Molecular characterisation and host specificity of canine distemper virus in selected wild carnivores of South Africa

by

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Submitted in partial fulfilment of the requirements for the degree
Philosophiae Doctor
in the Faculty of Veterinary Science
University of Pretoria
Pretoria

July 2017

To my husband Rob, you never stopped believing in me, and our little Daniel, may you always experience the joy and wonder of new discoveries

"The way of success is the way of continuous pursuit of knowledge"
~ Napoleon Hill ~

DECLARATION

I, Angelika Loots, declare that the thesis, which I hereby submit for the degree, Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

I, Angelika Loots, declare that for the research described in this work, the applicable research ethics approval has been obtained. I declare that I have observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

Signed: …….. Date: …18.07.2017…….

ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance and help of several individuals, who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study:

Professor Estelle Venter, Dr. Emily Lane and Dr. Desire Dalton for granting me the opportunity to complete my PhD under their supervision. This thesis would not have been possible without their continued support, guidance and encouragement. They have given me the opportunity to grow as a scientist and for that I am grateful.

Wildlife veterinarians Dr Peter Caldwell, and Dr Louis van Schalkwyk for their invaluable contribution in knowledge regarding the CDV outbreaks in South Africa.

Welgevonden Nature Reserve, Tswalu Kalahari Reserve and SANparks for their permission to collect samples and data for this study.

Dr Ashley Barnyard for providing us with the Vero.DogSLAM cells.

Karen Ebersohn for cell culture maintenance and growth of the CDV strains.

Dr Peter Coetzee, Dr Elaine Cardoso-Vermaak and Prudent Mokgokong for assistance in the laboratory.

Dr Morné Du Plessis for assistance with the whole genome analyses.

The National Research Foundation and University of Pretoria for financial assistance.

My husband, Rob, for his unequivocal support throughout, and for always being there with love and understanding.

My family and friends for their constant support, prayer, love and encouragement.

To Him, through whom all things are possible.

SUMMARY

Molecular characterisation and host specificity of canine distemper virus in selected wild carnivores of South Africa

by

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Canine distemper virus (CDV) has emerged as a significant disease of wildlife, is highly contagious and readily transmitted between susceptible hosts. Initially described as an infectious disease of domestic dogs, it is now recognised as a global multi-host pathogen, infecting and causing mass mortalities in a wide range of carnivore species. The last decade has seen the negative effect of numerous CDV outbreaks in various wildlife populations. Prevention of CDV infection requires a clear understanding of the potential as well as the dynamic pathways CDV uses to gain entry to its host cells and its ability to initiate viral shedding and disease transmission. Additionally, vaccination failure in CDV-infected wildlife is not uncommon, with several cases of disease outbreaks reported in vaccinated individuals. More studies on the genetic characteristics of CDV is thus required to evaluate the effectiveness of current CDV vaccines and to determine if there is a need to develop new vaccines against emergence of novel CDV strains.

The first chapter is a review on recent research conducted on CDV infection in wildlife, including the latest findings on the causes of host specificity and cellular receptors involved in distemper pathogenesis.

This is followed by a chapter on the whole genome sequence analyses of three CDV vaccines (Nobivac, Onderstepoort and Bucharest) and wild-type strains, isolated from African wild dog (*Lycaon pictus*) and spotted hyena (*Crocuta crocuta*). Each gene region was assessed through phylogenetic analyses and was evaluated for their usefulness in distinguishing strain diversity. Results showed that these two wild-type strains belong to the South African lineage, and all three vaccine strains to America I. Little is known about the CDV strains circulating in South Africa and these results constitute the first genomic sequences reported from isolates in South Africa.

Chapter three investigated the phylogenetic relationship of CDV strains recently isolated from four different wildlife species: lion (*Panthera leo*), African wild dog, spotted- and brown hyena (*Hyaena brunnea*) from three different regions in South Africa. This is the first report on genetic evidence of CDV isolated from clinical samples from wildlife species in South Africa. Variation in the H-gene of CDV world-wide and from various animal species has shown that the H-gene undergoes genetic drift related to geographical regions, resulting in several co-circulating genotypes. Phylogenetic analyses confirmed the presence of 12 previously described geographical lineages of CDV, with the newly sequenced strains from South African wildlife falling within the southern African lineage. The study also revealed two possible co-circulating sub-genotypes with CDV strains isolated from non-canid species distinct from, yet highly similar to CDV isolates from both domestic dog and wild canids. Phylogenetic results also indicated that CDV strains circulating in South African wildlife and domestic dogs were genetically distinct from commonly used vaccine strains. The molecular adaptation of CDV strains to different carnivore species was further examined by combining the resultant sequences with published data from CDV strains isolated in terrestrial carnivores and investigating the residues present at amino acid sites of the SLAM and Nectin-4 binding regions on the CDV H-protein. The importance of site 519 and 549 in the adaptation of strains to infect various hosts was confirmed. All non-canid strains isolated in this study presented the amino acid residue combination 519I/549H on the CDV H-protein. The amino acids present at site 530 in CDV strains infecting various carnivores globally were conserved within lineages regardless of host species, with South African

strains presenting 530N. No evidence of host adaptation or lineage grouping was observed in amino acid sites of the Nectin-4 binding region.

The final chapter investigated host susceptibility to CDV by identifying the presence of non-synonymous single nucleotide polymorphisms (SNPs) in the coding regions of Toll-like receptors (TLR) 2, 3, 4, 7 and 8 genes, using DNA from lion and African wild dog isolated during a recent outbreak of CDV in South Africa. Host specificity and viral pathogenesis depend on the susceptibility of CDV to its host's cells and its ability to initiate an immune response. Toll-like receptors are key recognition structures of the innate immune system, able to distinguish between different invading pathogens. Analysis of TLR diversity showed a higher rate of polymorphism in the African wild dogs within each of the TLR loci compared to lions. A single amino acid change (Met527Thr) within the leucine rich repeat of TLR2 was observed in a surviving lioness. This alteration resulted in a non-polar (M) to polar (T) group change, potentially influencing the expression and function of TLR2 which could result in an immune resistance to CDV infection. No specific amino acid variants could be associated with CDV susceptibility in the African wild dogs.

This research provides a good indication of the diversity and prevalence of CDV in South African carnivores and a better understanding of the strain diversity and host susceptibility of CDV in South African carnivores. Additionally, it is a critical starting point in facilitating the development of a South African-specific CDV laboratory tests e.g. the subsequent large scale *ante mortem* screening of wild carnivores for CDV. Information obtained can further enable researchers to make recommendations to conservation agencies, veterinarians and wildlife managers for the effective *ante mortem* diagnosis of CDV, as well as the management and prevention of this disease in wildlife. This study also provides a critical starting point in elucidating the mechanism involved in host immunity and therefore susceptibility towards CDV infection.

Thesis outline

Each chapter is written up as a manuscript for publication, due to this there may be instances of duplication across chapters. All publications and conference contributions are listed. References and appendices are provided at the end of the thesis. The summary and conclusion are combined from the respective chapters.

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PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

Publications

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Loots AK, Mitchell E, Dalton DL, Kotzé A, Venter E. (2017) Advances in canine distemper virus (CDV) pathogenesis research: a wildlife perspective. *Journal of General Virology.* 98: 311-321. DOI: 10.1099/jgv.0.000666.

Loots AK, Du Plessis M, Mitchell E, Dalton DL, Venter E. (2017) Genome sequences of three vaccine strains and two wild-type canine distemper virus strains from a recent disease outbreak in South Africa. *Genome Announcements*. 5: 1-2. 17. DOI: 10.1128/ genomeA.00603-17.

Loots AK, Cardoso-Vermaak E, Venter EH, Mitchell E, Kotzé A, Dalton DL. (2018) The role of Toll-like receptor polymorphisms in susceptibility to canine distemper virus. *Mammalian Biology.* 88: 94-99. DOI: 10.1016/j.mambio.2017.11.014.

Submitted (under review):

Loots AK, Mokgokong PS, Mitchell E, Venter EH, Kotzé A, Dalton DL. (2018) Phylogenetic analysis of canine distemper virus in South African wildlife. *PLOS ONE.* Revised and submitted.

Conference contributions

Oral presentations:

Veterinary Management of African Wildlife Conference, February 2017, Pretoria, SA
Title: "(Dis)temper tantrums: a tale of host idiosyncrasies"

7th Annual NZG Research Symposium, November 2016, Pretoria, SA
Title: "(Dis)temper tantrums: a tale of host idiosyncrasies"

6th Annual NZG Research Symposium, November 2015, Pretoria, SA
Title: "The use of retrospective data to solve a current problem: molecular identification of Canine Distemper Virus in South African wildlife"

5th Annual NZG Research Symposium, November 2014, Pretoria, SA
Title: "Molecular characterisation of canine distemper, parvo- and corona viruses in wild carnivores of South Africa"

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LIST OF ABBREVIATIONS

Ala (A)	-	Alanine (amino acid)
Arg (R)	-	Arginine (amino acid)
Asn (N)	-	Asparagine (amino acid)
Asp (D)	-	Aspartic acid (amino acid)
AWD	-	African wild dog
bp	-	Base pair
CD	-	Canine distemper
cDNA	-	Complementary DNA
CDV	-	Canine distemper virus
CNS	-	Central nervous system
DNA	-	Deoxyribonucleic acid
e.g.	-	Exempli gratia / for example
EDTA	-	Ethylenediamine tetra-acetic acid
ELISA	-	Enzyme-linked immunosorbent assay
F-protein	-	Fusion protein
Glu (E)	-	Glutamic acid (amino acid)
Gly (G)	-	Glycine (amino acid)
GTR+G	-	General time reversable with gamma distribution
His (H)	-	Histidine (amino acid)
H-protein	-	Haemaglutinin protein
IFAT	-	Indirect fluorescent antibody test
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
Ile (I)	-	Isoleucine (amino acid)
KNP	-	Kruger National Park
Leu (L)	-	Leucine (amino acid)
L-protein	-	Large protein
LRR	-	Leucine-rich repeat
MCMC	-	Metropolis-coupled Monte Carlo Markov Chain
Met (M)	-	Methionine
MeV	-	Measles virus
MHC	-	Major histocompatibility complex

MLV	-	Modified live vaccine
M-protein	-	Matrix protein
NCBI	-	National Centre for Biotechnology Information
NJ	-	Neighbour Joining
N-protein	-	Nucleocapsid protein
nt	-	Nucleotides
NZG	-	National Zoological Gardens of South Africa
PBS	-	Phosphate-buffered saline
PCR	-	Polymerase chain reaction
PDV	-	Phocine distemper virus
PP	-	Posterior probability
PPR	-	Peste des petits ruminants
P-protein	-	Phosphoprotein
PVRL4	-	Poliovirus-receptor-like 4 / Nectin-4
RNA	-	Ribonucleic acid
RNP	-	Ribonucleoprotein complex
RT-nqPCR	-	Reverse-transcription nested real-time polymerase chain reaction
RT-PCR	-	Reverse-transcription polymerase chain reaction
SA	-	South Africa
Ser (S)	-	Serine (amino acid)
SLAM / CD150	-	Signalling lymphocyte activation molecule
SNP	-	Single nucleotide polymorphisms
SNT	-	Serum-neutralisation test
Thr (T)	-	Threonine (amino acid)
TLR	-	Toll-like receptor
Trp (W)	-	Tryptophan (amino acid)
Tyr (Y)	-	Tyrosine (amino acid)
UTR	-	Untranslated regions
UV	-	Ultra violet
Val (V)	-	Valine (amino acid)
Vero.DogSLAM	-	Vero cells expressing canine SLAM receptor

Published in the Journal of General Virology

Chapter I: Literature Review

Advances in canine distemper virus pathogenesis research: a wildlife perspective

Advances in canine distemper virus pathogenesis research: a wildlife perspective

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Abstract

Canine distemper virus (CDV) has emerged as a significant disease of wildlife, is highly contagious and readily transmitted between susceptible hosts. Initially described as an infectious disease of domestic dogs, it is now recognised as a global multi-host pathogen, infecting and causing mass mortalities in a wide range of carnivore species. The last decade has seen the effect of numerous CDV outbreaks in various wildlife populations. Prevention of CDV requires a clear understanding of the potential hosts in danger of infection as well as the dynamic pathways CDV uses to gain entry to its host cells and its ability to initiate viral shedding and disease transmission. We review recent research conducted on CDV infections in wildlife, including the latest findings on the causes of host specificity and cellular receptors involved in distemper pathogenesis.

Keywords: Canine distemper virus, wildlife, infectious diseases, SLAM, Nectin-4

1. General introduction

Accurate identification and understanding the impact of infectious diseases on the morbidity and mortality of wildlife populations is vital, not only as a cautionary measure in the treatment of diseases, but also for the surveillance and risk assessments of disease outbreaks. Sufficient epidemiological information is rarely available to determine the level of threat diseases pose to the viability of many wildlife populations (Goller *et al.*, 2010; Smith *et al.*, 2006), with rapid identification of disease agents often not being an available option. In many cases treatment relies on tentative and inaccurate diagnosis (Daszak *et al.*, 2001; Munson & Karesh, 2002; Wobeser, 2007). This becomes even more important when considering the conservation of endangered species.

Despite the fact that viruses have been associated with several major declines in carnivore populations (Packer *et al.*, 1999; Young, 1994) detailed or long term investigations of virus-carnivore interactions in wildlife is limited (Grenfell & Gulland, 1995; McCallum & Dobson, 1995). One such virus infecting carnivores is the canine distemper virus (CDV). This highly contagious pathogen is the cause of canine distemper (CD), a severe systemic disease affecting carnivores worldwide. Initially diagnosed as a life-threatening disease in domestic dogs (*Canis familiaris*), it has subsequently been recognized in a wide range of hosts including some non-human primates, posing a conservation risk to several free-ranging and captive non-domestic carnivores (Beineke *et al.*, 2015; Deem *et al.*, 2000). The ability of CDV to switch hosts has raised concerns about the extinction threat it poses to several endangered wildlife species (Ripple *et al.*, 2014; Viana *et al.*, 2015; Woodroffe, 1999).

The aim of this review is to compile literature from the past decade (since the last comprehensive review in 2001) on CDV infections in wildlife, including the latest findings on the causes of host specificity and cellular receptors involved in distemper viral pathogenesis.

2. Viral properties

Canine distemper virus is a large (100-250 nm) single stranded RNA virus (Figure 1.1a), belonging to the *Morbillivirus* genus of the *Paramyxoviridae* family. Examples of diseases caused by members of the *Morbillivirus* genus are measles in primates, rinderpest in artiodactyls, peste des petits ruminants (PPR) in small ruminants and phocine and porpoise distemper in marine mammals (Barrett, 1999; Lamb & Kolakofsky, 2001; Osterhaus *et al.*,

1990; Pringle, 1999). Canine distemper virus has a lipoprotein envelope, containing a 15 690 nucleotides (nt) long, non-segmented negative-stranded RNA genome (Figure 1.1b), consisting of six genes that encode for a single envelope-associated protein (matrix (M)), two glycoproteins (the haemagglutinin (H) and fusion (F) proteins), two transcriptase-associated proteins (phosphoprotein (P) and large (L) protein) and the nucleocapsid (N) protein that encapsulates the viral RNA (Curran *et al.*, 1992; Diallo, 1990; Martella *et al.*, 2006). The organisation of the major gene codes in the CDV genome is 3'-N-P-M-F-H-L-5', each separated by untranslated regions (UTR) (Barrett *et al.*, 1985; Bellini *et al.*, 1986; Lamb & Parks, 2013; Sidhu *et al.*, 1993). Flanking the six genes are two control regions essential for transcription and replication known as the leader, a 3' extracistronic region of approximately 52 nt, and the trailer, a 5' extracistronic region of approximately 38 nt (Lamb & Parks, 2013; Marcacci *et al.*, 2014; Sidhu *et al.*, 1993).

