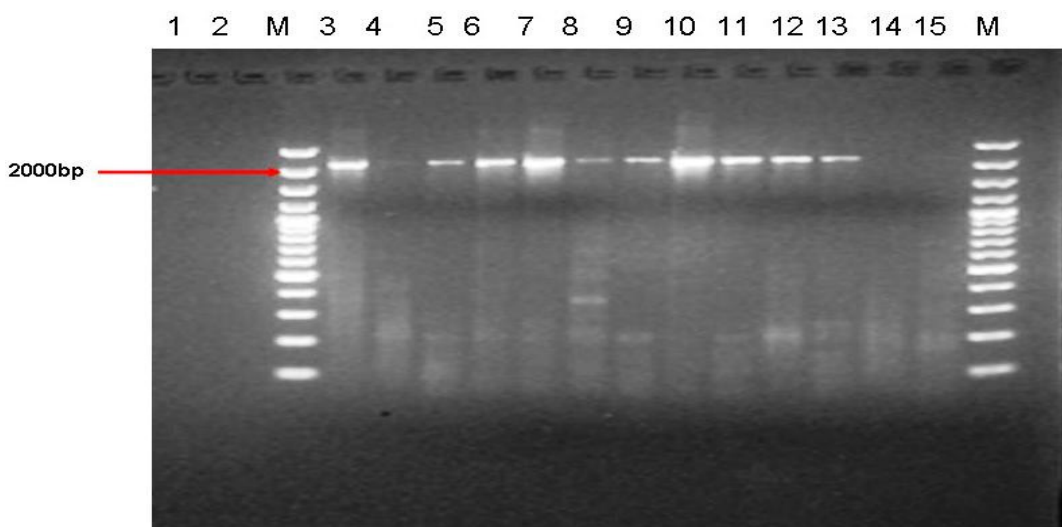
All 3 analysis types used showed trees with similar topology. The phylogeny inferred with the distance methods was consistent with those of the character based analysis (Figures 7 – 9). In the Neighbour joining tree (Fig. 7), the outgroup (PDV) splits first into the lineage America 1 (bootstrap value 100%), it then splits into the lineage Asia 2 (bootstrap value 67%) and a group consisting of the America 2 (bootstrap value 94%), Europe and Europe wildlife (bootstrap value 76%), and Asia 1 (95%). The tree then finally splits into the Arctic (97%) and the South African field isolates group (100%). The Bayesian tree (Fig. 8) gave the best bootstrap replicates of all. The outgroup splits first into the America 1, then a further split into a group consisting of the Arctic, Asia 2 and South African field isolates (Africa 1). The tree finally splits into a third group consisting of the European isolates, Asia 1 and America 2. The maximum likelihood tree (Fig. 9) first split from the outgroup and gave rise to the America 1. The Arctic group arose from a second split, a further split leads to the Asia 2 and South African field

isolates group and then a final split into the Europe, Europe wildlife, Asia 1 and America 2 groups.

The calculated genetic distance between unique CDV isolates are summarized in table 5. The mean CDV H gene distance within a clade (lineage) ( $\bar{x}=0.018$ ) was significantly less than between heterologous clades ( $\bar{x}=0.074$ ) ( $P < 0.001$ ). The CDV H genes of lineage Europe wildlife, America 2 and America 1 showed the greatest distance within each clade ( $\bar{x}= 0.040, 0.034$  and  $0.023$  respectively), while the least distance was within the lineage containing vaccine D ( $\bar{x}=0.007$ ). The European, African and Asia 2 lineages showed the same distance within each clade ( $\bar{x}=0.013$ ). Between lineages, the greatest distance was between lineage America 1 and Asia 2 ( $\bar{x}=0.107$ ), America 1 and Asia 1 ( $\bar{x}=0.105$ ), America 1 and Africa ( $\bar{x}=0.095$ ) and between America 1 and Europe ( $\bar{x}=0.091$ ). The least distance was between the lineage containing vaccine D and Europe ( $\bar{x}=0.030$ ), Europe wildlife ( $\bar{x}=0.032$ ), America 2 ( $\bar{x}=0.033$ ) and Arctic ( $\bar{x}=0.045$ ).



**Figure 4.** RT-PCR amplification of the 2100 bp complete H gene of CDV. M, marker (O'Gene Ruler™ 100 bp DNA ladder Plus, Fermentas Life Sciences); lane 1, 2, 9 & 10, positive clinical specimens used as positive controls for vaccines RT-PCR; lane 3, ultra sterile water used as template RNA; lane 4 – 8, vaccine A, B, C, D, and E respectively; lane 11, DNA extracted from Vero.DogSLAM cells also used as template RNA (both water and DNA extracted from Vero.DogSLAM cells were used as negative controls).



**Figure 5.** RT-PCR amplification of the 2100 bp complete H gene of CDV. M, marker (O'Gene Ruler™ 100 bp DNA ladder Plus, Fermentas Life Sciences); lane 1 and 2, ultra sterile water and DNA extracted from Vero.DogSLAM cells used as negative controls respectively. Lane 3, vaccine used as positive control for specimens RT-PCR. Lane 4 – 13, positive clinical specimens. Lane 14-15, negative (Nigerian) clinical specimens.

**Table 3:** Oligonucleotide primers used for RT-PCR amplification and subsequent nucleotide sequencing of the H gene\*

S/No	Primer	Orientation	Sequence (5' – 3')	Nucleotide Position
1	CDVff1	Sense	TCGAAATCCTATGTGAGATCACT	6897–6919
2	CDVHS2	Antisense	ATGCTGGAGATGGTTTAATTCAATCG	8994–8969
3	CDVHS1	Sense	AACTTAGGGCTCAGGTAGTCC	7054–7074
4	CDVHforD	Sense	GACACTGGCTTCCTTGTGTGTAG	7948–7970
5	CDVHr2	Antisense	GTTCTTCTTGTTTCTCAGAGG	8198–8178
6	CDVP2F	Sense	ACTTCCGCGATCTCCACT	7372–7389
7	CDVP3R	Antisense	ACACTCCGTCTGAGATAGC	7760–7742
8	CDVP5R	Antisense	GTGAACTGGTCTCCTCTA	8395–8378

\*Primers were used as described by Lan *et al.*, 2006 and Pardo *et al.*, 2005 with minor modifications.

**Table 4:** CDV isolates from South Africa, organs from which they were isolated and their GenBank accession numbers\*

Serial No	Isolate	Organ source	GenBank accession No
1	1sp	Spleen	FJ461696
2	1bn	Brain	FJ461713
3	2L	Blood	FJ461720
4	4L70214	Blood	FJ461693
5	4L70247	Blood	FJ461697
6	4L7039	Blood	FJ461714
7	4sp	Spleen	FJ461715
8	5L	Blood	FJ461698
9	5sp	Spleen	FJ461718
10	6L	Blood	FJ461703
11	7L	Blood	FJ461711
12	8L	Blood	FJ461699
13	9L	Blood	FJ461704
14	10L	Blood	FJ461705
15	11L	Blood	FJ461719
16	12sp	Spleen	FJ461721
17	13sp	Spleen	FJ461695
18	14L	Blood	FJ461724
19	15sp	Spleen	FJ461706
20	16L	Blood	FJ461716
21	17L	Blood	FJ461717
22	20L	Blood	FJ461707
23	21L	Blood	FJ461694
24	23L	Blood	FJ461722
25	23sp	Spleen	FJ461723
26	25L	Blood	FJ461712
27	26L	Blood	FJ461700
28	Vaccine A	Nobivac® DHPPI (Intervet)	FJ461701
29	Vaccine B	Nobivac® PuppyDP (Intervet)	FJ461709
30	Vaccine C	Galaxy DA <sub>2</sub> PPV (Schering-Plough/Fort Dodge®)	FJ461708
31	Vaccine D	Vanguard®Plus (Pfizer)	FJ461702
32	Vaccine E	Canigen DHPPI (Virbac Animal Health®)	FJ461710

\*The CDV sequences that were obtained in this study have been deposited in the National Center for Biotechnology Information database and assigned the above GenBank accession numbers.

**Table 5:** Mean, standard deviation and number of pairwise measurements of the genetic distances between CDV H gene sequences.

America 1	Arctic	Vaccine D	America 2	Asia 1	EUWD	Europe	Africa 1	Asia 2	Out-group	
<b>0.023</b>	<b>0.091</b>	<b>0.081</b>	<b>0.091</b>	<b>0.105</b>	<b>0.088</b>	<b>0.091</b>	<b>0.095</b>	<b>0.107</b>	<b>0.584</b>	America1
0.012	0.004	0.004	0.009	0.005	0.006	0.004	0.004	0.006	0.008	
91	56	28	168	364	42	140	378	126	14	Arctic
	<b>0.020</b>	<b>0.045</b>	<b>0.059</b>	<b>0.070</b>	<b>0.059</b>	<b>0.058</b>	<b>0.059</b>	<b>0.074</b>	<b>0.583</b>	
	0.009	0.002	0.007	0.005	0.006	0.004	0.004	0.005	0.006	
	6	8	48	104	12	40	108	36	4	VaccineD
		<b>0.007</b>	<b>0.033</b>	<b>0.046</b>	<b>0.032</b>	<b>0.030</b>	<b>0.048</b>	<b>0.061</b>	<b>0.588</b>	
		N/A	0.008	0.006	0.006	0.003	0.003	0.005	0.002	
		1	24	52	6	20	54	18	2	America2
			<b>0.034</b>	<b>0.067</b>	<b>0.050</b>	<b>0.053</b>	<b>0.063</b>	<b>0.076</b>	<b>0.598</b>	
			0.010	0.014	0.010	0.003	0.007	0.009	0.015	
			63	63	6	20	324	108	12	Asia1
				<b>0.019</b>	<b>0.059</b>	<b>0.057</b>	<b>0.071</b>	<b>0.086</b>	<b>0.596</b>	
				0.008	0.006	0.004	0.005	0.007	0.010	
				325	50	250	702	234	26	EUWD
					<b>0.040</b>	<b>0.049</b>	<b>0.062</b>	<b>0.073</b>	<b>0.586</b>	
					0.007	0.003	0.005	0.007	0.003	
					3	20	81	27	3	Europe
						<b>0.013</b>	<b>0.060</b>	<b>0.073</b>	<b>0.575</b>	
						0.006	0.003	0.006	0.008	
						45	270	90	10	Africa1
							<b>0.013</b>	<b>0.076</b>	<b>0.593</b>	
							0.010	0.005	0.005	
							351	243	27	Asia2
								<b>0.013</b>	<b>0.587</b>	
								0.006	0.015	
								36	9	Outgroup

The overall mean distance was 0.065 (standard deviation=0.027, n=5671), mean intralineaage distance 0.018 (standard deviation=0.011, n=921) and mean interlineage distance 0.074 (standard deviation=0.019, n=4750). EUWD = European wildlife lineage. N/A = not applicable.