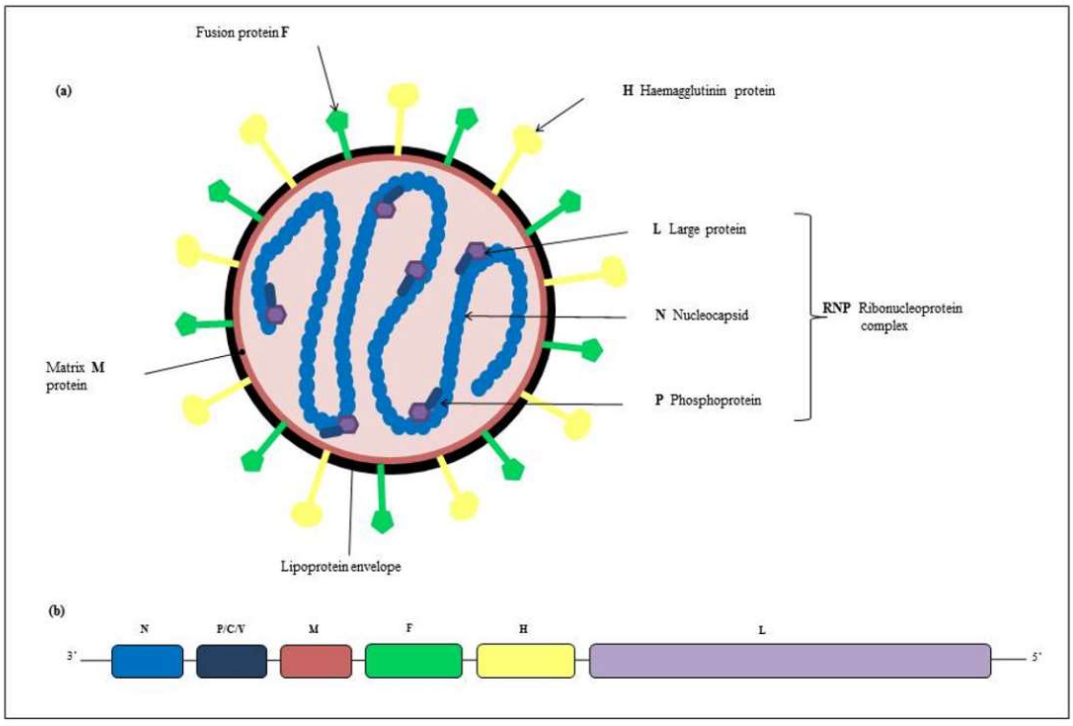

Figure 1.1. Schematic diagram of a **(a)** canine distemper virus with a lipoprotein envelope (black concentric circle), containing a non-segmented negative-sense single stranded RNA genome, consisting of six genes **(b)**. Underlying the lipoprotein is the viral matix protein (dark pink). Inserted through the viral membrane are the two glycoproteins, the haemagglutinin protein (H) (yellow) and fusion protein (F) (green). Together with the large protein (L) (purple), the nucleocapsid (N) (blue) and phosphoprotein (P) (dark blue) form the ribonucleoprotein complex (RNP). The relative abundance and scale of proteins are not illustrated. (Adapted from Sato *et al*. 2012)

Only one serotype of CDV is recognised with several co-circulating genotypes based on variation in the H-protein (Harder & Osterhaus, 1997; Ke *et al.*, 2015). Sequence analyses indicate that the H-protein undergoes genetic drift related to geographical regions, clustering into America I (includes almost all commercially available vaccine strains), America II, Asia I and -II, Europe/South America I, Europe wildlife, South America II and -III, Arctic, Rockborn-like, Africa and Africa II (Ke *et al.*, 2015; Martinez-Gutierrez & Ruiz-Saenz, 2016; Panzera *et al.*, 2015). Genotypes are defined on the basis of strains falling within the same clade sharing >95% amino acid similarity in their H-protein (Budaszewski *et al.*, 2014). Infection with CDV may be prevented by an adequate host immune response against the H-protein (Martella *et al.*, 2006), making the H-protein a suitable target for investigating polymorphism of CDV isolates and for molecular epidemiological studies (Budaszewski *et al.*, 2014; Haas *et al.*, 1997; Hashimoto *et al.*, 2001; Ke *et al.*, 2015; Panzera *et al.*, 2015).

3. Epidemiology

3.1. Host range & prevalence

Although CDV was initially described as an infectious disease of domestic dogs, it has increasingly become known as a worldwide multi-host pathogen, infecting a wide range of carnivores (Beineke *et al.*, 2015). Its ability to infect multiple species has led to mass mortalities in a range of carnivore species from wild canids, to felids, hyaenids, procyonids, ailurids, ursids, mustelids and viverrids. Distemper outbreaks have also been reported in marine mammals, including Baikal and Caspian seals (Kennedy *et al.*, 2000; Mamaev, 1995), with the viral strains likely originating from terrestrial carnivores (Forsyth *et al.,* 1998). More recently CDV was reported in non-human primates (rhesus monkey (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*)) with high mortality rates (Qiu, 2011; Sun *et al.*, 2010). Infections in these primates have raised several concerns of a potential zoonotic risk of CDV in humans. There are, however, no known reports of CDV infecting humans. Speculations regarding the potential adaptation of CDV to infect humans are outside the scope of this review and readers are referred to a review by Cosby (2012).

Reports of CDV outbreaks in large felids such as lions (*Panthera leo)*, leopards (*Panthera pardus*) and tigers (*Panthera tigris*), have challenged the belief that the Felidae group of animals is resistant to CDV infection (Appel *et al.*, 1994; Guiserix *et al.*, 2007; Harder *et al.*, 1996; Roelke-Parker *et al.*, 1996; Seimon *et al.*, 2013). When experimentally exposed to a highly virulent strain of CDV (Appel *et al.*, 1974) or inoculated with homogenized tissues

from a dead leopard infected with CDV (Harder *et al.*, 1996), domestic cats (*Felis catus*) were seropositive with no signs of clinical disease or viral shedding (Ikeda *et al.*, 2001). Recent studies on the seroprevalence of captive and free-ranging cheetah (*Acinonyx jubatus*) from Namibia to several viral pathogens have shown that cheetah are able to be infected by CDV (seropositive) but, similar to domestic cats, do not show clinical signs (Munson *et al.*, 2004; Thalwitzer *et al.*, 2010).

The last decade has seen numerous CDV outbreaks in various wildlife species worldwide. Outbreaks were confirmed in critically endangered species such as the Ethiopian wolf (*Canis simensis*), and Amur tiger (*Panthera tigris altaica*) (Gordon *et al.*, 2015; Seimon *et al.*, 2013). Concern for the conservation efforts of the giant panda (*Ailuropoda melanoleuca*) in China has also been raised due to several recent reports of CDV induced mortality in captive populations (Feng *et al.*, 2016; Hvistendahl, 2015). These outbreaks have highlighted the lack of knowledge on the extent of CDV susceptibility in wildlife species. This is even more evident for African wildlife with most studies originating from Tanzania, Kenya and Botswana. The CDV epidemic of 1994 that spread through the Serengeti National Park, Tanzania, is probably the best known of all, killing one-third of the lion (*Panthera leo*) population and causing deaths in several other species such as bat-eared fox (*Otocyon megalotis*), African wild dog (*Lycaon pictus*), silver-backed jackal (*Canis mesomelas*) and spotted hyena (*Crocuta crocuta*) (Roelke-Parker *et al.*, 1996). More recently CDV outbreaks occurred in several reserves within South Africa. Canine distemper in a lion population on a privately owned nature reserve in the Waterberg in December 2015 resulted in 95% mortality. This outbreak also infected other carnivore species, resulting in the first reported case of CDV mortality in an endangered brown hyena (*Hyaena brunnea*). Four months later the devastating effect of CDV was also observed in African wild dog populations of Kruger National Park and Tswalu Kalahari Reserve, South Africa, with the total eradication of two packs (26 animals in total).

3.2. Transmission & stability

Canine distemper is highly contagious and is readily transmitted between susceptible hosts through contact or aerosolized oral, respiratory and ocular fluids and exudates containing the pathogen. During the acute phase of infection other body excretions and secretions (e.g. urine, faeces, skin) can also contain the virus (Greene & Appel, 1990; Williams, 2001). Viral

shedding may follow infection for up to 90 days and occurs even if the animal was subclinically infected (Appel, 1987; Greene & Appel, 1990).

Canine distemper virus is extremely sensitive to UV radiation, heat, desiccation, oxidising agents, detergents and lipid solvents (Kingsbury *et al.*, 1978). At room temperature the virus is short lived, surviving between 20 minutes and 3 hours in tissues and exudates. Although the virus is able to survive for several days at temperatures below zero if protected by organic material (Greene & Appel, 1984), transmission of CDV is largely dependent on the close association between affected and susceptible animals. To sustain an epidemic of CD, dense populations of susceptible individuals and the continued presence of a biological reservoir are required (Alexander *et al.*, 2010; Williams, 2001). Owing to their wide distribution, domestic dogs (*Canis familiaris*) are key reservoirs for a variety of diseases and are considered as the primary reservoir for CDV infection (Alexander *et al.*, 2010; Berentsen *et al.*, 2013; Cleaveland *et al.*, 2000; Flacke *et al.*, 2013; Laurenson *et al.*, 1997). Domestic dogs, from communities surrounding protected wildlife areas are often unvaccinated and occur in high densities with a rapid population turnover. These and wildlife come into contact as both may wander several kilometers in and out of the protected areas (Butler *et al.*, 2004) increasing the risk of disease transmission, especially if these areas are unfenced. This risk of disease transmission between domestic dogs and wildlife is further augmented by a general lack of vaccination programs, particularly in rural areas. Pathogen maintenance in the system is further increased through interspecies transmission of CDV in a wide variety of hosts (Alexander *et al.*, 2010). Interactions among potential vectors of CDV, such as jackal, hyenas and lions at kills provide a potential mechanism for subsequent cross-species transmission (Cleaveland *et al.*, 2000). The amount of effort necessary to either prevent an epidemic or to eliminate an infection from a population could be determined by calculating the basic reproductive number ($R_{0)}$, an estimation of the number of secondary infections resulting from one infected individual (Dietz 1993, Dantzler *et al.*, 2016). This estimate may vary considerably for different populations infected as R_0 is influenced by several factors such as the duration of the infectious period, the probability of infection during contact and the number of susceptible individuals contacted per unit of time (Dietz 1993).

3.3. Clinical signs

Reports of clinical signs due to CDV infection in wildlife species largely resemble those in domestic dogs. However, the severity and the outcome of the infection may vary greatly

among species and depends on several factors, such as strain virulence, host age and host immune status. Initial signs of CDV infection are often subtle and rarely observed (Williams, 2001). If an animal develops a strong immune response, no clinical illness ensues. An estimated 50-70% of CDV infections in domestic dogs are thought to be subclinical (Greene & Appel, 1984). A weak immune response results in non-specific signs such as listlessness, appetite loss and fever. Despite a strong immune response that promotes recovery of the infected animal, CDV can persist for extended periods in the neurons, uvea, urothelium and skin (causing hyperkeratosis most dominantly seen in domestic dogs) (Appel, 1970, 1987; Schobesberger *et al.*, 2005). CDV infection during early developmental stages, before the eruption of permanent dentition, can also infect tooth buds and ameloblasts causing clear enamel hypoplasia (Bittegeko *et al.*, 1995; Dubielzig *et al.*, 1981) (Figure 1.2).

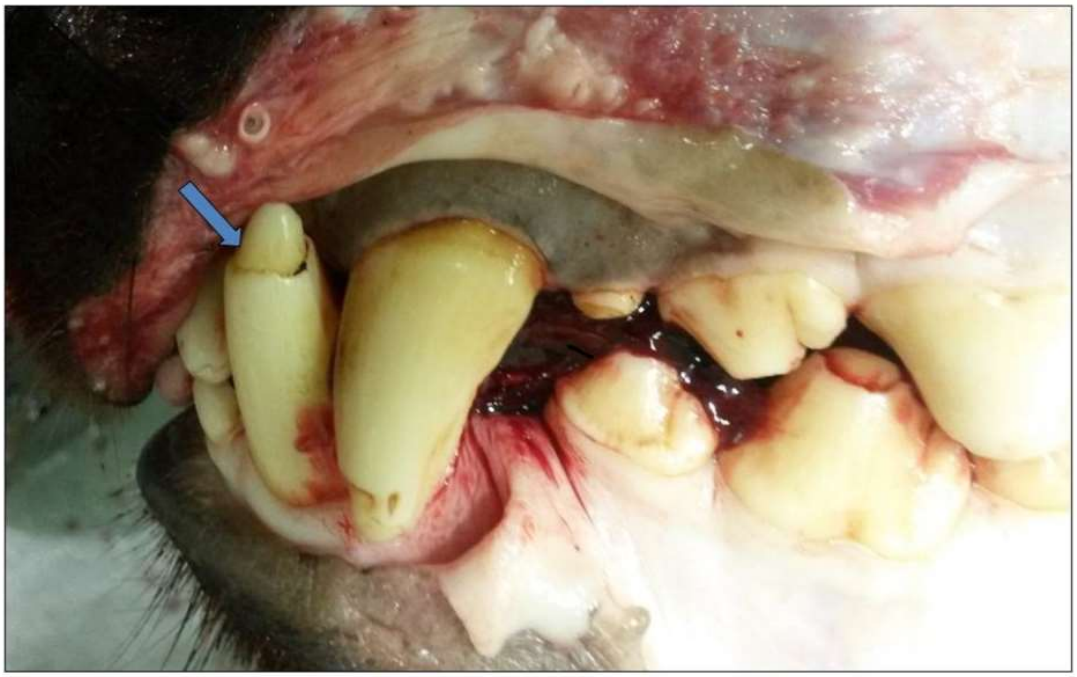

Figure 1.2. Teeth of a Brown Hyena (*Hyaena brunnea*) that died of CDV showing enamel hypoplasia due to presumed prior infection as a juvenile (Photo: AK Loots)

Two clinical forms of CDV can be distinguished in animals with minimal or no immune response, an acute systemic form and a chronic nervous form (Baumgärtner, 1993; Krakowka *et al.*, 1985). Acute systemic disease occurs 2-3 weeks post infection (Williams, 2001). The virus continues to replicate and spread throughout the body causing severe clinical signs which include fever, mucopurulent oculonasal discharge, coughing, dyspnoea, depression, anorexia, vomiting and diarrhoea (may be bloody) (Appel *et al.*, 1982; Winters *et al.*, 1983)

(Figure 1.3). During this stage of infection the virus is found in every secretion and excretion of the body (Krakowka *et al.*, 1985). Neurological signs may be concurrent or follow systemic disease within 2-3 weeks. Signs are progressive and varied depending on the area of the brain affected but commonly include abnormal behaviour, convulsions or seizures, chewing-gum movements of the mouth, blindness, cerebellar and vestibular signs, paresis or paralysis, incoordination and circling (Appel *et al.*, 1991; Williams, 2001). Infection in the central nervous system results in acute demyelinization, and most animals die 2-4 weeks after infection (Appel *et al.*, 1984; Winters *et al.*, 1983). Due to the immune compromising nature of CDV, clinical signs are often exacerbated by secondary bacterial infections of the skin and respiratory tract (Greene & Appel, 1990).

Figure 1.3. African wild dog (*Lycaon pictus*) afflicted by CDV showing clinical signs of mucopurulent oculonasal discharge **(a,b)** and weight loss **(c)**. (Photos: AK Loots)

4. Pathogenesis

Prevention of CDV requires knowledge of the potential hosts susceptible to infection as well as the dynamic pathways CDV uses to gain entry to host cells and its ability to initiate viral shedding. In domestic dogs, CDV may infect a new host by the nasal or oral route, coming into contact with the upper respiratory tract epithelium (Appel, 1970). There it multiplies in tissue macrophages, spreading, within 24 hours, via the lymphatics to the tonsils and respiratory lymph nodes, resulting in severe immunosuppression (Krakowka *et al.*, 1975; Leisewitz *et al.*, 2001; Winters *et al.*, 1983). Within two to four days other lymphoid tissues become infected and by day six the gastrointestinal mucosa, hepatic Kupffer cells and spleen are infected, resulting in a systemic reaction characterised by fever and leukopenia (Appel, 1970; Williams, 2001). Further spread of CDV occurs by cell-associated viraemia to other epithelial cells and the central nervous system (CNS) (Appel *et al.*, 1984; Winters *et al.*, 1983). Viral shedding from various host excretions and secretions begins approximately one week after infection (Appel, 1987).

4.1. Host range specificity

Host range specificity of a virus is determined by various mechanisms including the means by which viruses gain entry to host cells via cellular receptors and the ability of the host to respond to these viral infections through their innate and/or adaptive immune response (Hueffer *et al.*, 2003; Kaelber *et al.*, 2012; Qu *et al.*, 2005; Uematsu & Akira, 2006).