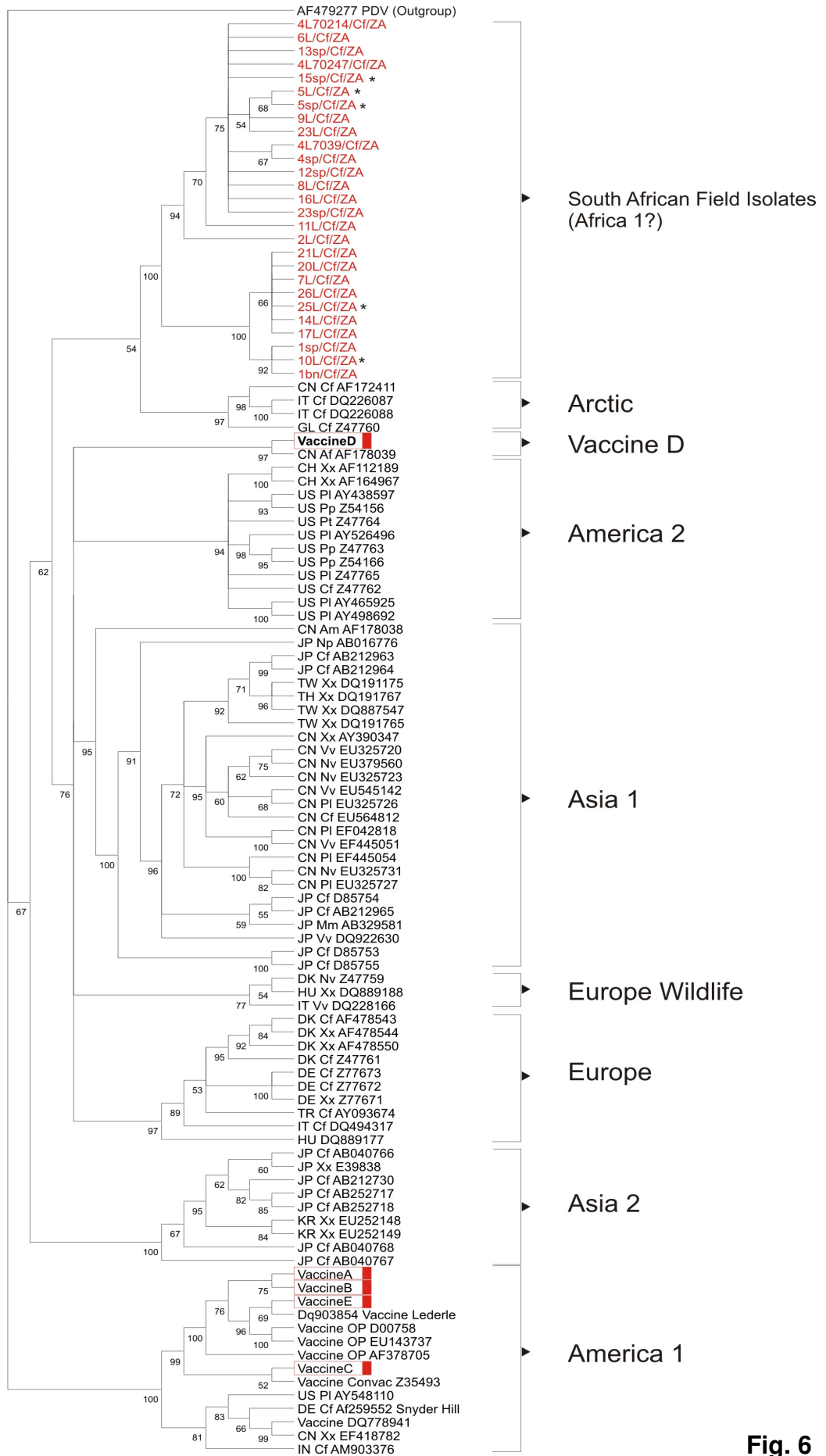


Fig. 6

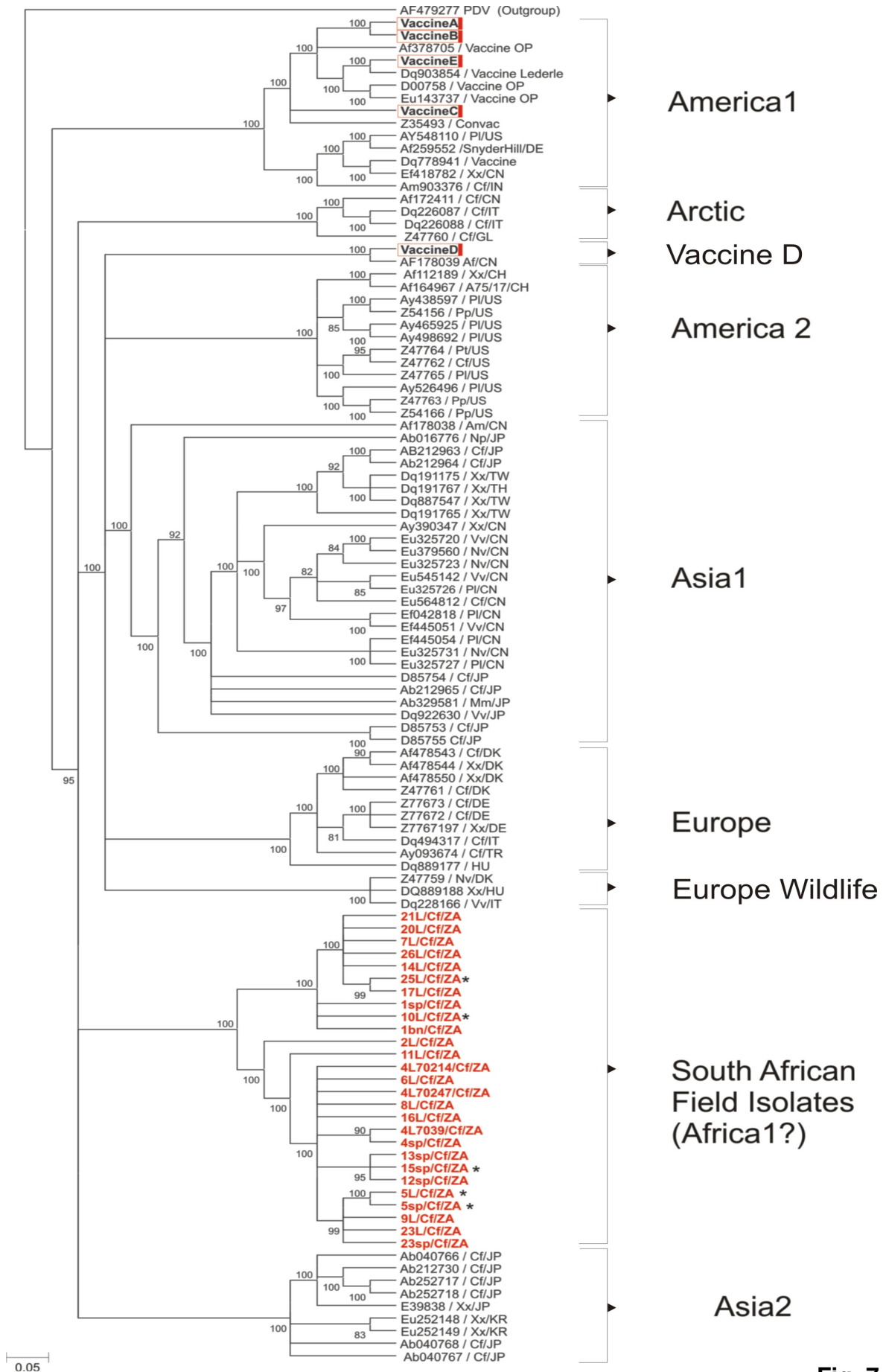


Fig. 7

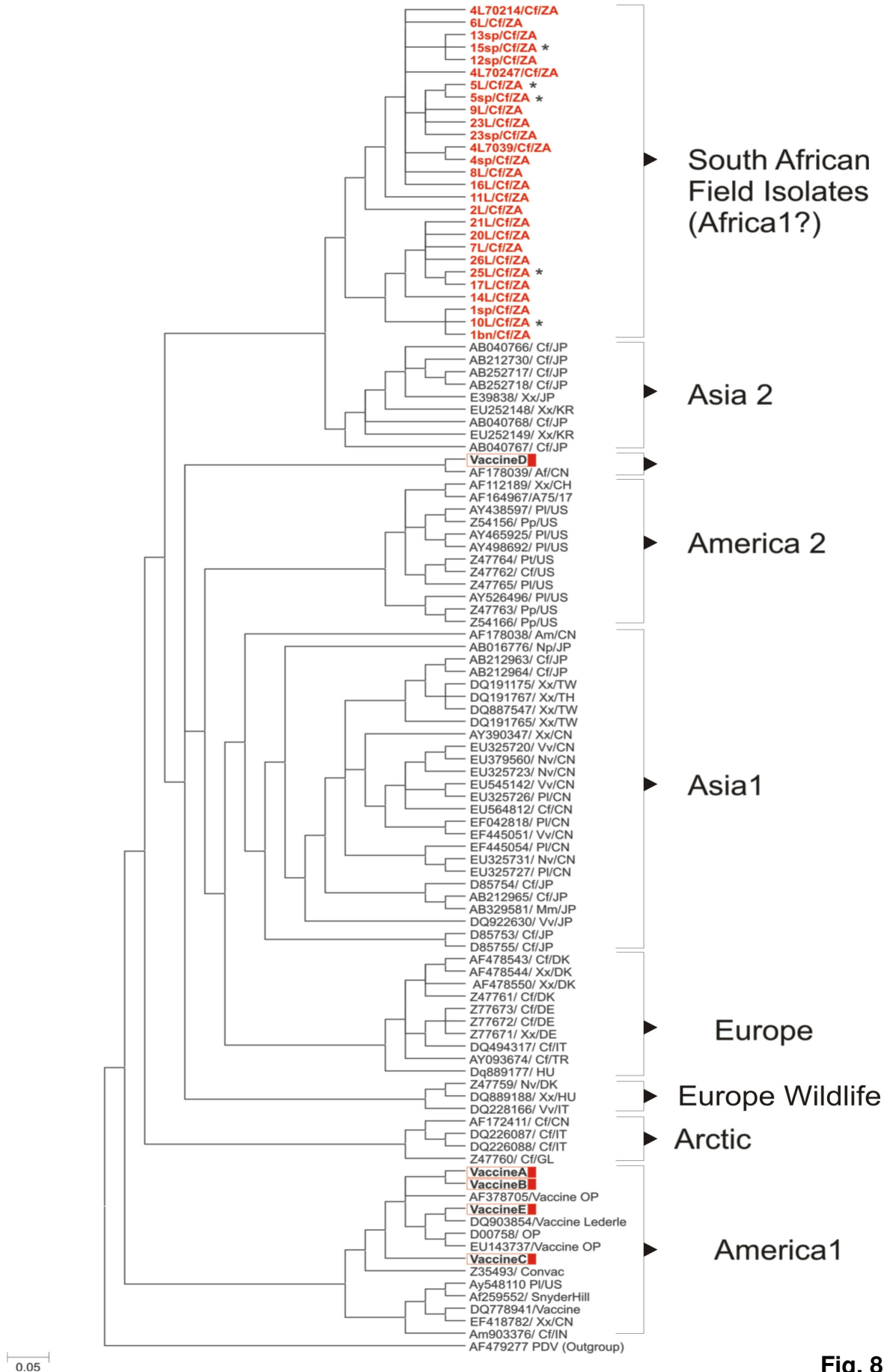


Fig. 8

**Figure 6.** Neighbour-joining rooted tree of the complete H gene sequences of CDV with the closely related PDV. The numbers at the nodes are bootstrap values obtained from 1000 re-samplings. The GenBank accession numbers/specie from which isolate was obtained/countries of origin are indicated. The isolates from this study are indicated by the lab number/specie/country. The sequences from vaccines used in South Africa are boxed. South African isolates from dogs with a history of vaccination are indicated with asterisks. *Abbreviations* Cf, *Canis familiaris* (dog); Np, *Nyctereutes procyonoides* (raccoon dog); Am, *Ailuropoda melanoleuca* (giant panda); Af, *Ailurus fulgens* (lesser panda); Xx, unspecified species; Pl, *Procyon lotor* (raccoon); Vv, *Vulpes vulpes* (fox); Nv, *Neovison vison* (mink); Mm, *Meles meles* (badger); Pt, *Pecari tajacu* (javelina); Pp, *Panthera pardus* (leopard). JP, Japan; CN, China; DK, Denmark; US, United states; TW, Taiwan; TH, Thailand; IT, Italy; HU, Hungary; KR, South Korea; IN, India; TR, Turkey; DE, Germany; GL, Greenland; CH, Switzerland; OP, Onderstepoort; ZA, South Africa.

**Figure 7.** A rooted cladogram of the complete H gene sequences of CDV and PDV (outgroup), as determined by Bayesian inference, using 3 heated chains and performing  $1 \times 10^6$  iterations. The GenBank accession numbers/specie from which isolate was obtained/countries of origin are indicated. The isolates from this study are indicated by the lab number/specie/country. The sequences from vaccines used in South Africa are boxed. South African isolates from dogs with a history of vaccination are indicated with asterisks. *Abbreviations* Cf, *Canis familiaris* (dog); Np, *Nyctereutes procyonoides* (raccoon dog); Am, *Ailuropoda melanoleuca* (giant panda); Af, *Ailurus fulgens* (lesser panda); Xx, unspecified species; Pl, *Procyon lotor* (raccoon); Vv, *Vulpes vulpes* (fox); Nv, *Neovison vison* (mink); Mm, *Meles meles* (badger); Pt, *Pecari tajacu* (javelina); Pp, *Panthera pardus* (leopard). JP, Japan; CN, China; DK, Denmark; US, United states; TW, Taiwan; TH, Thailand; IT, Italy; HU, Hungary; KR, South Korea; IN, India; TR, Turkey; DE, Germany; GL, Greenland; CH, Switzerland; OP, Onderstepoort; ZA, South Africa.