4.1.1. ***Cellular receptors***

Two major host cellular receptors have been identified that play a critical role in CDV pathogenesis: the signalling lymphocyte activation molecule (SLAM, CD150) and Nectin-4 (poliovirus-receptor-like-4, PVRL4) (Mühlebach *et al.*, 2011; Pratakpiriya *et al.*, 2012; Tatsuo *et al.*, 2001). Both of these receptors possess an immunoglobulin-like variable domain that provides a binding surface for morbilliviruses (Mühlebach *et al.*, 2011; Ono *et al.*, 2001). SLAM serves as an immune cell receptor and is expressed on the surface of activated T- and B-lymphocytes, dendritic cells and macrophages (Seki *et al.*, 2003; Tatsuo *et al.*, 2001). The second cellular receptor, Nectin-4, has only recently been recognised as the epithelial cell receptor for CDV (Mühlebach *et al.*, 2011; Noyce *et al.*, 2013; Pratakpiriya *et al.*, 2012). Nectin-4 is involved in the cell adhesion, participating in the organisation of epithelial and endothelial junctions of host cells (Reymond, 2001). It is thought to be an exit receptor,

functioning in the later stages of infection when the virus is amplified and released from epithelial cells (Noyce *et al.*, 2013).

Signalling lymphocyte activation molecule (SLAM, CD150)

Of the six structural proteins described for CDV, the H-protein has the greatest genetic variation and is a key protein in the attachment of the virion to receptors on the host cell surface (Budaszewski *et al.*, 2014). The specificity of CDV-H to interact with SLAM and its potential as a determinant of host range has been investigated (Bolt *et al.*, 1997; McCarthy *et al.*, 2007; Nikolin *et al.*, 2012b; Ohishi *et al.*, 2014). Amino acid residues Y525, D526, and R529 of CDV-H have been identified by site-directed mutagenesis to interact with SLAM (von Messling *et al.*, 2006; Zipperle *et al.*, 2010). Two other residues at amino acid sites 530 and 549 have also been studied and it is thought that these are important determinants of infectivity in carnivores. Both 530 and 549 fall into the receptor-binding domain located on propeller β-sheet 5 of CDV-H protein (McCarthy *et al.*, 2007). Previously suggested to be an adaptation of CDV to non-domestic dog hosts (McCarthy *et al.*, 2007), residues at site 530 have subsequently been shown as generally conserved within CDV lineages regardless of host species (Nikolin *et al.*, 2012b). Positive selection at site 549 of CDV-H and the specific substitution of Tyrosine (Y) by Histidine (H) is thought to have contributed to the spread of CDV from dog to non-dog host species (McCarthy *et al.*, 2007). The majority of CDV strains isolated from Canidae have Y at site 549, whereas CDV strains from other carnivore families mostly have H (Nikolin *et al.*, 2012a). Studies on the impact of specific amino acid substitutions within the H-protein are, however, speculative and several other factors could also have contributed to the spread of CDV. Conversely when comparing the amino acid sequences of the entire H binding site in SLAM among various carnivores, a high similarity among residues from Canidae species was found, suggesting a similar sensitivity to CDV among animals in this group (Nikolin *et al.*, 2012b). In contrast, comparing Felidae to Canidae, several residue differences were identified that ultimately led to electric charge differences in the SLAM interface of felids (Ohishi *et al.*, 2014). CDV strains that are well adapted to bind to dog SLAM receptors may thus be less adapted to bind to SLAM receptors from another non-canid host.

Nectin-4

The role of the epithelial receptor, Nectin-4, in CDV pathogenesis in the domestic dog, has only very recently been investigated. Six to nine days after infection with CDV, the virus

enters the epithelial cells of the respiratory, gastro-intestinal, urinary and endocrine system via an epithelial receptor (Ludlow *et al.*, 2012; von Messling *et al.*, 2004) now known as Nectin-4 (Delpeut *et al.*, 2014a; Noyce *et al.*, 2013). CDV amplification within the cells is promoted, after which the virus is released causing extensive respiratory, intestinal and dermatological symptoms (Iwatsuki *et al.*, 1995; von Messling *et al.*, 2004). In a host with a weakened immune response, CDV will move into the central nervous system, producing neurological symptoms (Beineke *et al.*, 2009). Nectin-4 has also been suggested to play a role in the neurovirulence of CDV (Pratakpiriya *et al.*, 2012) however other, thus far uncharacterised, receptors might also be involved (Ludlow *et al.*, 2014). Two protein variants of Nectin-4 have been identified, acting equally well for both viral entry and cell-to-cell spread (Delpeut *et al.*, 2014a, b; Noyce *et al.*, 2013).

5. Diagnosis

Ante mortem diagnosis of CDV is preferred due to the disease's high infectious potential, combined with a high mortality rate and fast progression. Initial diagnosis of CDV is mostly reliant on identifying the clinical signs associated with an infection. However, this form of diagnosis remains problematic and difficult due to the many varied clinical presentations of the disease. Differentiation from other diseases with respiratory, neurological, and/or gastrointestinal signs, such as rabies, feline panleukopenia, coronavirus, toxoplasmosis, bacterial enteritides and parvovirus, should be conducted. Several serological and immunological diagnostic tests have been developed for the detection of CDV in domestic animals. Diagnosis of CDV infection in wildlife is more difficult due to the challenges associated with acquiring cold-chain storage samples in the field for further testing in the laboratory. Diagnosis is mostly confirmed *post mortem* using histopathology and immunological tissue stains, although the specificity and sensitivity for the latter are not known for most wildlife species.

5.1. Molecular assays

The advent of molecular techniques brings diagnostic tools that are excellent with regards to sensitivity and specificity (Martella *et al.*, 2008; Soma *et al.*, 2013). One of several techniques that have been developed for the detection of CDV is the reverse-transcription polymerase chain reaction (RT-PCR) assay (Castilho *et al.*, 2007; Frisk *et al.*, 1999; Saito *et al.*, 2006; Yi *et al.*, 2012) which has been widely used predominantly targeting the highly

conserved N gene. While RT-PCR methods are more sensitive, specific and rapid compared to conventional culturing methods, they are still technically demanding and require several hours with additional post-PCR analyses (Elia *et al.*, 2006). Sensitivity also varies depending on the sample source, extraction method and choice of primers (Saito *et al.*, 2006).

A more rapid diagnostic technique for the detection of CDV is real-time RT-PCR (Elia *et al.*, 2006; Scagliarini *et al.*, 2007; Wilkes *et al.*, 2014). Real-time RT-PCR is used for both diagnostics and research and is especially useful for pathogen detection. Scagliarini *et al.*, (2007) developed a rapid and sensitive real-time RT-PCR assay based on TaqMan technology which is able to detect and quantify CDV in clinical samples and cell cultures. This assay is based on a highly conserved region of the P gene and is highly sensitive both as one-step and two-step reactions, confirming its suitability for research and diagnostic purposes.

Additionally, nested PCR techniques have been developed for the detection of CDV. Both Shin *et al.*, (2004) and Alcalde *et al.*, (2013) used a nested PCR with the product of a one-step conventional RT-PCR to detect the virus. Fischer *et al.*, (2013) took it one step further by developing a technique of reverse transcription followed by a nested real-time PCR (RT-nqPCR). The technique was performed on several clinical samples and proved to be two orders of magnitude more sensitive than RT-PCR.

5.2. Serological assays

Serological assays to detect and determine specific titers against CDV are the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), and the serum-neutralisation test (SNT). Both the IFAT and ELISA are used to detect IgM and IgG antibodies against CDV in domestic dogs and various non-dog hosts. The presence of IgM confirms not only current acute distemper infection, but is used to retrospectively diagnose distemper by detecting seroconversion in paired serum samples collected during the acute and recovering phase of the disease (Blixenkrone-Møller *et al.*, 1991; Haas *et al.*, 1999). However, there are not always suitable conjugated anti-species antibodies for wildlife species available for use with IFAT or ELISA. A systematic literature review of all possible non-dog hosts of CDV showed that ELISA was used 13.8% of the time as serological test, followed by IFAT (7.7%) (Martinez-Gutierrez & Ruiz-Saenz, 2016). The highly specific and sensitive SNT is more commonly used (75.4%) for the detection of CDV from serum samples and can be seen as the gold standard for detecting antibodies (Appel & Robson, 1973; Berentsen *et*

al., 2013; Martinez-Gutierrez & Ruiz-Saenz, 2016; Prager *et al.*, 2012). Serology as a diagnostic test is, however, not reliable in distinguishing between naturally acquired CDV infection (wild-type CDV strain), infection with attenuated virus vaccine strain (as used in modified-live vaccine) or immune response to recombinant, virus-vectored vaccine and should thus if possible be combined with other techniques, such as RT-PCR. (Frisk *et al.*, 1999; Kapil & Yeary, 2011; Kim *et al.*, 2001; Shin *et al.*, 1995).

5.3. Virus isolation

Virus isolation is typically conducted in pulmonary alveolar macrophages or by co-cultivation of infected tissues with mitogen-stimulated lymphocytes derived from healthy dogs (Appel *et al.*, 1992) or with the aid of ferret blood lymphocytes (Whetstone *et al.*, 1981; Williams, 2001; Woma & van Vuuren, 2009). These methods are demanding and time-consuming, taking several days to weeks (Elia *et al.*, 2006; Frisk *et al.*, 1999; Kim *et al.*, 2001; Shin *et al.*, 1995). In 2003, Vero cells expressing the canine SLAM, the principal receptor for morbilliviruses *in vivo* were engineered (Tatsuo *et al.*, 2001). These Vero.DogSLAM cells are highly sensitive for virus isolation, with cytopathic effects evident within 24 hours of inoculation (Seki *et al.*, 2003; Woma & van Vuuren, 2009).

5.4. Pathological examination

Routine *post mortem* diagnosis of CDV is by pathological examination of the spleen, lymph nodes, stomach, lung, small intestine, liver, pancreas, urinary bladder, kidney with renal pelvis and brain. Diagnosis is made by demonstration of typical histopathological lesions including the presence of viral inclusion bodies in lymphoid tissue, respiratory, urinary and gastro-intestinal tract epithelium and brain; by the presence of distinctive virions in negatively stained electron-microscopic preparations of faeces and through the detection of viral antigen in tissue by immunofluorescence or immunohistochemistry (Frisk *et al.*, 1999; Williams, 2001). Immunofluorescence has routinely been used as a diagnostic test, however, it is not sensitive and can detect CDV antigens only when the virus is still present in the epithelial cells (Appel, 1987; Elia *et al.*, 2006) with false negative results under certain clinical conditions (Fischer *et al.*, 2013; Jóźwik & Frymus, 2005).

6. Treatment & control

The treatment and control of infectious viral diseases is often difficult, especially in wildlife populations. Treatment of CDV infection is commonly based on symptomatic and supportive therapy as there is no specific antiviral drug available for the therapeutic use against CDV infection in any species, including domestic dogs. Studies on the *in vitro* effect of antiviral compounds in the treatment of CDV are ongoing and several future experiments are still required to determine their safety and efficacy in treating CDV in various species. Krumm *et al.,* (2014) evaluated an orally available, shelf-stable pan-morbillivirus inhibitor that targets viral polymerase. They found that treatment of CDV-infected ferrets at the onset of viraemia with the inhibitor, resulted in ferrets with low-grade viral loads, remaining asymptomatic and subsequently recovering from the infection. Other compounds such as fucoidan, a sulfated polysaccharide found in brown algae, have also been evaluated for their ability to act as antiviral drugs against CDV (Trejo-Avila *et al.*, 2014). *In vitro* results showed that fucoidan was able inhibit initial steps of the viral replication cycle, strongly suppressing the formation of syncytia in infected cells. Carvalho and colleagues (2013) evaluated the antiviral activity of several flavonoids (quercetin, morin, rutin, and hesperidin) and phenolic acids (cinnamic, trans-cinnamic, and ferulic acids), concentrating on their *in vitro* ability to inhibit stages of the CDV replication cycle. All flavonoids and phenolic acids demonstrated antiviral action against CDV infection. Other methods of treating CDV infection that have been explored include mesenchymal stem cells therapy (Pinheiro *et al.*, 2016) and the use of a veterinary pharmaceutical composition of silver nanoparticles (Bogdanchikova *et al.*, 2016).

An effective intervention strategy against CDV infection includes vaccination. In the 1960s two modified live vaccines (MLV) against CDV were introduced. The first, the Onderstepoort vaccine, was developed from a natural isolate, passaged in ferrets (*Mustela putorius furo*) and then adapted to chicken embryos (these were later replaced with chicken cell culture) (Haig, 1956). The second MLV was generated by adaptation of the CDV Rockborn-strain to canine kidney cells (Rockborn, 1959). These modified live virus vaccines are sufficient for management of CDV in domestic dogs, but can on rare occasions cause post-vaccination encephalitis and lead to vaccine induced illness (Hartley, 1974). The susceptibility of various species to vaccination with the MLV vaccine is largely unknown. Species differences in their response to vaccination have been observed, for example the avian cell adapted CDV vaccine can be fatal in European mink (*Mustela lutreola*) and ferrets (Carpenter *et al.*, 1976; Sutherland-Smith *et al.*, 1997), but was shown to give protection to

the maned wolf (*Chrysocyon brachyuru*), fennec fox (*Vulpes zerda*) and both red and grey fox (*Vulpes vulpes*) (Halbrooks *et al.*, 1981; Thomas-Baker, 1985). Concerns with differences in efficacy of MLV vaccines has led to the development of recombinant vaccines (Buczkowski *et al.*, 2014). Canarypox-vectored vaccines, developed for use in domestic canines, are incapable of replicating in the host cell, but can elicit an appropriate host immune response (Paoletti *et al.*, 1995; Taylor *et al.*, 1991, 1994). The canarypox-vectored vaccine has proven to be effective in challenge studies in various wildlife species including European ferrets (*Mustela putorius furo*), giant panda (*Ailuropoda melanoleuca*), fennec foxes (*Vulpes zerda*), meerkats (*Suricata suricatta*) and Siberian polecats (*Mustela eversmannil*) (Bronson *et al.*, 2007; Coke *et al.*, 2005; Stephensen *et al.*, 1997; Wimsatt *et al.*, 2003). A more recent study on vaccine efficiency in tigers (*Panthera tigris*) found that both the live attenuated and the recombinant canarypox-vectored vaccine appeared safe for use, although the live attenuated vaccine produced a significantly stronger and more consistent immune response in the tigers (Sadler *et al.*, 2016).

A general lack of quantitative data on the effect of CDV vaccine in wildlife has deemed it necessary to focus efforts on controlling CDV infection in the domestic dog reservoir surrounding conserved areas. While this approach benefits the domestic dog, vaccine coverage is rarely sufficient to reach the 95% target considered necessary to control CDV (Rikula *et al.*, 2007) and often fails to prevent infection in wildlife species that share their environment. Thus the question whether endangered wildlife should specifically be targeted for vaccination is raised. Several challenges associated with wildlife vaccination need to be considered including 1) knowledge on the safety and efficacy of the vaccine in the specific species targeted; 2) mode of vaccine delivery either during opportunistic animal handling (when fitting tracking collars, translocation or medical examination), or by hyperdermic dart (could cause injury and stress), or orally through laced bait (reduced efficacy if not eaten by target species); 3) the logistics of administering the required booster shots; and finally 4) the cost involved in initiating and implementing a vaccination program in wildlife (Cleaveland *et al.*, 2006; Coke *et al.*, 2005; Montali *et al.*, 1983; Viana *et al.*, 2015)

7. Conclusion

Canine distemper virus is an emerging pathogen posing a serious threat to the conservation of several captive and free-ranging wildlife populations. Its ability to infect multiple hosts considerably hampers disease eradication. Up to recently CDV had only been studied in

domestic dogs, with wildlife research greatly lacking. It is thus of great importance to study the factors influencing host susceptibility and CDV pathogenesis in all known and potential hosts of CDV. Further evaluation of the two known cellular receptors (SLAM and Nectin-4) in various wildlife species will aid in determining host specificity of the virus.

8. Acknowledgement

The authors would like to acknowledge wildlife veterinarians Dr Peter Caldwell, Old Chapel Vets, Gauteng and Dr Louis van Schalkwyk, State Veterinarian Department of Agriculture, Forestry and Fisheries, for their invaluable contribution in knowledge regarding the CDV outbreaks in South Africa. We also thank Welgevonden Nature Reserve, Tswalu Kalahari Reserve and SANparks for their contributions.

Chapter II:

Genome sequences of three vaccine strains and two wild-type canine distemper virus strains from a recent disease outbreak in South Africa

Genome sequences of three vaccine strains and two wild-type canine distemper virus strains from a recent disease outbreak in South Africa

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Abstract

Canine distemper virus is a global multi-host infectious disease. This report details the complete genome sequence of three vaccine and two new wild-type strains. The wild-type strains belong to the South African lineage, and all three vaccine strains to America I. This constitutes the first genomic sequences in South Africa.

Keywords: Canine distemper virus, wildlife, genome, South Africa

Canine distemper virus (CDV) (Family *Paramyxoviridae*) is an enveloped, non-segmented single-stranded negative sense RNA virus responsible for the disease canine distemper which has a high mortality rate in domestic and wild carnivore hosts (Appel, 1987; Beineke *et al.*, 2015). Vaccination has proven to be an effective intervention strategy against CDV (Bronson *et al.*, 2007; Sadler *et al.*, 2016), however, due to a general lack of domestic dog vaccination programs, particularly in rural areas, and the logistical and safety concerns of vaccinating wildlife, vaccine coverage is rarely sufficient to reach the 95% target to control the disease with several cases of vaccine-induced infections reported (Rikula *et al.*, 2007; Sutherland-Smith *et al.*, 1997). There is still a lack of quantitative data on the effects of CDV vaccines in wildlife (Loots *et al.*, 2017).

The RNA genome is 15 690 nucleotides (nt) long and consists of six genes that encode for a single envelope-associated protein (matrix (M)), two glycoproteins (the haemagglutinin (H) and fusion (F) proteins), two transcriptase-associated proteins (phosphoprotein (P) and large (L) protein) and a nucleocapsid (N) protein (Diallo, 1990). The organisation of the major genes are 3'-N-P-M-F-H-L-5', each separated by untranslated regions (Sidhu *et al.*, 1993). Only one serotype of CDV is recognised with several co-circulating genotypes clustering into America I and –II, Asia I and -II, Europe/South America I, Europe wildlife, South America II and -III, Arctic, Rockborn-like, Africa and Africa II, based on H-gene variation (Harder & Osterhaus, 1997; Ke *et al.*, 2015; Martinez-Gutierrez & Ruiz-Saenz, 2016; Panzera *et al.*, 2015).

Herein, the complete genome of the three CDV strains (CDV_Buc, CDV_Nobi, CDV_OVI) commonly found in vaccines and two wild-type strains (WT01, WT02) from a recent CDV outbreak in South Africa are reported. Both WT01 and WT02 were obtained from lung tissue collected from an infected African wild dog (*Lycaon pictus*) and spotted hyena (*Crocuta crocuta*), respectively. WT01 was from the Northern Cape Province, South Africa and WT02 from the Limpopo Province, South Africa. All samples were cultured and passaged between one and three times on Vero.DogSLAM cells grown in 25cm^2 tissue culture flasks (von Messling *et al.*, 2006). Viral RNA was extracted using Trizol (Invitrogen, catalog number: 15596026). Sequence-independent whole-genome reverse transcription-PCR amplification was used to prepare templates which were sequenced on an Illumina MiSeq sequencer using the TruSeq sample preparation kit (Illumina, catalog number: RS-122-2001). Data quality was assessed and poor quality sections were trimmed, using FastQC v0.11.2 software

(www.bioinformatics.babraham.ac.uk). Paired sequence reads were analysed in CLC Genomics Workbench v6 (CLC bio, Aarhus, Denmark). Full length genome sequences were assembled using a combination of mapping and *de novo*.

The genome of WT01, WT02, CDV_Buc, CDV_OVI and CDV_Nobi strains are 15 690nt, 15649nt, 15673nt, 15670nt, and 15649nt respectively. Amino acid lengths of the six proteins encoded by each of the four genomes were 522 (N), 506 (P), 334 (M), 662 (F), 604 (H) and 2183 (L). Comparing each individual gene the following mean amino acid identities (%) were obtained for WT01 and WT02 compared to the three vaccine strains, respectively: 83.6/84.6 (N), 86.6/88.6 (P), 84.3/84.6 (M), 84.6/83.6 (F), 81.4/81 (H), and 84/85 (L). WT01 is 97% identical to WT02. Multiple sequence alignments and Bayesian phylogenetic analysis (Figure 2) according to the H-gene revealed WT01 and WT02 belong to the South African cluster. The three vaccine strains grouped in the lineage America I, consistent with other known vaccine strain groupings.

The two wild-type CDV strains constitute the first report of a genomic sequence in South Africa and the first for South African wildlife. Data suggests the need for the formulation of new and updated vaccines, considering the level of genetic variability obtained. This data contributes to the knowledge of CDV which may be beneficial in determining effective preventative, diagnostic, and control measures for canine distemper in South Africa.

Nucleotide sequence accession numbers

The WT01, WT02, CDV_Buc, CDV_Nobi, and CDV_OVI sequences have been deposited in GenBank under accession numbers KY971528, KY971532, KY971529, KY971530, and KY971531, respectively.

Acknowledgement

This study was supported by the National Zoological Gardens of South Africa and funded by the National Research Foundation (NRF) Professional Development Program. The authors would like to acknowledge wildlife veterinarian Dr Peter Caldwell (Old Chapel Veterinary Clinic, Tshwane) for his invaluable contribution in collecting samples as well as Tswalu Kalahari Reserve for their permission to collect samples and data for this study. Karen Ebersohn and Dr Peter Coetzee are recognised for their assistance in the lab and the Agricultural research council biotechnology platform for sequencing on the MiSeq.

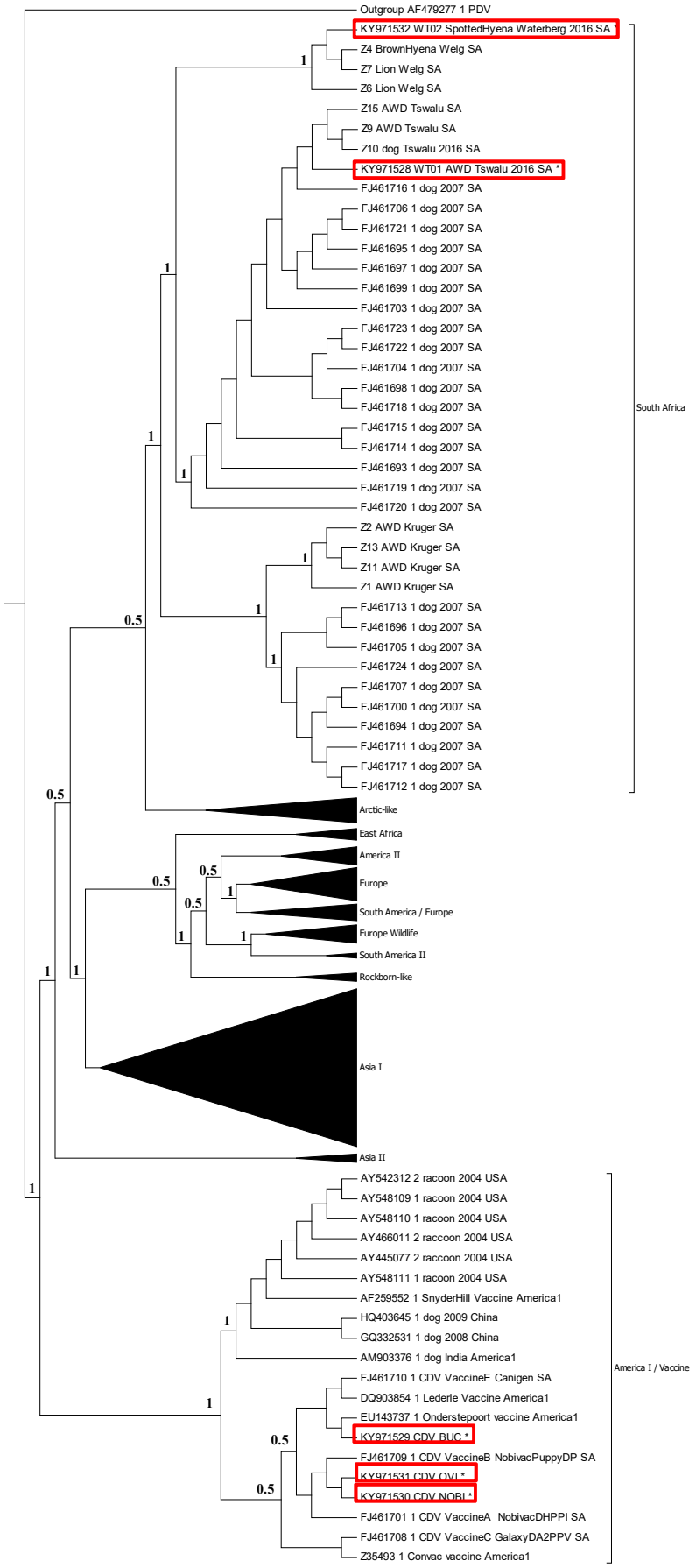

Figure 2. Rooted cladogram of the H-gene sequences of CDV and PDV (outgroup) with nodal support values above 0.5 Bayesian PP. Samples obtained in the present study are highlighted.

Chapter III:

Phylogenetic analysis of canine distemper virus in South African wildlife

Phylogenetic analysis of canine distemper virus in South African wildlife

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Abstract

Canine distemper virus (CDV) causes a severe contagious disease in a taxonomically broad range of immune-naïve hosts, including several endangered carnivores. The present study characterises CDV recently isolated from four different wildlife species, including lion (*Panthera leo*), African wild dog (*Lycaon pictus*), spotted- (*Crocuta crocuta*) and brown hyena (*Hyaena brunnea*), obtained from three different areas in South Africa (SA). It is also the first report on genetic evidence of CDV isolated in clinical samples from various wildlife species in SA. The phylogenetic diversity of CDV is examined, using sequence data from the complete H-gene as amplified by nested RT-PCR. The newly sequenced SA wildlife CDV isolates showed a high degree of similarity to CDV in domestic dogs previously isolated from SA. Phylogenetic analyses inferred by both distance (NJ) and character (MrBayes) approaches confirmed the presence of 12 previously described geographical lineages with the newly sequenced CDV strains from SA wildlife falling within the Southern African lineage. The study also reveals two possible co-circulating sub-genotypes. CDV strains isolated from the non-canid species were distinct, but highly similar to CDV isolates from both domestic dog and wild canids. Additionally, residues at amino acid sites of the SLAM and Nectin-4 binding regions on the H-protein were investigated and compared, confirming the importance of site 519 and 549 in the adaptation of strains to infect various hosts. It also confirms the notion that amino acids present at site 530 in CDV strains infecting various carnivores globally are conserved within lineages regardless of host species. Strains isolated from SA

wild carnivores showed no difference between host species with all strains presenting 530N. All non-canid strains isolated in this study presented the amino acid residue combination 519I/549H on the CDV H-protein. No evidence of host adaptation or lineage grouping was observed in amino acid sites of the Nectin-4 binding region. The data also shows that CDV strains circulating in SA wildlife and domestic dogs are genetically distinct from commonly used vaccine strains. Results are limited to available sequence data and in the Southern African lineage there is a clear bias towards CDV strains isolated in domestic dogs from one area. Further studies should include CDV strains isolated from various hosts from a wider geographical range in SA and vaccine efficacy should be tested by field trials.

Keywords: Canine distemper virus, hemagglutinin (H) gene, molecular phylogeny, lion, hyena, African wild dog, South Africa

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1. Introduction

Canine distemper virus (CDV; family *Paramyxoviridae*, genus *Morbillivirus*) is a single-stranded, enveloped RNA virus that is reported to cause a severe systemic disease called canine distemper (CD) globally (Deem *et al.*, 2000). This contagious disease is characterised by high morbidity and mortality in a taxonomically broad range of immune-naïve hosts, including some non-human primates and several endangered carnivores (Beineke *et al.*, 2015; Martinez-Gutierrez & Ruiz-Saenz, 2016). The development of vaccines against CDV infection in the late 1950s, has considerably reduced the mortality rates, partially controlling the disease in domestic dogs (*Canis lupus familiaris*) (Haig, 1956; Rockborn, 1959; Taylor *et al.*, 1994). Yet, there are still several reports on the regular occurrence of CD outbreaks amongst domestic dogs as well as several wildlife species (Feng *et al.*, 2016; Gordon *et al.*, 2015; Munson *et al.*, 2008, Zacarias *et al.*, 2016).

The CDV genome encodes for six structural proteins including the nucleocapsid (N), encapsidating the viral RNA; the phosphor- (P) and large protein (L), together forming the transcriptase/replicase complex; the matrix protein (M), important in the budding of virus particles; and the fusion (F) and haemagglutinin (H) protein, important in facilitating viral entry into host cells (Curran *et al.*, 1992; Diallo, 1990; Martella *et al.*, 2006; Nikolin *et al.*, 2016). Based on the genetic variability and the phylogenetic relationship of the H-protein, CDV is classified into several co-circulating genotypes (Ke *et al.*, 2015). Genotype clusters largely follow a geographical pattern and include America I, America II, Asia I and II, South America I/ Europe, Europe wildlife, South America II, Arctic-like, Rockborn-like, South Africa and East Africa (Ke *et al.*, 2015; Martinez-Gutierrez & Ruiz-Saenz, 2016; Nikolin *et al.*, 2016; Panzera *et al.*, 2015). These clusters are defined on the basis of strains falling within the same clade showing an amino acid divergence of less than 4% in their H-protein region (Budaszewski *et al.*, 2014; Martella *et al.*, 2006). Budaszewski et al. (2014) further suggested that sub-genotypes can be classified within a single clade based on strains with less than 2% divergence within their H-protein.

It is proposed that the H-protein is involved in cell tropism and is associated with host shift and adaptability, due to its ability to attach to cellular receptors such as the signalling lymphocyte activation molecule (SLAM, CD150), and Nectin-4 (PVRL4), facilitating viral entry (Budaszewski *et al.*, 2014; Ke *et al.*, 2015; Nikolin *et al.*, 2012a, 2016; Panzera *et al.*, 2015). The importance of an amino acid substitution at site 530 of the CDV H-protein was

first highlighted by Seki *et al.* (2003). CDV strains isolated from domestic dog showed a single amino acid substitution at site 530 conferring them the ability to infect both canine or human SLAM-expressing Vero cells as well as B95a (marmoset) cells *in vitro*. Amino acid sites 530 and 549, within the SLAM binding region of the CDV H-protein were later identified to be under positive selection (McCarthy *et al.*, 2007). This was confirmed by Nikolin *et al.* (2016), with the addition of site 519 that also showed evidence of episodic positive selection in some genotypes. Differences in residues at these sites have been associated with an adaptation of CDV to non-domestic dog hosts, as is shown with the amino acid substitution of Tyrosine (Y) with Histidine (H) at site 549. Canine distemper virus strains isolated from Canidae showed a majority of 549Y substitutions, whereas the 549H substitution was dominantly associated with other carnivore families (McCarthy *et al.*, 2007; Nikolin *et al.*, 2012a). A combination of amino acids in the CDV H-protein of Isoleucine (I) at site 519 together with H at 549 (519I/549H) was also reported to only occur in infections of non-canid hosts, such as lion (*Panthera leo*) and spotted hyena (*Crocuta crocuta*) (Nikolin *et al.*, 2016). Further investigation of site 530 however found the site to be generally conserved within lineages regardless of host species (Liao *et al.*, 2015; Nikolin *et al.*, 2012b, 2016). Conversely, amino acids of the H-protein considered responsible for viral attachment to the Nectin-4 receptor (478, 479, 537, and 539) (Langedijk *et al.*, 2011; Sawatsky *et al.*, 2012) showed no evidence for adaptation to non-canid or canid hosts (Nikolin *et al.*, 2016).

Canine distemper virus is thought to have spread from the United Stated to South Africa (SA) in the 1920s by way of migration routes (Panzera *et al.*, 2015), leading to the now known South African clade (Woma *et al.*, 2010). However, these results are only based on CDV strains isolated from domestic dogs. Research into the occurrence and diversity of CDV in wildlife species in SA is still severely lacking. Until recently the only research available on other African carnivores infected with CDV originated from Kenya, Tanzania and Botswana (Alexander *et al.*, 1996; Van De Bildt *et al.*, 2002; Goller *et al.*, 2010; Roelke-Parker *et al.*, 1996), with the only H-gene sequences isolated from Tanzania (Nikolin *et al.*, 2016). Thus, in order to obtain a better understanding of CDV dynamics in SA, virus strains isolated from wildlife should also be investigated.