**Figure 8.** Maximum likelihood rooted tree of the complete H gene sequences of CDV with the closely related PDV. The GenBank accession numbers/specie from which isolate was obtained/countries of origin are indicated. The isolates from this study are indicated by the lab number/specie/country. The sequences from vaccines used in South Africa are boxed. South African isolates from dogs with a history of vaccination are indicated with asterisks. *Abbreviations* Cf, *Canis familiaris* (dog); Np, *Nyctereutes procyonoides* (raccoon dog); Am, *Ailuropoda melanoleuca* (giant panda); Af, *Ailurus fulgens* (lesser panda); Xx, unspecified species; Pl, *Procyon lotor* (raccoon); Vv, *Vulpes vulpes* (fox); Nv, *Neovison vison* (mink); Mm, *Meles meles* (badger); Pt, *Pecari tajacu* (javelina); Pp, *Panthera pardus* (leopard). JP, Japan; CN, China; DK, Denmark; US, United states; TW, Taiwan; TH, Thailand; IT, Italy; HU, Hungary; KR, South Korea; IN, India; TR, Turkey; DE, Germany; GL, Greenland; CH, Switzerland; OP, Onderstepoort; ZA, South Africa.

#### 4.4 Discussion

The primary objective of this work was to genetically characterize South African field CDV strains obtained from infected and clinically sick dogs and to study the genetic variability between these field strains with commercial distemper vaccines used in South Africa, as well as to determine the geographical lineage(s) to which the current South African field strains belong. This represents the first study on the genetic characterization of CDV in South Africa.

The field isolates from this study clustered within a well-defined clade and form a distinct “lineage Africa 1” separate from other established lineages and vaccine viruses (Figures 6 - 8). Many other studies have demonstrated that phylogenetic analysis of the H gene of CDV shows geographically distinct lineages (Bolt *et al.*, 1997; Haas *et al.*, 1997; Hashimoto *et al.*, 2001; Martella *et al.*, 2006; 2007; Ozkul *et al.*, 2004; Pardo *et al.*, 2005). Phylogenetic analysis of the H gene nucleotide and predicted amino acid sequences by both distance and parsimony methods demonstrated that the field strains examined in this study are genetically distinct. A high degree of identity was detected among the South African wild-type strains. All of them grouped in a phylogenetic cluster clearly separate from the vaccine strains and from other wild-type CDV strains from GenBank. Furthermore, phylogenetic analysis of the sequences suggested that none of the strains detected were closely related to lineages previously detected either in the United States, Europe, Asia or those of the Arctic ecosystem. The field isolates examined in this study form a distinct clade of their own but sub-dividing into two clusters of approximately 100% identity. However, all the isolates were collected from Gauteng province and within the same time frame. These limitations mean that we

have to get isolates from other parts of the country and indeed the continent to determine if other lineages also exist in Africa. The present classification of CDV H gene into America 1 and 2, Asia 1 and 2, Europe, Europe wildlife and Arctic should include the present lineage Africa 1.

The thirteen blood samples from Nigerian dogs with clinical signs compatible with CDV did not yield any amplicons on RT-PCR. Lack of amplification of CDV in these blood samples does not exclude distemper. The lack of nucleic acid amplification from the Nigerian samples may have been due to a 6-week period between collection and transport to South Africa, sample source (blood and not virus isolated in cell cultures), and the viral nucleic acid extraction method. Other reasons for the failure to detect viruses in blood samples from Nigerian dogs could be due to the enzyme used in the RT step and the type of DNA polymerase used for PCR (Hoyland *et al.*, 2003).

Sequence comparisons, which result from the direct sequencing of PCR products, are an important tool for further understanding of the molecular epidemiology of CDV infections (Maes *et al.*, 2003). The results of this study showed that despite the sub-clustering observed within the field strains, the percent identity is high enough for us to conclude that they belong to one lineage. Further studies taking into consideration the limitations stated above will clarify whether more than one lineage is circulating in South Africa. Some non-specific bands were observed sporadically in some isolates during amplification. The nature of the non-specific but discrete products was not characterized but it probably resulted from mispriming events during amplification of the nucleic acids (Martella *et al.*, 2007).

For the purpose of this phylogenetic analysis, we used the Onderstepoort (GenBank accession no AF378705), the Convac (Z35493), the Snyder Hill (AF259552) and the A75/17 (AF164967) strains as references. The Onderstepoort CDV was derived from the so called Green's distemperoid virus (Green & Carlson, 1945) which had been isolated from a natural distemper case and serially passaged in ferrets. The ferret-passaged virus was then adapted to chicken eggs and passaged in this system numerous times, after which it was called Onderstepoort-CDV (Haig, 1956). Onderstepoort-CDV is considered to be completely apathogenic and is used in vaccines (Stettler *et al.*, 1997). The Convac strain is also used as a vaccine strain but details on its origin are scanty. The Snyder Hill strain is also used for vaccine production. Snyder Hill CDV was isolated from a natural case of distemper and has been serially passaged through direct intracerebral inoculation in dogs (Stettler *et al.*, 1997). Several features of experimental Snyder Hill-CDV infection are similar to the wild type CDV infection (Stettler *et al.*, 1997). The A75/17 CDV is a virulent wild strain isolated from a dog with spontaneous distemper. It is regarded as the virulent prototype of field CDV isolates. Under experimental conditions, this virus causes a demyelinating disease and persistent infection of the CNS as seen in natural distemper (Appel, 1969). Current South African field isolates examined in this study did not cluster with the Onderstepoort strain in lineage America 1. However, it did show about 95% nucleotide identity to the A75/17 strain on NCBI BLAST. Considering the more than 60-year interval between the isolation of the Onderstepoort strain and our present field isolates, such distant genetic relationship is not unexpected.

There are other distemper vaccines in the global market but the most frequently used products in South Africa are manufactured by Fort Dodge, Pfizer, Intervet, Merial and Virbac. This study sequenced some of these commercial vaccines used in South Africa to see if there are major genetic differences compared to current field isolates. Four (vaccine A, B, C, and E) out of the 5 vaccine viruses sequenced showed 99% nucleotide identity to each other and to the Onderstepoort and Lederle CDV and group together with the Onderstepoort strain in lineage America 1. The other one (vaccine D) showed 99% nucleotide identity to a Hungarian vaccine strain (EF095750) and to a Chinese CDV isolate from a lesser panda (AF178039). Vaccine D grouped with the lesser panda isolate in a distinct clade of its own but closely related with CDV strains in lineage America 2 which has many CDVs of non-canine species. These vaccines used in South Africa are genetically distinct from the circulating wild-type strains reported in other parts of the world (Calderon *et al.*, 2007; Lan *et al.*, 2006). However, it is reasonable to assume that the efficacy of the currently used vaccines is not partially compromised by the extent of genetic variation observed since only one serotype of the virus is currently recognized.

According to the vaccine package insert, Intervet and Fort Dodge (vaccines A, B and C) use the Onderstepoort strain for CDV vaccine production, while Pfizer (vaccine D) use the Snyder Hill strain and Virbac (vaccine E) uses the Lederle strain. Expectedly, the phylogenetic trees (Fig 6– 8) showed that vaccines A, B and C originated from the Onderstepoort strain. The phylogenetic analysis also confirmed that vaccine E originated from the Lederle strain. However, though the product package insert stated that vaccine D was produced from the Snyder Hill



strain, the data from this study showed that they are genetically not similar. It is not known if repeated attenuation or the presence of other agents (adenovirus type 2, parainfluenza, parvovirus) influenced the genetic relationship between vaccine D and the original Snyder Hill strain.

Vaccination with modified-live vaccines were performed in some of the dogs from which viruses were obtained in this study (showed with asterisks in figures 6 - 8). The CDV strains isolated from these clinical cases were genetically distinct from known vaccine strains, as has been demonstrated in previous studies of other CDV positive cases with a history of CDV vaccination (Bolt *et al.*, 1997; Calderon *et al.*, 2007; Haas *et al.*, 1999; Iwatsuki *et al.*, 2000; Lednicky *et al.*, 2004b; Maes *et al.*, 2003; Martella *et al.*, 2006). These observations suggest that a recent reversion of vaccine virus to virulence was not the cause of the clinical signs seen in dogs with a history of previous vaccination. The reasons for this apparent failure of vaccination may include interference by maternal immunity at the time of vaccination or the emergence of new strains that are divergent enough to evade immune protection elicited by the vaccines used (Pardo *et al.*, 2007). More studies on genetic characteristics are required to evaluate the efficacy of current CDV vaccines and to determine if there is a need to develop new vaccines against emergence of novel CDV strains. A thorough characterization of the genetic diversity of CDVs can improve our understanding of its emergence, epidemiology, and aid development of new vaccines and diagnostic tests.

In conclusion, the phylogenetic analysis of the complete H genes of CDV isolates from different parts of the world (available in GenBank) with wild-type South African isolates revealed nine clades. All South African isolates form a separate African clade of their own and are clearly separated from the American, European, Asian, Arctic and vaccine virus clades. It is concluded that only one lineage of CDV may currently be circulating in South Africa, and that the viruses isolated from dogs with a history of prior vaccination against CDV were not vaccine strains that reverted to virulence but a wild-type strain.

## CHAPTER 5

### GENERAL DISCUSSION

Canine distemper viruses isolated from dogs following natural infection cluster according to geographical distribution (Bolt *et al.*, 1997; Carpenter *et al.*, 1998). Some workers have reported genetic variation between vaccine strains and current circulating wild-type strains (Lan *et al.*, 2006; Martella *et al.*, 2006). There has not been any report of CDV isolations in southern Africa, justifying the need to isolate field strains from clinical specimens, and provide a pool of local strains for genetic characterization and comparison with vaccine and other strains from other parts of the world. This study therefore, reports for the first time, the isolation, identification and characterization of wild-type canine distemper viruses in Africa isolated in cell cultures. This also represents the first documented study on the genetic characteristics of canine distemper viruses in South Africa.

During the course of this study, we collected 162 clinical specimens from 124 domestic dogs. The clinical specimens were obtained from veterinary practices and diagnostic laboratories in Gauteng province, South Africa. Thirty three (20%) specimens from 26 (21%) dogs yielded viruses in Vero.DogSLAM cell cultures. Cytopathic effects (CPE) in the form of syncytia formation was observed in positive specimens within 24 hours of inoculation and the identity of the viruses was confirmed by immunofluorescence and electronmicroscopy. The findings confirmed that Vero.DogSLAM cell cultures facilitate the isolation of virulent CDV from infected dogs as early as 1 day post-inoculation with clinical material, with a significant reduction in the costs associated with the isolation of wild-type CDV.

The ability of laboratories to isolate and store current field isolates of CDV has improved considerably with this methodology.

The positive specimens used in this study were obtained from dogs showing either one or a combination of the respiratory, systemic, and nervous signs of the disease. Only 1 dog showed digital hyperkeratosis (so called “hardpad disease”). A clinical history of affected animals is important in understanding the epidemiology of CDV. Twenty two (85%) of the CDV positive dogs had no known vaccination history or were never vaccinated. It is likely that after several years of the absence of outbreaks of distemper, dog owners become complacent about vaccination. A significant reservoir of susceptible animals is a prerequisite for the proliferation and circulation of CDV in a population. More than 70% herd immunity is considered adequate to control infectious diseases as has been documented for measles, a human viral infection caused by a related morbillivirus (Rikula *et al.*, 2007).

Four (15%) of the CDV positive dogs examined in this study had a history of prior vaccination. Outbreaks of canine distemper have been reported among vaccinated dogs (Harder *et al.*, 1996; Harder & Osterhaus, 1997; Liermann *et al.*, 1998; Momberg-Jorgensen, 1951; Ott *et al.*, 1957; Simon-Martinez *et al.*, 2007), and some workers report that morbillivirus vaccination failures are common (Blixenkrone-Moller *et al.*, 1993; Povey, 1986). Clusters of clinical outbreaks have been recorded among human populations with documented measles vaccination coverage of more than 90% (Gustafson *et al.*, 1987). Assuming that quality, handling and administration of the vaccines were appropriate in the present cases

of apparent vaccination failure, an extraordinarily high infection pressure or immune dysfunctions of the dogs, which may be inherent or due to stress or interference by concurrent infections at the time of vaccination or at the time of natural exposure may have been involved. It is possible that part of the apparent immunization failures in properly vaccinated dogs may indicate changes in the biological properties of the current field viruses (Blixenkron-Moller *et al.*, 1993).

Nineteen (73%) of the CDV positive dogs were aged between 1.5 to 12 months. Two (8%) were 24 months old, and a further 2 were between 25 to 36 months of age, while 3 (12%) were aged between 60 to 72 months. The data reflect the tendency of the majority of confirmed cases to be found among young dogs. This trend is characteristic of morbillivirus infections in partly immune populations (Black L, 1989; Gorham, 1966). Twelve (46%) of the CDV positive dogs were males while 14 (54%) were females. The findings support the theory that the sex of the host does not have any effect on the prevalence. Infected dogs (26) in this study consisted of 15 different breeds and it is concluded that the prevalence of CDV in dogs does not have a breed predisposition. (Gorham, 1966) also did not find any breed predisposition to CDV infection.

In this study, we analyzed nucleic acid sequences of South African wild-type CDV strains isolated in Vero.DogSLAM cell cultures, viruses included in canine distemper vaccines used in South Africa, and other CDV sequences deposited in GenBank. Since genetic characterization of the H gene is useful for phylogenetic analyses (Haas *et al.* 1997; Harder & Osterhaus 1997; Martella *et al.* 2006), a primer pair that allowed amplification of the H gene (Lan *et al.*, 2006) was used

and the complete H gene was sequenced and phylogenetic analysis was performed. It was possible to clarify the H gene phylogenetic relationships among South African field and vaccine viruses and other CDV strains from GenBank.

It was found that the isolates examined in this study represent a distinct African lineage separate from the vaccine viruses and other established lineages. The variation in sequences is most likely due to changes that have occurred in the field viruses over the past 50 years since the first CDV vaccines became available. Cell culture adaptation is unlikely to have radically changed the virus sequences since, in the case of the related rinderpest virus it has been shown that the vaccine strain derived by multiple cell culture passages was more than 99% similar to the virulent parental virus (Baron & Barrett, 1995). Geographically distinct lineages have been described for other morbilliviruses such as rinderpest (Chamberlain *et al.*, 1993; Wamwayi *et al.*, 1995) and measles viruses (Taylor *et al.*, 1991). This study supports earlier work that RT-PCR of H genes in combination with fast sequencing methods is a sensitive tool to characterise canine distemper viruses.

## **5.1 Conclusions and recommendations**

Virus isolation remains a useful tool for the diagnosis of canine distemper. The formation of multinucleated giant cells (syncytia) accompanies the replication of CDV in Vero.DogSLAM cells as in a variety of other cell cultures. The use of Vero.DogSLAM cells for the isolation of CDV is efficient and fast. The efficiency, and cost effectiveness of using Vero.DogSLAM cells for confirmatory diagnosis (by viral isolation) of distemper should encourage wider use especially in some

developing countries where cost and technical expertise may limit the use of present day molecular diagnostic tools.

The CDV negative samples in this study were obtained mostly from animals with respiratory and systemic signs compatible with clinical distemper. Clinicians may over diagnose distemper when animals show signs due to other related diseases but compatible with canine distemper. Accurate diagnostic assays for CDV are important when considering its global distribution, its broad host range, myriad of clinical presentations, its history of mass mortality events, and the availability of vaccines that may curtail local epizootics.

A significant proportion of the South African dog population is unvaccinated against canine distemper and is reproductively active. This is an ideal condition for sporadic large scale distemper outbreaks. The infected dogs with a history of vaccination were infected with wild-type CDV as revealed by genetic relationships distant from the vaccine strain. Reversion to virulence of vaccine virus can therefore be ruled out.

The South African viruses isolated during this project are genetically distinct from the Onderstepoort strain and forms a distinct African lineage separate from other established lineages. Only one lineage of CDV may be circulating in South Africa currently, but further investigation may reveal a different picture.

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## APPENDIX 1

### Nucleotide Sequence Alignment of the H Gene of CDV Reference Strains\*, South African Field Strains and Commercial Vaccines use in the Country†.