In this study the phylogenetic diversity of CDV strains recovered from four wild carnivore species including lion, African wild dog (*Lycaon pictus*, AWD), spotted- and brown hyena (*Hyaena brunnea*), and one domestic dog recently isolated in SA is examined (n=12), using

sequence data from the CDV H-protein. Additionally, to examine the molecular adaptation of CDV strains to different carnivore species, residues at amino acid sites of the SLAM and Nectin-4 binding regions on the H-protein were investigated and compared to data available on the National Centre for Biotechnology Information (NCBI) nucleotide database.

2. Materials and methods

2.1. Samples

Canine distemper virus strains were recovered from three different regions in SA and were isolated from AWD and domestic dog from the Tswalu Kalahari Reserve, Northern Cape Province; AWD from Kruger National Park (KNP), Mpumalanga Province; brown hyena, lion and spotted hyena from Welgevonden Nature Reserve and a neighbouring nature reserve, Limpopo Province (Figure 3.1, Table 3.1). Samples were collected from animals that succumbed due to various clinical signs associated with CDV. Initial positive diagnosis was confirmed by physical examination, typical histopathology and immunohistochemical staining of formalin-fixed paraffin-embedded samples. Ethical approval was obtained from the Animal Ethics Committee, University of Pretoria, SA (V072-14) and the National Zoological Gardens of SA Research, Ethics and Scientific Committee (P14/26). All samples were obtained under Section 20 permit from the Department of Agriculture, Forestry and Fisheries, SA.

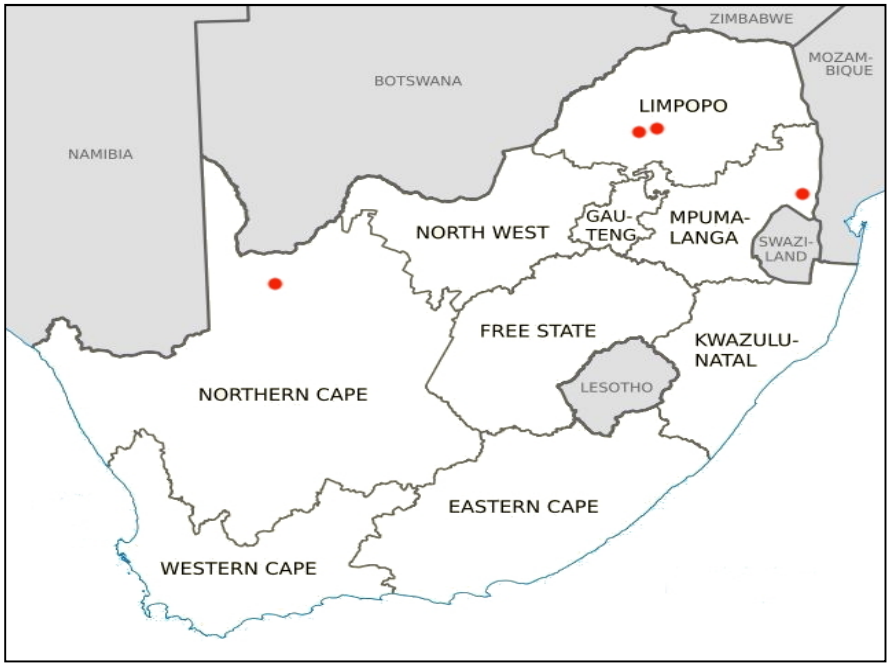

Figure 3.1. Map of South Africa depicting the different regions were canine distemper virus was isolated from wildlife in 2015/2016. Red circles indicate different reserves.

Table 3.1. Canine distemper virus strains from wild carnivores and one domestic dog isolated from South Africa in the summer/autumn months of 2015/2016.

Host species	Location	Year sampled	Sequence label	Accession number
African wild dog	Kruger National Park, Mpumalanga, South Africa	2016	Z1_African wild dog_Kruger	MF467742
African wild dog	Kruger National Park, Mpumalanga, South Africa	2016	Z2_ African wild dog _Kruger	MF467740
African wild dog	Kruger National Park, Mpumalanga, South Africa	2016	Z11_ African wild dog _Kruger	MF467743
African wild dog	Kruger National Park, Mpumalanga, South Africa	2016	Z13_ African wild dog _Kruger	MF467741
African wild dog	Tswalu Kalahari Reserve, Northern Cape, South Africa	2016	Z9_ African wild dog _Tswalu	MF467739
African wild dog	Tswalu Kalahari Reserve, Northern Cape, South Africa	2016	Z15_ African wild dog _Tswalu	MF467738
African wild dog	Tswalu Kalahari Reserve, Northern Cape, South Africa	2016	WT01_ African wild dog _Tswalu	KY971528
Domestic dog	Tswalu Kalahari Reserve, Northern Cape, South Africa	2016	Z10_dog_Tswalu	MF467747
Lion	Welgevonden Reserve, Limpopo, South Africa	2015	Z6_Lion_Welg	MF467745
Lion	Welgevonden Reserve, Limpopo, South Africa	2015	Z7_Lion_Welg	MF467746
Brown Hyena	Welgevonden Reserve, Limpopo, South Africa	2015	Z4_BHyena_Welg	MF467744
Spotted Hyena	Marakele, Limpopo, South Africa	2016	WT02_SHyena_Waterberg	KY971532

2.2 RNA extraction

Tissue samples were homogenized in phosphate-buffered saline (PBS) using the Precellys Homogenization system (Bertin Technologies). Subsequent RNA extraction was performed by means of TRIzol LS Reagent (Invitrogen) according to the manufacturer's instructions and stored at -80°C until used. Two cultured CDV strains commonly used in vaccines, Onderstepoort (OVI) and Nobivac, and RNase-free water were used as positive and negative controls in each reaction cycle, respectively.

2.3 Amplification of the H-gene by nested RT-PCR

Complementary DNA (cDNA) was synthesised with PrimeScript RT Mastermix (Takara) according to the manufacturer's instructions. Template cDNA was immediately stored at -20°C until used for PCR. Primers were designed based on South African strains previously amplified and sequenced by Woma *et al.*, (2010). The complete H-gene was amplified by nested RT-PCR, using a combination of the newly designed primers and primers as previously published, with minor modifications (Table 3.2). The first round of amplification was achieved using the primer pair RH3-F2 and RH4-R. The inner primer pairs H1F/CDVH1, CDVH2/R1R4, CDVH3/H2RB, CDVH4/CDVH5, CDVH6/CDVH7, H5F/CDVH8, CDVH9/CDVH10, CDVH11/CDVH12, and CDVH13/H7R were used for nested PCR, generating overlapping fragments. Amplification conditions consisted of an initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 1 min). Final extension was achieved at 72°C for 10 min. All reactions were performed in an ABI 2720 thermal cycler (Applied Biosystems).

2.4 Sequence and phylogenetic analysis of the H-gene

Amplicons were visualised by electrophoresis in a 1.5% Tris acetate-EDTA-agarose gel stained with ethidium bromide. Amplified PCR products generated with sets of inner primers were subsequently purified with Exonuclease I and FastAP (Thermo Fisher Scientific Inc.) according to manufacturer's instructions. Purified products were sequenced on an ABI PRISM 3100 Genetic Analyser using the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). Sequencing was conducted in both the forward and reverse direction. Generated overlapping sequences were aligned and contigs constructed in BioEdit Sequence Alignment Editor v.7.2.5 (Hall, 1999). Resulting contigs were aligned using the multiple alignment method (ClustalW) as implemented in MEGA6 software (Tamura *et al.*, 2013).

Table 3.2. Oligonucleotide primers used in the PCR assays of canine distemper virus H-gene.

Primer	Sequence (5'-3')	Template length (bp)	Reference
RH3-F2 (RH3-F[a])	AGG GCT CAG GTA **G**TC CAG C	Full H-gene	Harder *et al.* 1996
RH4-R	AAT GCT AGA GAT GGT TTA ATT		Harder *et al.* 1996
H1F	ATG CTC TCC **Y**AC CAA GAC AA	384	An *et al.* 2008
CDVH1	GCT CGG ATT GAA GAA GTT TG		Present study
CDVH2	CAA ACT TCT TCA ATC CGA GC	425	Present study
H1R4 (H1R[a])	CAT **R**T**Y** ATT CAG CCA CCG TT		An *et al.* 2008
CDVH3	CAA ACG GTG GCT GAA TGA CA	410	Present study
H2RB	TTT GGT TGC ACA TAG GGT AG		Budaszwenski *et al*. 2014
CDVH4	CGC TCA YCC ATC AGT AGA AA	163	Present study
CDVH5	GTT GCA CAT AGG GTA GGA TT		Present study
CDVH6	AAT CCTA CCC TAT GTG CAA C	159	Present study
CDVH7	CCA TAC CRT CTC CAT TCA GT		Present study
H5F	GGA CAG TTG CCA TCT TAC GG	165	Present study
CDVH8	CTT RGG AGG AAT GGT RAG CC		Present study
CDVH9	ACT GAA TGG AGA YGG TAT GG	159	Present study
CDVH10	CTA GGC GAA AAT GTC AAC AC		Present study
CDVH11	GTG TTG ACA TTT TCG CCT AG	245	Present study
CDVH12	CGT ATA AGA AAT CGT CCG G		Present study
CDVH13	ACG TCG TAG CAA CAT ATG AT	266	Present study
H7R	TCA AGG TYT TGA ACG GTT AC		Present study

Modifications introduced to original published sequence indicated in bold, [a] Original primer name in reference

Phylogenetic relationships for the SA CDV H-gene sequences generated in this study, and 229 previously published H-gene sequences from GenBank (http://www.ncbi.nlm.nih.gov) (Appendix A, Table A1) were inferred by the Neighbour Joining (NJ) and Metropolis-coupled Monte Carlo Markov Chain (MCMC) methods. Phocine distemper virus (PDV; Genbank AF479277) was selected as outgroup. The NJ trees were constructed using MEGA6 (Tamura *et al.*, 2013) with Tamura-3-parameter distance correction. Rate variation among sites was modelled with gamma distribution. Tree reliability was estimated by 1000 non-parametric bootstrap analyses. MrBayes v 3.2.6 (Ronquist *et al.*, 2012) with 1,000,000 iterations, subsampling every 1000 trees and a burnin of 10,000 iterations was used for MCMC tree analysis. The general time reversible nucleotide substitution model with gamma distributed rate variation among sites (GTR+G), as selected by MrModeltest v. 2.3 (Nylander, 2004), was used. Nodal support was estimated by calculating posterior probabilities (PP). Trees were produced and visualised in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). A subset of aligned H-gene sequences were used to calculate the nucleotide distance matrix and CDV genotypes distinguished based on a 95% similarity at the nucleotide level (Budaszewski *et al.*, 2014; Mochizuki *et al.*, 1999).

2.5 Analysis of amino acid sites

Amino acids of the H-protein present at sites 519, 530, and 549 of the SLAM binding region, together with amino acids 478, 479, 493, 537 and 539 of Nectin-4 binding region were determined for the 12 CDV H-protein sequences generated in this study, and 209 strains available from GenBank for which information on host, location and date of collection was available (Appendix A, Table A2).

3. Results

3.1. Phylogenetic relationship of the H-gene

A 1815 base pair (bp) fragment of the CDV H-gene was amplified and sequenced for 12 clinical specimens obtained from seven AWD, one domestic dog, one spotted hyena, two lions and one brown hyena. All sequences were submitted to GenBank (Table 3.1). The newly sequenced SA wildlife CDV isolates showed a high degree of similarity to CDV in domestic dogs previously isolated from SA ranging from 97% to 98% nucleotide identity. Comparing these wildlife strains to sequence data of known vaccine strains available from GenBank resulted in a 89-95% maximum nucleotide identity (Table 3.3).Isolates from SA

Table 3.3. Maximum identity of CDV isolated in South Africa in 2015/2016 compared to known vaccine strains from GenBank.

CDV isolates from South Africa	Maximum nucleotide identity (%)												
	Nobivac DHPPI[a]	Nobivac PuppyDP[b]	Galaxy DA2PPV[c]	Vanguard Plus[d]	Canigen[e]	NOBI[f]	OVI[g]	BUC[h]	Rockborn Candur[i]	Convac[j]	Lederle[k]	Snyder-Hill[l]	Onderste-Poort[m]
Z1	90	91	91	94	90	91	91	90	94	91	90	91	90
Z2	91	91	91	94	91	91	91	90	94	91	91	91	90
Z11	90	90	89	93	89	90	90	89	93	89	89	89	89
Z13	91	91	91	94	90	91	91	90	94	91	91	91	90
Z9	91	91	91	94	90	91	91	90	94	90	90	90	90
Z15	91	91	91	95	91	91	91	91	95	91	91	91	91
WT01	92	92	92	95	92	92	92	91	95	92	92	92	92
Z10	91	91	91	95	90	91	91	90	95	91	91	91	90
Z6	91	91	91	94	90	91	91	90	94	91	90	90	90
Z7	91	91	91	95	90	91	91	90	95	91	91	91	90
Z4	91	91	91	94	90	91	91	90	95	91	91	91	90
WT02	92	92	92	95	91	92	92	91	95	92	92	92	91

Genbank: [a] FJ461701.1, [b] FJ461709.1, [c] FJ461708.1, [d] FJ461702.1, [e] FJ461710.1, [f] KY971530, [g] KY971531, [h] KY971529, [i] GU266280.1, [j] Z35493.1, [k] DQ903854.1, [l] AF259552.1, [m] EU143737.1

consistently showed the highest similarity to the Rockborn-Candur (GU266280.1) and Vanguard® Plus (FJ461702.1) vaccine strains (93-95%).

Phylogenetic analyses of the H-gene inferred by the distance (NJ) and character (MrBayes) approaches resulted in trees with similar topology. Figure 3.2 depicts a rooted cladogram of the H-gene sequences of CDV and PDV (outgroup) with nodal support values above 0.5 Bayesian PP and 50% NJ bootstrap indicated. Nodal support of 0.9 PP and 70% bootstrap, respectively, are considered as strongly supported. The analyses identified 12 lineages. The outgroup (PDV) first splits into lineage America I (containing most vaccine strains), before splitting into lineage Asia II and a group consisting of the lineages Asia I, Rockborn-like, South America II, Europe Wildlife, South America I/Europe, Europe, America II, East Africa, Arctic-like and Southern Africa. Within the Southern Africa lineage two clades can clearly be defined (indicated as Clade A and Clade B). Clade A splits into two sister clades (A1 and A2). A1 consists of the spotted hyena, brown hyena and lion samples from Limpopo Province and A2 of previously isolated domestic dogs and the newly isolated AWD and dog from the Northern Cape Province. Clade B also splits into two sister clades (B1 and B2). The AWD isolated from Mpumalanga Province group together into B1. B2 exclusively consists of previously isolated domestic dogs. The overall mean genetic distance between unique CDV clusters within the Southern African lineage showed a 3.1% difference between Clade A and Clade B.

3.2 Amino acid variation

Sequenced H-gene fragments from each of the SA field isolates (n=12) were translated into a 605 amino acid long polypeptide and compared to H-protein strains (n=209), representing known geographical lineages and various host species (domestic dog, wild canid and non-canid) as sourced from GenBank. The amino acid residue at site 530 was identical (530N) for all SA field isolates obtained in this study, matching all previously sequenced SA domestic dog strains (Table 3.4). The overall dataset however included an additional seven amino acid residues (A, D, E, G, R, and V) at site 530 (Appendix A, Table A2). The majority of CDV retrieved from domestic dogs displayed 530G (60%) followed by 530N (30%, n=127). Similarly wild canids, including AWD, bat-eared fox, red fox, golden jackal, wolf and raccoon dog, displayed mostly 530G (74%) followed by 530N (19%, n=42). CDV strains retrieved from non-canid hosts displayed 48% 530G and 24% 530N (n=42). Residues 530A and 530E are only represented in domestic dogs from Asia I (3%, n=127) and Asia II (2%,

n=127), respectively. All vaccines, grouping into lineage America I, displayed 530S (78%, n=9), barring Snyder Hill (530N) and Vanguard Plus (530D). Residues 530R and 530V are represented by raccoon in America II (7%, n=42) and Europe Wildlife (2%, n=42), respectively.