	10	20	30	40	50	60	70	80	90	100	110	120
<u>Z35493_convac</u>	atgctctcctaccaagacaaggcgggtgccttctacaaggataaatgcaagagccaattcaaccaagctgtccttagtgacagaagaacatgggggcaggagaccaccttatttggtg	t	t	t	t	t	t	t	t	t	t	t
VaccineC		t	t	t	t	t	t	t	t	t	t	t
AF378705_Op		t	t	t	t	t	t	t	t	t	t	t
VaccineA		t	t	t	t	t	t	t	t	t	t	t
VaccineB		t	t	t	t	t	t	t	t	t	t	t
VaccineE		t	t	t	t	t	t	t	t	t	t	t
AF259552_SynderHill		t	t	t	t	t	t	t	t	t	t	t
AF164967_A75/17	g	t	a	t	t	t	a	t	a	c	c	
VaccineD		t	t	a	t	t	t	g	a	c	c	
08_ZA_4L70214		t	c	t	t	t	t	g	a	at	g	c
08_ZA_6L		t	c	t	t	t	t	g	a	at	g	c
08_ZA_13sp		t	c	t	t	t	t	g	a	at	g	c
08_ZA_4L70247		t	c	t	t	t	t	g	a	at	g	c
08_ZA_15sp		t	c	t	t	t	t	g	a	at	g	c
08_ZA_12sp		t	c	t	t	t	t	g	a	at	g	c
08_ZA_5L		t	c	t	t	t	t	g	a	at	g	c
08_ZA_5sp		t	c	t	t	t	t	g	a	at	g	c
08_ZA_9L		t	c	t	t	t	t	g	a	at	g	c
08_ZA_23L		t	c	t	t	t	t	g	a	at	g	c
08_ZA_4L7039		t	c	t	t	t	t	g	a	at	g	c
08_ZA_8L		t	c	t	t	t	t	g	a	at	g	c
08_ZA_4sp		t	c	t	t	t	t	g	a	at	g	c
08_ZA_16L		t	c	t	t	t	t	g	a	at	g	c
08_ZA_2L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_23sp		t	c	t	t	t	t	g	a	at	g	c
08_ZA_11L		t	c	t	t	t	t	g	a	at	g	c
08_ZA_21L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_20L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_7L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_26L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_25L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_1sp		a	t	t	t	t	t	g	a	at	g	c
08_ZA_10L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_1bn		a	t	t	t	t	t	g	a	at	g	c
08_ZA_14L		a	t	t	t	t	t	g	a	at	g	c
08_ZA_17L		a	t	t	t	t	t	g	a	at	g	c

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed





	250	260	270	280	290	300	310	320	330	340	350	360
<u>Z35493_convac</u>	tcagaggccg	tacatcaccaagt	catagatgtc	ttgacacccgct	cttcaagattat	cggagatgagatt	gggtcacgg	ttgccacaaaag	ctaaacgagat	caacaatttat	ccttcaa	360
<u>VaccineC</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
<u>AF378705_Op</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	359
<u>VaccineA</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
<u>VaccineB</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
<u>VaccineE</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
<u>AF259552_SynderHill</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
<u>AF164967_A75/17</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
<u>VaccineD</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_4L70214	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_6L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_13sp	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_4L70247	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_15sp	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_12sp	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_5L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_5sp	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_9L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_23L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_4L7039	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_8L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_4sp	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_16L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_2L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_23sp	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_11L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_21L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_20L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_7L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_26L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_25L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_1sp	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_10L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_1bn	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_14L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360
08_ZA_17L	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	360

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed



	490	500	510	520	530	540	550	560	570	580	590	600													
<u>Z35493_convac</u>	aaagc	tattg	catc	ggcag	caaat	cctat	cctttt	atcag	ccctc	ctggg	ggcgag	gagtg	acatatt	cccac	caacag	atg	ggag	ctact	actt	cagtag	gcaag	tttt	cccc	600	
<u>VaccineC</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
<u>AF378705_Op</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
<u>VaccineA</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
<u>VaccineB</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
<u>VaccineE</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
<u>AF259552_SynderHill</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
<u>AF164967_A75/17</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
<u>VaccineD</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_4L70214	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_6L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_13sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_4L70247	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_15sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_12sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_5L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_5sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_9L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_23L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_4L7039	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_8L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_4sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_16L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_2L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_23sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_11L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_21L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_20L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_7L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_26L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_25L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_1sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_10L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_1bn	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_14L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
08_ZA_17L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed





	850	860	870	880	890	900	910	920	930	940	950	960																		
<u>Z35493_convac</u>	aagg	tatg	tact	atag	cagtg	gggt	gagtt	gacact	ggctt	cctt	gtgt	gtaga	agagag	cactg	tatt	attat	atcat	gacag	cagtg	ggtt	caca	agat	ggtatt	ctag	tagt	gacact	g	960		
<u>VaccineC</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
<u>AF378705_Op</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	959
<u>VaccineA</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
<u>VaccineB</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
<u>VaccineE</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
<u>AF259552_SynderHill</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
<u>AF164967_A75/17</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
<u>VaccineD</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_4L70214	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_6L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_13sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_4L70247	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_15sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_12sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_5L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_5sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_9L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_23L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_4L7039	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_8L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_4sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_16L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_2L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_23sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_11L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_21L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_20L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_7L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_26L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_25L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_1sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_10L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_1bn	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_14L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960
08_ZA_17L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	960

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed

	970	980	990	1000	1010	1020	1030	1040	1050	1060	1070	1080					
<u>Z35493_convac</u>	gggatatttggga	caacacctat	ggatcacatt	gaggaagt	gatcctgtcgct	caccatcaat	ggagaaaata	catataacta	accaccgt	ggttttata	aaaagatt	caattgcaacc	1080				
<u>VaccineC</u>	.....g	.....a	.....a	.....a	.....a	.....a	.....a	.....a	.....a	.....a	.....a	.....a	1080				
<u>AF378705_Op</u>	.....t	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	1079				
<u>VaccineA</u>	.....t	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....ac	.....g	.....g	1080				
<u>VaccineB</u>	.....t	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	1080				
<u>VaccineE</u>	.....t	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....a	.....g	.....g	1080				
<u>AF259552_SynderHill</u>	.....g	.....t	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	.....g	1080				
<u>AF164967_A75/17</u>	..a	.....g	.....ag	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....g	.....c	.....a	1080		
<u>VaccineD</u>	..a	.....g	.....ag	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....g	.....c	.....a	1080		
<u>08_ZA_4L70214</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_6L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_13sp</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_4L70247</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_15sp</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_12sp</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_5L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_5sp</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_9L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_23L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_4L7039</u>	..a	.....g	.....c	.....a	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_8L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_4sp</u>	..a	.....t	.....g	.....c	.....a	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080
<u>08_ZA_16L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_2L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_23sp</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_11L</u>	..a	.....g	.....c	.....ag	.....g	.....a	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_21L</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_20L</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_7L</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_26L</u>	..a	.....g	.....gg	.....a	.....g	.....c	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....a	1080	
<u>08_ZA_25L</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_1sp</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_10L</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_1bn</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_14L</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....a	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	
<u>08_ZA_17L</u>	..a	.....g	.....gg	.....a	.....g	.....t	.....g	.....aa	.....a	.....a	.....t	.....c	.....c	.....c	.....a	1080	

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed



	1090	1100	1110	1120	1130	1140	1150	1160	1170	1180	1190	1200											
Z35493_convac	tggatgg	tgctgc	ctgcct	ggcctct	gagaa	caaga	agaac	aaaaagg	ttgtct	ggagtc	agctt	gtcaa	agaaa	ccctac	cccat	gtgca	accaa	cgatc	atggg	aac	ccctc	oggagga	
<u>VaccineC</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AF378705_Op	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<u>VaccineA</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<u>VaccineB</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<u>VaccineE</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AF259552_SynderHill	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AF164967_A75/17	.	at	ta	.	g	.	aa	.	g	.	t	t	.	.	.	.	.	.	.	.	.	t	
<u>VaccineD</u>	.	at	t	.	g	.	aa	.	g	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_4L70214	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_6L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_13sp	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_4L70247	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_15sp	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_12sp	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_5L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_5sp	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_9L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_23L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_4L7039	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_8L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_4sp	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_16L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_2L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_23sp	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_11L	.	at	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_21L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_20L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_7L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_26L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_25L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_1sp	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_10L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_1bn	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_14L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	
08_ZA_17L	.	tt	t	.	g	.	at	.	.	.	t	t	.	.	.	.	.	.	.	.	.	t	

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed

	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300	1310	1320
<u>Z35493_convac</u>	agacagttg	ccatccttat	ggggcgttg	acattacctct	agatgcaagt	gttgaccttca	accttaacata	tcgttcacata	cgggtccgg	ttatactgaat	ggagatgg	tatggattattat
<u>VaccineC</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
<u>AF378705_Op</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
<u>VaccineA</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
<u>VaccineB</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
<u>VaccineE</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
<u>AF259552_SynderHill</u>	g.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	t.....
<u>AF164967_A75/17</u>	g.....	.....	.....	c.....	ca.....	.....	c.....	t.....	.....	.....	c.....	.....
<u>VaccineD</u>	g.....	.....	.....	c.....	a.....	.....	t.....	.....	.....	.....	c.....	.....
08_ZA_4L70214	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_6L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_13sp	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_4L70247	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_15sp	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_12sp	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_5L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_5sp	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_9L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_23L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_4L7039	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_8L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_4sp	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_16L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_2L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_23sp	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_11L	g.....	c.....	c.....	t.....	ca.....	t.....	t.....	t.....	t.....	t.....	t.....	t.....
08_ZA_21L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_20L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_7L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_26L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_25L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_1sp	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_10L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_1bn	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....
08_ZA_14L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	g.....	t.....	t.....
08_ZA_17L	g.....	c.....	c.....	t.....	ca.....	t.....	g.....	a.....	t.....	t.....	t.....	t.....

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed

	1330	1340	1350	1360	1370	1380	1390	1400	1410	1420	1430	1440
<u>Z35493_convac</u>	gaaagcccacttttgaactccggatggccttaccattcctcccaaaaacggacaatccttgattgataaacaaagcaggtagaggagaccagttcactgtaatcccccatgtgttaaca											1440
<u>VaccineC</u>						t						1440
<u>AF378705_Op</u>						g				c		1439
<u>VaccineA</u>						tc				c		1440
<u>VaccineB</u>						tc				c		1440
<u>VaccineE</u>			t		g		at			c		1440
<u>AF259552_SynderHill</u>				t				a			a	1440
<u>AF164967_A75/17</u>	g			c	g	g		a		a		1440
<u>VaccineD</u>	g				g	g		a				1440
08_ZA_4L70214	g			t	g			a				1440
08_ZA_6L	g			t	g			a				1440
08_ZA_13sp	g			t	g			a				1440
08_ZA_4L70247	g			t	g			a				1440
08_ZA_15sp	g			t	g			a				1440
08_ZA_12sp	g			t	g			a				1440
08_ZA_5L	g			t	g			a				1440
08_ZA_5sp	g			t	g			a				1440
08_ZA_9L	g			t	g			a				1440
08_ZA_23L	g			t	g			a				1440
08_ZA_4L7039	g			t	g			a				1440
08_ZA_8L	a	g		t	g			a				1440
08_ZA_4sp	g			t	g			a				1440
08_ZA_16L	g			t	g			a				1440
08_ZA_2L	g			t	g			a				1440
08_ZA_23sp	g			t	g			a				1440
08_ZA_11L	g			t	g			a				1440
08_ZA_21L	g			t	g			a				1440
08_ZA_20L	g			t	g			a				1440
08_ZA_7L	g			t	g			a				1440
08_ZA_26L	g			t	g			a				1440
08_ZA_25L	g			t	g			a				1440
08_ZA_1sp	g			t	g	g		a				1440
08_ZA_10L	g			t	g	g		a				1440
08_ZA_1bn	g	g		t	g	ga	g	a				1440
08_ZA_14L	g	a		t	g			a				1440
08_ZA_17L	g			t	g			a				1440

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed

	1450	1460	1470	1480	1490	1500	1510	1520	1530	1540	1550	1560
<u>Z35493_convac</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>VaccineC</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>AF378705_Op</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>VaccineA</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>VaccineB</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>VaccineE</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>AF259552_SynderHill</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>AF164967_A75/17</u>	t	t	t	g	c	c	c	a	a	t	t	t
<u>VaccineD</u>	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_4L70214	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_6L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_13sp	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_4L70247	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_15sp	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_12sp	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_5L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_5sp	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_9L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_23L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_4L7039	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_8L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_4sp	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_16L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_2L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_23sp	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_11L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_21L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_20L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_7L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_26L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_25L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_1sp	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_10L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_1bn	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_14L	t	t	t	g	c	c	c	a	a	t	t	t
08_ZA_17L	t	t	t	g	c	c	c	a	a	t	t	t

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed

	1570	1580	1590	1600	1610	1620	1630	1640	1650	1660	1670	1680	
<u>Z35493_convac</u>	gt	catagcaacg	tatgacatatc	cacgaagt	gatcatg	cgattgttt	tattatgttt	atgacccaat	ccggacg	atcttatac	gcaccatttag	actaccaagggtagacctgat	1680
<u>VaccineC</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1680
<u>AF378705_Op</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	g.....	1679
<u>VaccineA</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1680
<u>VaccineB</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1680
<u>VaccineE</u>	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1680
<u>AF259552_SynderHill</u>	.....	t.....	.....	a.....	.....	.....	.....	t.....	.....	t.....	.....	t.....	1680
<u>AF164967_A75/17</u>	.....	a.....	t.....	c.gg.c	.....	.....	.....	g.....	.....	t.....	.....	t.....	1680
<u>VaccineD</u>	.....	a.....	t.....	c.ggac	.....	.....	.....	a.....	.....	t.....	.....	t.....	1680
<u>08_ZA_4L70214</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_6L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_13sp</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_4L70247</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_15sp</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_12sp</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_5L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_5sp</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_9L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_23L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_4L7039</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_8L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_4sp</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_16L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_2L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_23sp</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	t.....	1680
<u>08_ZA_11L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	a.....	1680
<u>08_ZA_21L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_20L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_7L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_26L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_25L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_1sp</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_10L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_1bn</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_14L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680
<u>08_ZA_17L</u>	g.....	a.....	t.....	c.g.ac	.....	.....	.....	.....	.....	t.....	.....	c.....	1680

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed

	1690	1700	1710	1720	1730	1740	1750	1760	1770	1780	1790	1800			
<u>Z35493_convac</u>	ttcctaaggatt	gaatg	ttttgt	gtgggat	gacaattt	gtggtgt	caccaatttt	acagattc	gaggct	aacatc	gccaaact	ctacaacc	agtggtgagaatttagt	cogtataagattc	1800
<u>VaccineC</u>	.....												1800		
<u>AF378705_Op</u>	.....												1799		
<u>VaccineA</u>	.....												1800		
<u>VaccineB</u>	.....												1800		
<u>VaccineE</u>	.....												1800		
<u>AF259552_SynderHill</u>	.....												1800		
<u>AF164967_A75/17</u>	.....												1800		
<u>VaccineD</u>	.....												1800		
08_ZA_4L70214	.....												1800		
08_ZA_6L	.....												1800		
08_ZA_13sp	.....												1800		
08_ZA_4L70247	.....												1800		
08_ZA_15sp	.....												1800		
08_ZA_12sp	.....												1800		
08_ZA_5L	.....												1800		
08_ZA_5sp	.....												1800		
08_ZA_9L	.....												1800		
08_ZA_23L	.....												1800		
08_ZA_4L7039	.....												1800		
08_ZA_8L	.....												1800		
08_ZA_4sp	.....												1800		
08_ZA_16L	.....												1800		
08_ZA_2L	.....												1800		
08_ZA_23sp	.....												1800		
08_ZA_11L	.....												1800		
08_ZA_21L	.....												1800		
08_ZA_20L	.....												1800		
08_ZA_7L	.....												1800		
08_ZA_26L	.....												1800		
08_ZA_25L	.....												1800		
08_ZA_1sp	.....												1800		
08_ZA_10L	.....												1800		
08_ZA_1bn	.....												1800		
08_ZA_14L	.....												1800		
08_ZA_17L	.....												1800		

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed



## APPENDIX 1 (CONTINUE)

	1810	1820	
	... ... ... ... ...		
<u>Z35493_convac</u>	tcatgtaaccggttcaaatccctga		1824
<u>VaccineC</u>	.....t...		1824
<u>AF378705_Op</u>	.....a.		1814
<u>VaccineA</u>	.....a.		1815
<u>VaccineB</u>	.....a.		1815
<u>VaccineE</u>	.....a.		1815
<u>AF259552_SynderHill</u>	.....		1824
<u>AF164967_A75/17</u>	.....t.....a.t.		1824
<u>VaccineD</u>	.....t.....a.t.		1824
08_ZA_4L70214	.....a.t.		1824
08_ZA_6L	.....a.t.		1824
08_ZA_13sp	.....a.t.		1824
08_ZA_4L70247	.....a.t.		1824
08_ZA_15sp	.....a.t.		1824
08_ZA_12sp	.....a.t.		1824
08_ZA_5L	.....a.t.		1824
08_ZA_5sp	.....a.t.		1824
08_ZA_9L	.....a.t.		1824
08_ZA_23L	.....a.t.		1824
08_ZA_4L7039	.....a.t.		1824
08_ZA_8L	.....a.t.		1824
08_ZA_4sp	.....a.t.		1824
08_ZA_16L	.....a.t.		1824
08_ZA_2L	.....a.t.		1824
08_ZA_23sp	.....a.t.		1824
08_ZA_11L	.....ga.t.		1824
08_ZA_21L	.....ga.t.		1824
08_ZA_20L	.....ga.t.		1824
08_ZA_7L	.....ga.t.		1824
08_ZA_26L	.....ga.t.		1824
08_ZA_25L	.....a.t.		1824
08_ZA_1sp	.....a.t.		1824
08_ZA_10L	.....a.t.		1824
08_ZA_1bn	.....a.t.		1824
08_ZA_14L	.....ga.t.		1824
08_ZA_17L	.....a.t.		1824

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Nucleotides of Commercial CDV Vaccines use in South Africa are boxed

## Protein Sequences alignment of the H Gene of CDV Reference Strains\*, South African Field Strains and Commercial Vaccines use in the Country†.