The CDV strain obtained from the domestic dog (Z10/dog/2016/SA) in this study specified 519R, 530N and 549Y, identical to former domestic dog CDV strains isolated from SA (Table 3.4). The wild canids analysed from SA showed majority 519R (86%, n=7). A majority of 71% 549Y was also observed in these wild canid isolates. Only one strain from the KNP, designated Z1/African wild dog/2016/SA, presented with 519I and 549N (Table 4). Overall analyses of domestic dog and wild canid CDV strains globally showed a majority 519R (98%, n=127 and 93%, n=42, respectively). Of the 127 domestic dog CDV strains analysed 95% presented 549Y and 5% 549H. Wild canids overall (n=42) had 74% 549Y, 21% 549H and 5% 549N. Non-canid species isolated in this study, that included two lions, a spotted and a brown hyena, all had the combination of 519I and 549H (n=4) (Table 3.4). Overall the combination of 519I and 549H was only present in 19% (n=42) of the non-canid species analysed. Residues 519R and 519I were presented 79% and 21%, respectively and both 549H (55%) and 549Y (45%) were present in the overall non-canid analyses.

Amino acid residues thought to be crucial in CDV attachment to the cellular receptor Nectin-4 were generally conserved across species and geographical lineages. All CDV strains isolated in this study presented majority 478V, 479L, 537Y, and 539Y. Two strains from AWD in KNP however resulted in 479S (Table 4). Overall analyses of the Nectin-4 binding sites in CDV strains across geographic lineages also gave majority 478V, 479L, 539Y, and 539Y, although the CDV strain isolated from the javelina (Family: Tyassuidae) from Denmark in 1995 showed 479W.

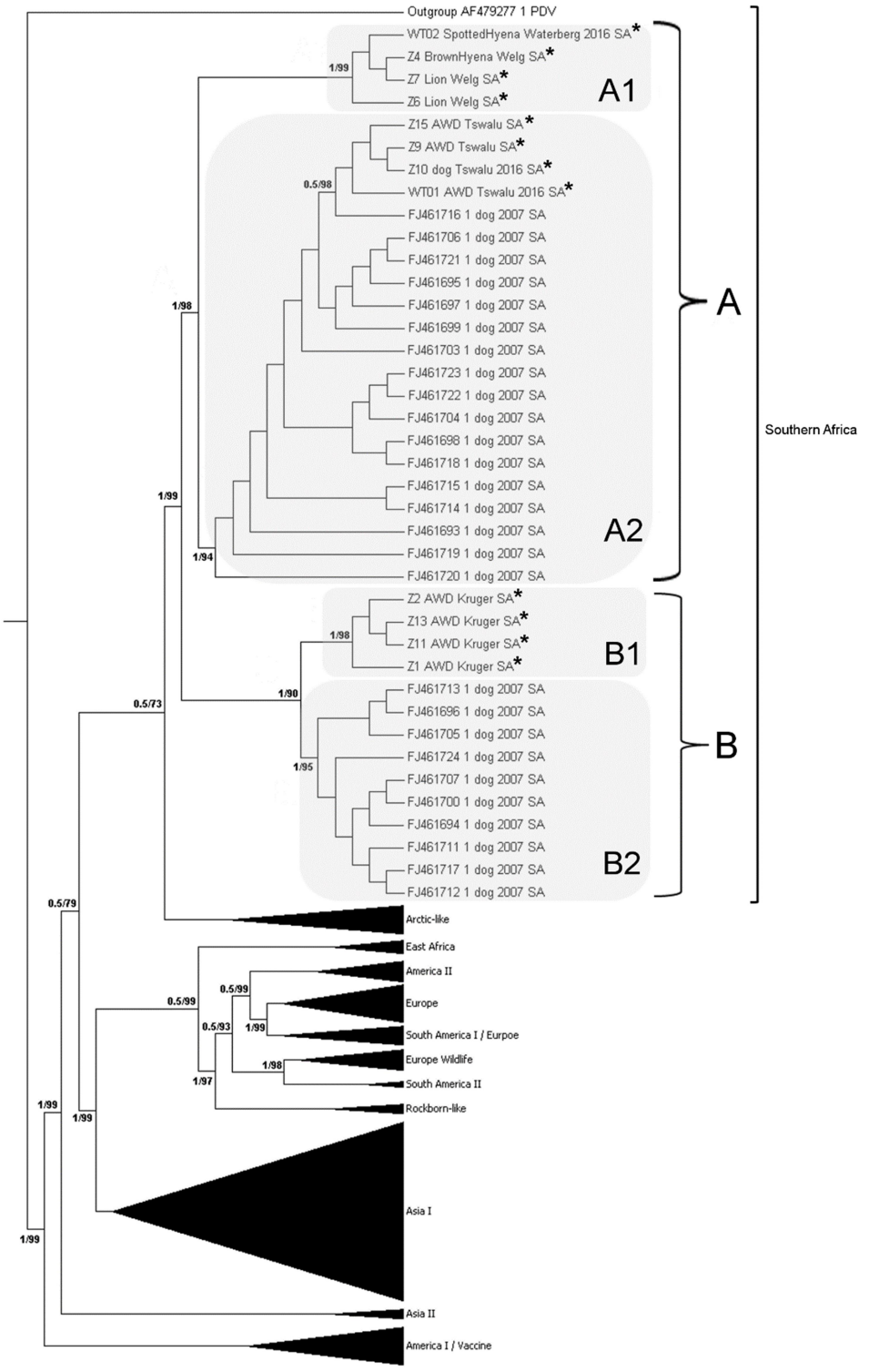

Figure 3.2. Rooted cladogram of the H-gene sequences of CDV and PDV (outgroup) with nodal support values above 0.5 Bayesian PP and 50% NJ bootstrap indicated. Samples obtained in the present study are highlighted with an asterisk (*).

Table 3.4. Residues at amino acid sites of the SLAM and nectin-4 cell binding regions on the Canine distemper virus H-protein isolated in South Africa in 2015/2016. The accession number, host species, year and country of origin are indicated for each strain. Identical amino acids are indicated with a dash (-), varying amino acids are indicated by single letter amino acid codes

Accession number/species/year/origin	SLAM binding region			Nectin-4 binding region			
	519	530	549	478	479	537	539
SOUTHERN AFRICA							
Domestic dog							
Z10/dog/2016/SA	R	N	Y	V	L	Y	Y
[a] FJ461723.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461698.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461718.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461722.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461704.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461706.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461721.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461695.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461697.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461693.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461703.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461715.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461714.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461699.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461716.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461719.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461720.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461713.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461705.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461696.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461724.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461707.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461711.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461694.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461700.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461717.1/dog/2007/SA	-	-	-	-	-	-	-
[a] FJ461712.1/dog/2007/SA	-	-	-	-	-	-	-
Wild canid							
Z15/African wild dog/2016/SA	R	N	Y	V	L	Y	Y
Z9/African wild dog /2016/SA	-	-	-	-	-	-	-
Z2/African wild dog /2016/SA	-	-	-	-	-	-	-
Z13/African wild dog /2016/SA	-	-	-	-	S	-	-
Z1/African wild dog /2016/SA	I	-	N	-	-	-	-
Z11/African wild dog /2016/SA	-	-	N	-	S	-	-
WT01/African wild dog /2016/SA	-	-	-	-	-	-	-

Table 3.4. (continued)

Accession number/species/year/origin	SLAM binding region			Nectin-4 binding region			
	519	530	549	478	479	537	539
Non-canid							
WT02/SpottedHyena/2016/SA	I	N	H	V	L	Y	Y
Z4/BrownHyena/2016/SA	-	-	-	-	-	-	-
Z6/Lion/2015/SA	-	-	-	-	-	-	-
Z7/Lion/2015/SA	-	-	-	-	-	-	-

[a] South African CDV strains isolated by Woma *et al.* (2010) and deposited in GenBank

4. Discussion

The present study characterises CDV from four different wild carnivore species, obtained from three different areas in SA. It is also the first report on genetic evidence of CDV isolated in clinical samples from various wildlife species in SA. Earlier reports of CDV in SA are very limited and it was not until 2010 that CDV strains isolated from domestic dogs were sequenced and phylogenetically characterised (Woma *et al.*, 2010). The aforementioned study was however limited to local CDV outbreaks isolated from one species (domestic dog) occurring in one area (Gauteng Province) of SA. The present study reports on the status of CDV infection in SA wildlife and how it relates to global CDV outbreaks.

Phylogenetic analyses of the H-gene sequences of the newly isolated SA strains, together with several globally isolated CDV strains, confirmed the presence of 12 previously described geographical lineages (Ke *et al.*, 2015; Martinez-Gutierrez & Ruiz-Saenz, 2016; Nikolin *et al.*, 2016; Panzera *et al.*, 2015) with the newly sequenced CDV strains from SA wildlife falling within the Southern African lineage. This grouping is further supported by the high degree of nucleotide similarity that was observed between the CDV wildlife strains in comparison to the domestic dog strains isolated from SA in 2007. Geographical lineages (genotypes) are defined based on a nucleotide difference of 5% between clades (Budaszewski *et al.*, 2014; Martella *et al.*, 2006), whereas sub-genotypes can be classified as clades that have a nucleotide difference of more than 2% but less than 5% (Budaszewski *et al.*, 2014). Sub-genotypes have thus far only been described in the South America-I / Europe lineage of CDV, showing clear clustering according to distinct geographical areas (Budaszewski *et al.*, 2014). The present study revealed the co-circulation of two distinct clades of CDV within the Southern African lineage (Figure 3.2) with a mean nucleotide difference of 3%, suggesting

the co-circulation of two sub-genotypes in SA. A correlation between sub-genotype grouping in SA and geographical origin of the CDV strains could however not clearly be determined. The first sub-genotype, designated Clade A, comprises sequence data isolated in Limpopo, Northern Cape and Gauteng areas, respectively. The second sub-genotype, designated Clade B, contains mainly isolates from Mpumalanga and Gauteng provinces. It is thus hypothesised that CDV isolates from Clade A are predominantly from the northern parts of SA and isolates from Clade B from further south, with both sub-genotypes circulating in Gauteng. This hypothesis should however be confirmed by extending phylogenetic studies to other areas in SA.

Focusing on the Southern Africa lineage, it becomes apparent that the phylogenetic relationship of CDV strains isolated from the non-canid species (Felidae and Hyenidae) are distinct, grouping in a separate sister clade (A1), but highly similar to CDV isolates from both domestic dog and wild canids. Biological and sequence data obtained in previous studies did not indicate the existence of a CDV lineage adapted for non-canine species (Harder *et al.*, 1996; Nikolin *et al.*, 2016). All non-canid CDV strains isolated in this study originated from one outbreak in the Limpopo Province area, thus explaining the grouping and supporting previous studies. The addition of a CDV strain isolated from a canid species in the same geographical area will give a better understanding as to the current observed groupings.

Analysis of amino acid substitutions at known functional positions on the SLAM binding region of the CDV H-gene confirmed the importance of sites 519 and 549 in the adaptation of strains to infect various hosts. It also confirms the notion that amino acids present at site 530 in CDV strains infecting various carnivores globally are conserved within lineages regardless of host species. The present analyses showed that the majority of CDV strains exhibit 530G or 530N in the CDV H-protein of wild-, domestic- and non-canine hosts. Strains isolated from SA wildlife also showed no difference between host species with all strains presenting 530N, corresponding to the amino acid residue observed in previously isolated domestic dogs from SA. Our analyses further confirms the notion that at site 530 in certain CDV strains there is a bias towards A or V in lineages Asia I and Europe wildlife, respectively (Liao *et al.*, 2015; McCarthy *et al.*, 2007).

This study shows that the arrangement of amino acid residues at site 549 of the CDV H-protein differed in canid and non-canid species, with strains from canids (both domestic and wild) showing a clear bias towards 549Y. CDV strains from non-canid species globally

however were equally likely to exhibit H or Y at site 549. These findings are consistent with previous studies and supports the assumption that both canids and non-canid hosts are just as likely to encounter CDV strains with 549Y or 549H, but that canids are more likely to be infected by CDV strains with 549Y (McCarthy *et al.*, 2007; von Messling *et al.*, 2003; Nikolin *et al.*, 2012a). This is also consistent with the findings of Nikolin *et al.* (2012b) that showed an *in vitro* antagonistic pleiotropic effect of site 549, with CDV strains encoding 549Y performing significantly better in cells expressing dog SLAM receptors than those encoding 549H. The current study also presents the first evidence of CDV strains with 549H in the Southern Africa lineage; with all non-canid strains isolated in this study presenting residue H at this site. The current study also reports the presence of the amino acid residue combination 519I/549H on the CDV H-protein isolated from three non-canid species (lion, spotted- and brown hyena). This is consistent with the findings of Nikolin *et al.* (2016) that showed strains encoding 519I/549H causing fatal CDV infection only in non-canid hosts during the 1993/1994 Serengeti epidemic.

No evidence of host adaptation or lineage grouping was observed in the four amino acid H-protein sites of the Nectin-4 binding region in CDV. Sites 478, 537 and 539 were all conserved. However, a CDV strain isolated in a Javelina (Family: Tyassuidae) from Denmark in 1995 showed 479W. This could be an indication of site 479 as significant in CDV spread to other mammals outside the order Carnivora, but will have to be substantiated with more data from non-carnivore hosts infected with CDV. As such our data supports Nikolin *et al.* (2016) in the notion that residues responsible for the binding of CDV to Nectin-4 have no influence on host adaptation.

Canine distemper virus is known as a monotypic virus, with only one serotype of the virus currently recognised (Bolt *et al.*, 1997). Thus a single exposure to the virus normally confers long-lasting immunity and the control of infection can be significantly minimised through vaccination. Several reports of vaccine 'failures' have however emerged amongst domestic dogs and several wildlife species (Feng *et al.*, 2016; Gordon *et al.*, 2015; Munson *et al.*, 2008) bringing into question the effectiveness of currently used vaccines. It is suggested that vaccines are partially compromised due to the genetic/antigenic differences between vaccine strains and wild-type isolates (Hashimoto *et al.*, 2001; Martella *et al.*, 2006; Pardo *et al.*, 2005; Si *et al.*, 2010). All sequenced CDV vaccines strains group within the America I lineage, apart from the Rockborn strain separating into a distinct Rockborn-like lineage

(Martella *et al.*, 2011). The South African CDV strains isolated in this study showed a nucleotide variation of 8-11% with vaccine strains in lineage America I, that included the commonly used vaccines Galaxy® DA2PPV (FJ461708.1) from Schering-Plough/Forte Dodge, Nobivac® DHPPI (FJ461701.1) and Nobivac® PuppyDP (FJ461709.1) from Intervet, and Canigen® DHPPI (FJ461710.1) from Virbac Animal Health (Woma *et al.*, 2010). The highest similarity in vaccinal strain identity with SA CDV wild-type strains was observed for the RockbornCandur strain (GU266280.1) and the Vanguard® Plus (FJ461702.1) strain, both grouping within the Rockborn-like lineage showing a 93-95% nucleotide identity. Our data supports the notion that CDV strains circulating in SA wildlife and domestic dogs are genetically distinct from commonly used vaccines.

In conclusion, the current study presents the first sequence data of CDV infections in Southern African wild carnivores. The presence of one CDV lineage circulating in SA is confirmed, with all wildlife isolates grouping within the Southern African lineage. The study also reveals two possible co-circulating sub-genotypes with a possible geographical pattern at regional level; however more data is needed to confirm this association. The importance of the amino acid residue combination at site 519 and 549 on the SLAM binding region of CDV H-gene in non-canid hosts is also revealed. Comparing wild-type CDV strains to vaccine strains currently in use in SA also showed clear genetic differences. Conclusions are, however, limited to available sequence data and in the South African lineage there is a clear bias towards CDV strains isolated in domestic dogs from one particular area. Further studies should thus include CDV strains isolated from various hosts from a wider geographical range in SA and vaccine efficacy should be tested by field trials.

5. Acknowledgment

The authors would like to acknowledge wildlife veterinarians Dr Peter Caldwell (Old Chapel Veterinary Clinic, Tshwane), and Dr Louis van Schalkwyk (State Veterinarian, Department of Agriculture, Forestry and Fisheries, Skukuza) for their invaluable contribution in knowledge and samples of the CDV outbreaks in South Africa. We also thank Welgevonden Nature Reserve, Tswalu Kalahari Reserve and SANParks for their permission to collect samples and data for this study. This study was funded by the National Zoological Gardens of South Africa and supported by the National Research Foundation (NRF) Professional Development Program.