	10	20	30	40	50	60	70	80	90	100	
<u>Z35493_convac</u>	ML	SY	QDKAGAFYKDNARANSTKLSLVT	EEHGRRPPYLLFVLLVLLVGI	LALLAITGVRFHQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIG	100					
<b>VaccineC</b>	.	.	.	.	.	.	.	.	.	.	.
<u>AF378705_Op</u>	.	.	.	.	.	.	.	.	.	.	.
<b>VaccineA</b>	.	.	.	.	.	.	.	.	.	.	.
<b>VaccineB</b>	.	.	.	.	.	.	.	.	.	.	.
<b>VaccineE</b>	P	.	.	.	.	.	.	.	.	.	.
<u>AF259552_SynderHill</u>	.	.	.	.	.	.	.	.	.	.	.
<u>AF164967_A75/17</u>	.	.	.	.	.	.	.	.	.	.	.
<b>Vaccined</b>	.	.	.	.	.	.	.	.	.	.	.
08_ZA_4L70214	.	.	.	.	.	.	.	.	.	.	.
08_ZA_6L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_13sp	.	.	.	.	.	.	.	.	.	.	.
08_ZA_4L70247	.	.	.	.	.	.	.	.	.	.	.
08_ZA_15sp	.	.	.	.	.	.	.	.	.	.	.
08_ZA_12sp	.	.	.	.	.	.	.	.	.	.	.
08_ZA_5L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_5sp	.	.	.	.	.	.	.	.	.	.	.
08_ZA_9L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_23L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_4L7039	.	.	.	.	.	.	.	.	.	.	.
08_ZA_8L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_4sp	.	.	.	.	.	.	.	.	.	.	.
08_ZA_16L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_2L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_23sp	.	.	.	.	.	.	.	.	.	.	.
08_ZA_11L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_21L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_20L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_7L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_26L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_25L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_1sp	.	.	.	.	.	.	.	.	.	.	.
08_ZA_10L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_1bn	.	.	.	.	.	.	.	.	.	.	.
08_ZA_14L	.	.	.	.	.	.	.	.	.	.	.
08_ZA_17L	.	.	.	.	.	.	.	.	.	.	.

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Proteins of Commercial CDV Vaccines use in South Africa are boxed







	310	320	330	340	350	360	370	380	390	400																										
<u>Z35493_convac</u>	TVLLYHDS	SSGSQD	GILVVT	LGIFGT	TPMDHIE	EVIPVA	HPSMEKI	HITNHR	GFIKDS	IATWMP	PALASEK	QEEQK	GCLES	A	C	Q	R	K	P	Y	P	M	C	N	Q	T	S	W	E	P	F	G	G	400		
<u>VaccineC</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400
<u>AF378705_Op</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400
<u>VaccineA</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
<u>VaccineB</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
<u>VaccineE</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
<u>AF259552_SynderHill</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
<u>AF164967_A75/17</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
<u>Vaccined</u>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_4L70214	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_6L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_13sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_4L70247	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_15sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_12sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_5L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_5sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_9L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_23L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_4L7039	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_8L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_4sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_16L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_2L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_23sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_11L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_21L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_20L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_7L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_26L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_25L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_1sp	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_10L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_1bn	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_14L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	
08_ZA_17L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	400	

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Proteins of Commercial CDV Vaccines use in South Africa are boxed



	510	520	530	540	550	560	570	580	590	600			
<u>Z35493_convac</u>	DRDVLIESNLVVLPTQSF	RYVIATYDIS	RS	DHAI	VYVYDPI	RTIS	YTHPF	RLLTKGR	P	DFLRI	ECFVWDDNLWCHQFYRFEANIANSTTSVENLVRIRF	600	
<u>VaccineC</u>	.	.	.	.	.	.	.	.	.	.	.	M.	600
<u>AF378705_Op</u>	.	I.	.	.	.	.	.	.	.	.	.	D.	600
<u>VaccineA</u>	.	I.	.	.	.	.	.	.	N.	.	.	D.	600
<u>VaccineB</u>	.	I.	.	.	.	.	.	.	N.	.	.	D.	600
<u>VaccineE</u>	.	I.	.	.	.	.	.	.	.	C.	G.	D.	600
<u>AF259552_SynderHill</u>	.	.	.	.	N.	.	F.	F.	Y.	.	.	Y.	600
<u>AF164967_A75/17</u>	K.	T.	N.	G.	.	A.	Y.	.	.	D.	.	DST.	600
<u>Vaccined</u>	K.	T.	K.	D.	.	K.	F.	Y.	.	D.	.	D.TD.	600
<u>08_ZA_4L70214</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_6L</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_13sp</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_4L70247</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_15sp</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_12sp</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_5L</u>	K.	T.	N.	V.	N.	.	Y.	.	H.	D.	.	TD.T.	600
<u>08_ZA_5sp</u>	K.	T.	N.	V.	N.	.	Y.	.	H.	D.	.	TD.T.	600
<u>08_ZA_9L</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_23L</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_4L7039</u>	K.	T.	N.	V.	N.	.	Y.	.	G.	D.	.	TD.T.	600
<u>08_ZA_8L</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TDTP.	600
<u>08_ZA_4sp</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_16L</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_2L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_23sp</u>	K.	T.	N.	V.	N.	.	Y.	.	.	D.	.	TD.T.	600
<u>08_ZA_11L</u>	K.	T.	N.	V.	N.	.	Y.	.	T.	D.	.	D.T.	600
<u>08_ZA_21L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_20L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_7L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_26L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_25L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	DTT.	600
<u>08_ZA_1sp</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_10L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_1bn</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_14L</u>	K.	T.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600
<u>08_ZA_17L</u>	K.	S.	N.	V.	N.	.	Y.	.	K.	D.	.	D.T.	600

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Proteins of Commercial CDV Vaccines use in South Africa are boxed



## APPENDIX 2 (CONTINUE)

	..... ....	
<u>Z35493_convac</u>	<u>SCNRSNP</u> *	608
<u>VaccineC</u>	.....*	608
<u>AF378705_Op</u>	.....*	605
<u>VaccineA</u>	.....*	605
<u>VaccineB</u>	.....*	605
<u>VaccineE</u>	.....*	605
<u>AF259552_SynderHill</u>	.....*	608
<u>AF164967_A75/17</u>	.....K.*	608
<u>Vaccined</u>	.....K.*	608
08_ZA_4L70214	.....K.*	608
08_ZA_6L	.....K.*	608
08_ZA_13sp	.....K.*	608
08_ZA_4L70247	.....K.*	608
08_ZA_15sp	.....K.*	608
08_ZA_12sp	.....K.*	608
08_ZA_5L	.....K.*	608
08_ZA_5sp	.....K.*	608
08_ZA_9L	.....K.*	608
08_ZA_23L	.....K.*	608
08_ZA_4L7039	.....K.*	608
08_ZA_8L	.....K.*	608
08_ZA_4sp	.....K.*	608
08_ZA_16L	.....K.*	608
08_ZA_2L	.....K.*	608
08_ZA_23sp	.....K.*	608
08_ZA_11L	.....R.*	608
08_ZA_21L	.....R.*	608
08_ZA_20L	.....R.*	608
08_ZA_7L	.....R.*	608
08_ZA_26L	.....R.*	608
08_ZA_25L	.....K.*	608
08_ZA_1sp	.....K.*	608
08_ZA_10L	.....K.*	608
08_ZA_1bn	.....K.*	608
08_ZA_14L	.....R.*	608
08_ZA_17L	.....K.*	608

Dots (.) indicates similarities

\*Reference Strains are underlined

† H Gene Proteins of Commercial CDV Vaccines use in South Africa are boxed