Published in Mammalian Biology

Chapter IV

The role of Toll-like receptor polymorphisms in susceptibility to canine distemper virus

The role of Toll-like receptor polymorphisms in susceptibility to canine distemper virus

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Abstract

Canine distemper virus (CDV) has emerged as a significant global multi-host pathogen of wildlife, causing severe systemic disease. Host specificity and viral pathogenesis depend on the susceptibility of host cells to virus infection and CDVs ability to initiate an immune response. Toll-like receptors (TLR), as key recognition structures of the innate immune system, are able to distinguish between invading pathogens. To investigate host susceptibility to CDV, the presence of non-synonymous single nucleotide polymorphisms (SNPs) in the coding regions of TLR 2, 3, 4, 7 and 8 genes were investigated in two recent CDV outbreaks in South Africa. The first case consisted of five lions (*Panthera leo*), diagnosed with CDV by physical examination, histopathology, immunohistochemical staining and PCR amplification of the virus. Four of the lions died following exposure to the virus. The second case consisted of six African wild dogs (*Lycaon pictus*) with CDV and one surviving African wild dog. Analysis of TLR diversity showed a higher rate of polymorphism in the African wild dogs within each of the TLR loci compared to lions. A single amino acid change (Met527Thr) within the leucine rich repeat of TLR2 was observed in the single surviving lioness. This alteration resulted in a non-polar (M) to polar (T) group change, potentially influencing the expression and function of TLR2 which could result in an immune resistance to CDV infection. No specific amino acid variants could be associated with CDV susceptibility in the African wild dogs. This study provides a critical starting point in elucidating the mechanism involved in host immunity and therefore susceptibility towards CDV infection. Future studies

should include targeting a larger area of the TLR genes, increasing sample size and expression analyses.

Key words: Canine distemper virus, Toll-like receptors, wildlife diseases, immunology, host susceptibility

1. Introduction

Infectious diseases are increasingly recognised as a potential threat to the conservation and biological diversity of wildlife (Daszak *et al.*, 2000; Smith *et al.*, 2009). To fully understand this threat and to implement successful management strategies for wildlife populations, knowledge of host ecology, pathogen characteristics, and host-pathogen interactions are required (Joseph *et al.*, 2013). Information on the complex interactions between pathogen and host is however difficult to study and is often lacking for wildlife diseases. An example is the infectious viral disease canine distemper (CD) caused by the canine distemper virus (CDV; family *Paramyxoviridae*; genus *Morbillivirus*). First isolated from domestic dogs (*Canis familiaris*, family Canidae) in 1905 (Carré, 1905), CDV has subsequently been shown to infect a wide range of non-domestic carnivores, as well as some non-human primates (Beineke *et al.*, 2015). Canine distemper virus infection ranges from subclinical to severe systemic disease, characteristically exhibiting lympho-, neuro- and epitheliotropism (Beineke *et al.*, 2009; Iwatsuki *et al.*, 1995; von Messling *et al.*, 2004), resulting in the infection of the lymphatic, respiratory, endocrine, digestive, urinary, cutaneous, skeletal and central nervous systems (Lempp *et al.*, 2014; von Messling *et al.*, 2004). Survival of infection provides lifelong immunity in domestic dogs (Appel & Summers, 1995).

The ability of CDV to infect multiple species and its broad and expanding host range has been a significant area of research interest. The past two decades have accrued several publications on CDV's ability to infect a wide range of canine and non-canine carnivorous hosts with a focus on the mechanisms involved in viral entry of a host cell and how it relates to host range specificity (Cuthill & Charleston, 2013; Ludlow *et al.*, 2014; Nikolin *et al.*, 2012a; Ohishi *et al.*, 2014). Other factors influencing host range, such as the ability of a host to respond to viral infections have, however, not been explored in detail for CDV. Clinical and pathological characteristics of CDV infection in a variety of species largely resembles the disease in dogs, however, mortality and morbidity may vary greatly among different species infected (Beineke *et al.*, 2015). Observed differences in the infection rate are especially evident in felids, with only 49% of reported records of felid species infected with CDV presenting with clinical disease (Martinez-Gutierrez & Ruiz-Saenz, 2016).

Research on the immune responses in wildlife has thus far been generally conducted on the major histocompatibility complex (MHC), a multigene family crucial to the adaptive immune response of vertebrates (Acevedo-Whitehouse & Cunningham, 2006). However, immunity is

a complex system and studies have revealed that approximately half of genetic variability for resistance to infection is reliant on non-MHC immune-relevant genes such as cytokines and toll-like receptors (TLRs) (Castro-Prieto *et al.*, 2011; Jepson *et al.*, 1997). Toll-like receptor molecules are a first line of defence against a variety of pathogens, including bacteria, protozoa, fungi and viruses (Melchjorsen *et al.*, 2005; Uematsu & Akira, 2006). They can be expressed either on the cell surface or membrane compartments of immune (macrophages, dentritic cells, mast cells, eosinophils, neutrophils, B lymphocytes) and non-immune (epithelial, endothelium, cardio-myocytes and adipocytes) cells (Jin & Lee, 2008; Takeda *et al.*, 2003). Toll-like receptor genes encode Type I transmembrane glycoproteins consisting of cytoplasmic, transmembrane, and extracellular regions. The cytoplasmic region of the TLR is related to the interleukin-1 receptor family, designated Toll-IL-IR. These sequences are highly conserved between species and are required for initiating intracellular signalling and inducing biological responses towards specific microorganisms (Akira *et al.*, 2006; Kawai & Akira, 2005; Takeda *et al.*, 2003; Xu *et al.*, 2000). Leucine-rich repeats (LRRs) within the ectodomain of TLRs are responsible for directly interacting with microbes and microbial components (Uematsu & Akira, 2007).

Thirteen mammalian TLR members have been identified, each responsible for selectively recognising distinct invariant microbial structures (Hopkins & Sriskandan, 2005). Of these, only six TLRs have been implicated in viral recognition in mammals by means of distinctive pathogen-associated molecular patterns that include glycoproteins (TLR2, TLR4), double stranded RNA (TLR3), single stranded RNA (TLR7, TLR8) and unmethylated viral CpG DNA (TLR9) (Boehme & Compton, 2004; Mogensen & Paludan, 2005). Polymorphisms in TLR genes are associated with the variability of a hosts immune response against specific pathogens (Bharti *et al.*, 2014; Bochud *et al.*, 2007; Heng *et al.*, 2011; Saçkesen *et al.*, 2005; Xue *et al.*, 2010). TLR2 is able to initiate an immune response by recognising glycoproteins from various viruses including measles virus (MeV), human cytomegalovirus and herpes simplex virus type 1 (Bieback *et al.*, 2002; Compton *et al.*, 2003; Kurt-Jones *et al.*, 2004), whereas West Nile virus triggers an inflammatory response via TLR3 (Wang *et al.*, 2004). TLR4 was shown to be involved in the innate immunity of mice to respiratory syncytial virus (Haynes *et al.*, 2001; Kurt-Jones *et al.*, 2000), while TLR7 and TLR8 are able to detect viral guanosine- and uridine-rich single stranded RNA of Sendai virus, human immunodeficiency virus and influenza virus (Beignon *et al.*, 2005; Diebold *et al.*, 2004; Heil *et al.*, 2003; Melchjorsen *et al.*, 2005). TLR9 has been demonstrated to induce antiviral responses via CpG

DNA of viruses such as herpes simplex virus type 1 and type 2 and murine cytomegalovirus (Lund *et al.*, 2003; Tabeta *et al.*, 2004).

No published studies have investigated the involvement of TLRs in the immune response of animals susceptible to CDV. The aim of this study was to characterise viral-associated TLRs using samples obtained from two separate case and control groups from recent CDV outbreaks in lion and African wild dog populations in South Africa. We hypothesise that single nucleotide polymorphisms (SNPs), which potentially influence the expression and function of TLRs, contribute to differential infection outcomes.

2. Materials and methods

2.1. Samples

All biological materials used in the present study were collected for diagnostic purposes and were stored at the Biobank of the National Zoological Gardens of South Africa (NZG). Samples from two case studies of recent CDV outbreaks from two privately owned reserves within South Africa were included. These consisted of a pride of lions from Welgevonden Nature reserve and a single pack of African wild dogs from Tswalu Kalahari Reserve that succumbed to CDV in December 2015 to May 2016. Ethical approval was obtained from the Animal Ethics Committee (V072-14), University of Pretoria, South Africa and the NZG Research, Ethics and Scientific Committee (P14/26). Samples were obtained under a Section 20 permit from the Department of Agriculture, Forestry and Fisheries, South Africa.

2.2. Selection of TLR and primers

Toll-like receptors were selected based on their known involvement in viral recognition in mammals and included TLR2, TLR3, TLR4, TLR7 and TLR8 (Haynes *et al.*, 2001; Kurt-Jones *et al.*, 2000; Wang *et al.*, 2004). Three of these TLRs (TLR2, TLR7 and TLR8) have additionally been reported to be involved in human measles virus (MeV) infection (Bieback *et al.*, 2002; Clifford *et al.*, 2012). Canine distemper virus shares clinicopathological features with the paramyxovirus MeV (de Vries *et al.*, 2014) and although TLRs have been studied for MeV, they have not yet been characterised for CDV. Primers previously developed for use in felids and hyenids (Flies *et al.*, 2014; Ignacio *et al.*, 2005) were used (Appendix B, Table B.1).

2.3. Genomic DNA isolation, amplification, and sequencing

Genomic DNA from blood and tissue samples was extracted using the MagMAX^{TM}-96 DNA Multi-Sample kit (Ambion), according to the manufacturer's protocol. Conventional polymerase chain reaction (PCR) was carried out at an annealing temperature of 53-58°C using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.) Successful PCR products were subsequently purified with Exonuclease I and FastAP (Thermo Fisher Scientific Inc.). Purified gene fragments were sequenced, in both the forward and reverse directions, using the BigDye Terminator v3.1 Cycle sequencing kit and visualised on a 3500 Genetic Analyser (Applied Biosystems). Sequence chromatograms were edited and assembled in BioEdit Sequence Alignment Editor v7.0.9.0 (Hall, 1999).

2.4 Identification of SNPs

Synonymous and non-synonymous SNP variations were determined by translating the TLR gene nucleotide sequences to the longest open reading frames. The identity and integrity of the respective amino acid sequences were confirmed by standard protein BLAST (blastp as implemented on the National Centre for Biotechnology Information platform). Amino acid variations were visually inspected using BioEdit v.7.0.9.0 (Hall, 1999).

2.5 Identification of polymorphisms associated with canine distemper virus

The possible association between TLR non-synonymous SNPs and differential infection outcomes was assessed in lions and African wild dogs during a CDV outbreak. Diagnosis of CDV was made on the basis of typical clinical signs and histopathology, immunohistochemical staining of formalin fixed paraffin embedded samples and PCR amplification of the H-gene of the virus (data not shown). In December 2015, the carcasses of three lions were observed on the Welgevonden reserve. Upon post mortem examination no clear cause of death could be determined. However, in the weeks that followed other lions in the reserve were observed with severe seizures (a neurological symptom associated with CDV). Blood tests and post mortem analyses confirmed the presence of CDV. One lioness showed no clinical signs and has consistently tested negative for CDV, however, serological evidence suggested that she had been exposed to the virus. She was kept in isolation and monitored closely for two months before being released back into the reserve after all subsequent tests (including the screening of cerebrospinal fluid) were all negative for CDV. None of the lions had been vaccinated against CDV. In the second case , three African wild dogs from Tswalu Kalahari Reserve succumbed to CDV infection and one survived even

though all four wild dogs had been previously vaccinated against CDV with Recombitek® C4/CV [Merial]. A case was defined as an individual showing clinical signs of infection and that was confirmed positive for current CDV infection. A control was defined as an individual exposed to CDV without clinical signs and negative tests for current CDV infection. Observed amino acid changes were assessed in both case/control groups to determine if these (1) had functional consequences (alteration in TLR structure); (2) were found in LRR regions (http://www.lrrfinder.com); and (3) were present/absent in control versus case individuals.

3. Results

Partial gene/DNA sequences for the five TLRs of samples of all species were successfully amplified and included: TLR2 (166 bp), TLR3 (256 bp), TLR4 (208 bp), TLR7 (172 bp) and TLR8 (167 bp). All loci consisted of unique polymorphic sites. The wild dog showed the highest rate of polymorphism across all loci with 80 (non-synonymous: 45, synonymous: 35) observed variants. TLR4 had the highest rate of non-synonymous SNPs in the wild dog population (n=23), followed by TLR2 (n=8), TLR3 (n=8) and TLR7 (n=5). The lowest rate of polymorphisms was observed in TLR8 (n=1). The lion showed lower rates of polymorphisms across all loci with two non-synonymous and one synonymous alteration observed in TLR2 and TLR3. No polymorphisms were observed in TLR4, TLR7 and TLR8 (Table 4.1).

Table 4.1. Polymorphisms in carnivore TLRs. Synonymous SNPs indicated inside of brackets and non-synonymous SNPs in the coding regions indicated outside brackets.

Species	n	TLR2	TLR3	TLR4	TLR7	TLR8
Wild dog	7	8 (12)	8 (6)	23 (15)	5 (2)	1 (0)
Lion	3	1 (1)	1 (0)	0 (0)	0 (0)	0 (1)

Overall, we detected 39 amino acid substitutions in the two species across all loci (Table 4.2). Substitutions often (35.9%) involved a non-polar to non-polar amino acid change. Substitutions resulting in a change in amino acid properties mostly consisted of a change from non-polar to polar (12.8%) and polar to basic (12.8%). Leucine-rich repeat regions were identified in TLR2 and TLR4. A single methionine>threonine (M527T) variant was observed in TLR2 that was present in the CDV positive lions and was absent in the control lion (Figure

4.1). An absence of specific amino acid variants that may be associated with CDV was observed in the African wild dog case and control group (results not shown).

Table 4.2. Amino acid deviations in TLRs of carnivores naturally infected with CDV. LRRs: Leucine Rich Repeats

Locus	Species	Amino acid change	Group change	Structural influence	Present in cases and not controls
			Within LRRs		
TLR2	Wild dog	Leu>Val	non-polar>non-polar	No	No
		Thr>Ala	polar>non-polar	Yes	No
		Gln>Thr	polar>polar	No	No
	Lion	Met>Thr	non-polar>polar	Yes	Yes
TLR4	Wild dog	Lys>Asp/Arg	basic>acidic/basic	Yes/No	No
		Gln>His	polar>basic	Yes	No
		Leu>Ile	non-polar>non-polar	No	No
		Asn>His/Ser	polar>basic/polar	Yes/No	No
			Outside LRRs		
TLR2	Wild dog	Ala>Thr	non-polar>polar	Yes	No
		His>Gln	basic>polar	Yes	No
		Leu>Ile	non-polar>non-polar	No	No
TLR3	Wild dog	Pro>Ala	non-polar>non-polar	No	No
		Ile>Leu	non-polar>non-polar	No	No
		Val>Ile	non-polar>non-polar	No	No
		Thr>Ile	polar>non-polar	Yes	No
		Ile>Thr	non-polar>polar	Yes	No
		Ile>Val	non-polar>non-polar	No	No
	Lion	Tyr>His	polar>basic	Yes	No
TLR4	Wild dog	Leu>Ser/Phe	non-polar>polar/non-polar	Yes/No	No
		Asp>Asn	acidic>polar	Yes	No
		Lys>Met/Thr	basic>non-polar/polar	Yes	No
		Glu>Gly	acidic>non-polar	Yes	No
		Gln>Arg	polar>basic	Yes	No
		Ala>Glu/Val	non-polar>acidic/non-polar	Yes/No	No
		Ala>Ser	non-polar>polar	Yes	No
		His>Val/Asn	basic>non-polar/polar	Yes	No
		Leu>Phe	non-polar>non-polar	No	No
		Pro>Ala	non-polar>non-polar	No	No
TLR7	Wild dog	Arg>Lys	basic>basic	No	No
		Val>Leu	non-polar>non-polar	No	No
		Ile>Val	non-polar>non-polar	No	No
TLR8	Wild dog	Phe>Ile	non-polar>non-polar	No	No
	Lion	Tyr>His	polar>basic	Yes	No

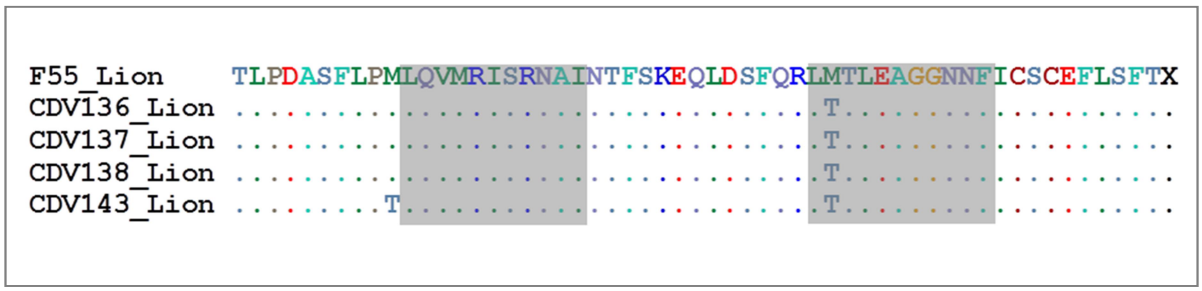

Figure 4.1. Partial TLR2 alleles of CDV-infected African lion aligned to a CDV-negative African lioness (F55_Lion). Identical amino acids are indicated with a dash, varying amino acids are indicated by single letter amino acid codes. Leucine rich repeats (LRR) are highlighted.

4. Discussion

4.1. Species differences in Toll-like receptor diversity

To our knowledge this is the first report on TLR polymorphism and diversity in lion and African wild dog. African wild dogs are reported to be highly susceptible to diseases of common sympatric canids such as domestic dog and jackal (Marsden *et al.*, 2009; Woodroffe *et al.*, 2012). When exposed to CDV, African wild dogs have been observed to be highly susceptible to developing signs and succumbing to infection (Van De Bildt *et al.*, 2002; Durchfeld *et al.*, 1990; Goller *et al.*, 2010). During an outbreak in the Serengeti-Mara ecosystem of East Africa in 1994 approximately one third of the lion population died or disappeared (Munson *et al.*, 2008; Roelke-Parker *et al.*, 1996). Subsequently several more instances of CDV infections in lions have been reported; most recently (2015/2016) in the Waterberg area of South Africa.

Low TLR diversity observed in the study presented here in lion was expected. Lack in genetic diversity of lions is enhanced by the growing number of lions in peripherally isolated populations or in wildlife parks with little to no gene flow (Bertola *et al.*, 2011). However, in contrast to lions, the African wild dog consistently showed the highest rate of polymorphism within each of the TLR loci (Tables 4.1, 4.2) even though both species were from single populations. African wild dogs are amongst the most endangered carnivores in Africa with <8000 individuals remaining in the wild (IUCN/SSC, 2009). Formally widely distributed across sub-Saharan Africa, their populations have been at a dramatic declined due to increased habitat loss, hunting and fragmentation. This has led to a decrease in gene flow which is reflected in their limited genetic variation in their allozymes and microsatellites

(Girman *et al.* 2001). In addition, Marsden et al. (2009) reported that African wild dogs are genetically depauperate at MHC genes in comparison to other canid species. The incongruity in levels of genetic diversity at adaptive loci for the MHC loci and TLRs in wild dogs warrants further investigation. The discrepancy may, however, have arisen due to different ecological and evolutionary forces driving variation at these loci. It has been previously reported that diversifying (or positive) selection plays a role in the evolution of MHC variability (Apanius *et al.*, 1997), whereas in TLRs, purifying (or negative) selection is dominant with specific sites displaying diversifying selection (Fornůsková *et al.*, 2013). In addition, pathogen mediated selection may play a role in variation at TLR loci (Basu *et al.*, 2010).

Non-synonymous SNPs identified in the various TLRs were compared between CDV-infected lions and a CDV-negative lioness. A single methionine>threonine (M527T) variant was observed in TLR2 (Figure 4.1). A hydrophobic, non-polar amino acid (M) was observed for the control group (F55_Lion), whereas the affected lions exhibited a hydrophilic, polar amino acid (T). This mutation falls within an identified LRR motif. The highly conserved 11-residue hallmark sequence LxxLxLxxNxL (with 'x' being any amino acid) is a defining feature of a LRR region (Matsushima *et al.*, 2007). The variable 'x' residues are hydrophilic and exposed to the concave surface of the horseshoe-like structure responsible for directly interacting with microbes and microbial components (Uematsu & Akira, 2007; Werling *et al.*, 2009). The observed M527T variable was found at the first 'x' position of the LRR motif. The observed amino acid change could influence the protein structure. In contrast, no specific amino acidvariants could be associated with CDV susceptibility in the African wild dogs and there were no clear differences in TLRs between the African wild dogs that succumbed to CDV and the one that survived.

5. Conclusion

In conclusion, our study provides evidence of a possible role of an alteration in TLR2 and differences in CDV outcomes in lions. However, variations at adaptive loci not tested in this study may play an additional role. Immunity should also not be regarded as an unambiguous event and depends on several factors, including recognition of cell surface structures, intensity of exposure to the pathogen, prior vaccination and the generation of a specific and definitive immune response (Acevedo-Whitehouse & Cunningham, 2006). In addition, it is not known if genetic variants in these TLR genes are functional or specifically affect the host

response to CDV. As experimental exposure to pathogens is not always possible or ethical for wildlife species, it is difficult to measure differential levels of disease resistance. Sample size of the number of individuals surviving or dying from exposure to disease is also generally small. Evidence presented in this study merits further consideration and future studies should include targeting a larger area of the TLR genes, increasing sample size and expression analyses. This study, however, provides a critical starting point in elucidating the mechanism involved in host immunity towards CDV infection.

6. *Acknowledgements*

The authors would like to acknowledge wildlife veterinarians Dr Peter Caldwell (Old Chapel Veterinary Clinic, Tshwane), and Dr Louis van Schalkwyk (State Veterinarian, Department of Agriculture, Forestry and Fisheries, Skukuza) for their invaluable contribution in knowledge regarding the CDV outbreaks in South Africa. We also thank Welgevonden Nature Reserve and Tswalu Kalahari Reserve for their permission to collect samples and data for this study. This study is supported by the National Zoological Gardens of South Africa and funded by the National Research Foundation (NRF) Professional Development Program.

Chapter V:

General conclusion

For the effective control of infectious diseases in wildlife, sufficient epidemiological information on the disease and agent are required (Goller et al., 2010; Smith et al., 2006). This becomes even more important when considering the conservation of endangered species. Canine distemper virus (CDV) is an emerging infectious disease posing a serious threat to several captive and free-ranging wildlife populations. Outbreaks of CDV have been confirmed in several species worldwide including highly endangered species such as the Ethiopian wolf (*Canis simensis*), Amur tiger (*Panthera tigris altaica*) and African wild dog (*Lycaon pictus*) (Gordon et al., 2015; Martinez-Gutierrez and Ruiz-Saenz, 2016; Seimon et al., 2013). Current data on the epidemiology of CDV in wildlife is, however, not sufficient to apply effective disease control strategies and with each new outbreak, this lack of knowledge on the extent of CDV susceptibility in wildlife species is increasingly emphasised.

The aim of the present study was thus to investigate host receptors influencing susceptibility to CDV infection, with a specific focus on selected wild carnivores in South Africa (SA). This aim was achieved by:

- Obtaining the whole genome sequence analyses of three CDV vaccines (Nobivac, Onderstepoort and Bucharest) and two wild-type strains isolated from African wild dog and spotted hyena (*Crocuta crocuta*) (Chapter II).

- Determining the phylogenetic analysis of the H-gene region of CDV isolated from four different wildlife species, including lion (*Panthera leo*), African wild dog, spotted- and brown hyena (*Hyaena brunnea*), obtained from three different areas in SA (Chapter III).

- Investigating the involvement of Toll-like receptors (TLR) in the immune response of lions and African wild dog populations to CDV infection (Chapter IV).

Until recently CDV research in SA had only been focused on infections in domestic dogs, with information on wildlife infections greatly lacking. The present study resulted in the first report on genetic evidence of CDV isolated from clinical samples from various wildlife species in SA. It also resulted in the first genomic sequences of CDV in SA. The phylogenetic study showed, in combination with results obtained by Woma *et al*., (2010) on CDV in domestic dogs, the presence of one CDV lineage circulating in SA. Additionally, a possible geographical pattern at regional level was observed with two co-circulating sub-genotypes of CDV identified. When compared to current vaccine strains, CDV isolates from

SA showed clear genetic differences, suggesting the possible value of formulation of new and updated vaccines for use in especially wildlife in SA.

Furthermore, the present study resulted in the first report of the H-gene encoding protein in CDV isolates from SA, and the amino acid regions thought to be responsible for attachment to the host cellular receptors SLAM and Nectin-4. This is also the first report on the role of TLRs in the susceptibility of various carnivores to CDV infection. Results of the study revealed the importance of the amino acid residue combination at site 519 and 549 on the SLAM binding region of CDV H-gene in non-canid hosts. Residues responsible for the binding of CDV to Nectin-4, however, did not seem to have an influence on host adaptation. The study further provided evidence of a possible role of TLR2 in the outcome of CDV infection in lions.

The data obtained gives a good indication of the diversity and prevalence of CDV in South African wild carnivores and allows for a better understanding of the host range and strain diversity of CDV in SA. Various deficiencies and/or challenges in the study were, however, identified and ranged from the availability of adequate samples in wildlife, to the difficulty in isolation and characterisation of a single-stranded RNA virus such as CDV. At the onset of this study a general lack in knowledge of handling and storage of samples for RNA research was observed. Fresh and frozen samples are the preferred sample source for RNA extraction and subsequent molecular work. However, these are not always easily obtained especially in situations with a lack of cold-chain facilities such as in the field and where laboratory equipment is scarce. Additionally, as CDV is not a notifiable disease in SA, disease outbreaks on wildlife reserves are often not reported. As a result the acquisition of appropriate samples for the isolation of CDV in this study was difficult. These challenges can largely be overcome through researchers communicating with conservation agencies and veterinarians on various platforms, educating them on the disease and the appropriate handling of samples. This was in part achieved during the current study, through the presentation of the research at conferences and engaging with various wildlife veterinarians.

In light of these findings, it is suggested that future considerations in terms of CDV research in SA should include:

- obtaining CDV strains isolated from various hosts from a wider geographical range in SA.

- developing methods to use retrospective samples, such as formalin-fixed paraffin-embedded tissues, as a potential alternative source for molecular diagnostics and pathogen identification.
- determining the effect of current CDV vaccines on wildlife by means of field trials and testing antibody responses.
- expanding on the variations observed in the TLR analyses by targeting a larger area of the TLR genes, increasing sample size and performing expression analyses.
- investigating the host specificity of CDV with relation to the involvement of Nectin-4 as epithelial receptor in felids.

Results of this study have been published and/or submitted to various journals and include the most recent review on CDV in wildlife (published in Journal of General Virology), the first whole genome sequence of CDV strains in SA (published in Genome Announcements), the phylogenetic relationship of CDV in SA wildlife (submitted to Journal of General Virology) and the role of TLR polymorphisms in CDV susceptibility (submitted to Molecular Immunology).

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ETHICS REPORTS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

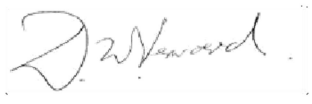
PROJECT TITLE	**Molecular characterization of canine distemper, parvo- and corona viruses in wild carnivores of South Africa**
PROJECT NUMBER	**V072-14 (REVISED)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**AE Switala**

STUDENT NUMBER (where applicable)	**043 616095**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Carnivores (8 species)**	
NUMBER OF ANIMALS	**200 of each species**	
Approval period to use animals for research/testing purposes		**October 2014-October 2015**
SUPERVISOR	**Prof. EH Venter**	

KINDLY NOTE:

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

APPROVED	Date	27 October 2014
CHAIRMAN: UP Animal Ethics Committee	Signature	

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NZG/RES/P/001/F/07

09 December 2014

Ms Angelika Switala
National Zoological Gardens of South Africa
PO Box 754
Pretoria
0001

Dear Ms Angelika Switala

APPROVAL OF RESEARCH PROPOSAL

This letter serves to inform you that your research proposal “Molecular characterization of canine distemper, parvo- and corona viruses in wild carnivores of South Africa” has been approved by the NZG Research Ethics and Scientific Committee (RESC) on November 26, 2014 with the following provisos:

1. Inform the RESC of completion or termination (with reason) of the research at the NZG.
2. Submission of an annual progress report on request. Failure to submit a progress report may result in approval to be withdrawn.
3. Submission of a written request for an extension or for any changes within the research project.
4. Acknowledgement of the NZG in all research outputs emanating from this research project (please include PDF documents of all publications).
5. Submission of a final report on completion of the study.

The research proposal has been registered on the database as P14/26. Please use this number in all future correspondence.

Thank you for making use of the NZG as a research platform.

Yours sincerely

Prof Antoinette Kotze
Chair: NZG Research Ethics & Scientific Committee

APPENDICES

Appendix A

Table A1. H-gene sequence isolates used in determining the phylogenetic relationship of Canine distemper virus. The accession number, host species, year and country of origin (when available) are indicated for each strain. South African strains isolated for this study indicated with asterisk (*)

Sample name	Lineage	Family	Species
*WT02/SpottedHyena/Waterberg/2016/SA	Southern Africa	Hyenidae	Spotted Hyena
*Z15/AWD/Tswalu/SA	Southern Africa	Canidae	African wild dog
*Z9/AWD/Tswalu/SA	Southern Africa	Canidae	African wild dog
*Z2/AWD/Kruger/SA	Southern Africa	Canidae	African wild dog
*Z13/AWD/Kruger/SA	Southern Africa	Canidae	African wild dog
*Z1/AWD/Kruger/SA	Southern Africa	Canidae	African wild dog
*Z11/AWD/Kruger/SA	Southern Africa	Canidae	African wild dog
*Z4/BrownHyena/Welg/SA	Southern Africa	Hyenidae	Brown Hyena
*Z6/Lion/Welg/SA	Southern Africa	Felidae	Lion
*Z7/Lion/Welg/SA	Southern Africa	Felidae	Lion
*Z10/dog/Tswalu/2016/SA	Southern Africa	Canidae	Domestic dog
*WT01/AWD/Tswalu/2016/SA	Southern Africa	Canidae	African wild dog
FJ461723.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461698.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461718.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461722.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461704.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461706.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461721.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461695.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461697.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461693.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461703.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461715.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461714.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461699.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461716.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461719.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461720.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461713.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461705.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461696.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461724.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461707.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog

Sample name	Lineage	Family	Species
FJ461711.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461694.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461700.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461717.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
FJ461712.1/dog/2007/SA	Southern Africa	Canidae	Domestic dog
JN812975.1/lion/1994/Tanzania	East Africa	Felidae	Lion
KC916716.1/bat-earedfox/1994/Tanzania	East Africa	Canidae	Bat-eared fox
JN812976.1/dog/1994/Tanzania	East Africa	Canidae	Domestic dog
KC916715.1/AWD/2007/Tanzania	East Africa	Canidae	African wild dog
KC916714.1/goldenjackal/2011/Tanzania	East Africa	Canidae	Golden Jackal
KC916717.1/spottedhyena/1994/Tanzania	East Africa	Hyenidae	Spotted Hyena
Z47760.1//Greenlandic/dog/1995/Denmark	Arctic-like	Canidae	Domestic dog
Z47763.1/blackleopard/1995/Denmark	America II	Felidae	Black leopard
Z47765.1/raccoon/1995/Denmark	America II	Procyonidae	Raccoon
Z47764.1/javelina/1995/Denmark	America II	Tayassuidae	Javelina
Z47762.1/dog/1995/Denmark	America II	Canidae	Domestic dog
HM563057.1/wolf/Portugal/1998	Europe	Canidae	Wolf
HM563058.1/wolf/2008/Portugal	Europe	Canidae	Wolf
HM563059.1/dog/2007/Portugal	Europe	Canidae	Domestic dog
Z54156.1/Chineseleopard/1995/Netherlands	America II	Felidae	Chinese leopard
FJ416339.1/fox/2008/Germany	Europe	Canidae	Fox
FJ416337.1/fox/2008/Germany	Europe	Canidae	Fox
FJ416336.1/fox/2008/Germany	Europe	Canidae	Fox
FJ416338.1/badger/2008/Germany	Europe	Mustelidae	Badger
JN153020.1/raccoon/2007/Germany	Europe Wildlife	Procyonidae	Raccoon
JN153021.1/raccoon/2007/Germany	Europe Wildlife	Procyonidae	Raccoon
JN153023.1/raccoon/2007/Germany	Europe Wildlife	Procyonidae	Raccoon
JN153019.1/raccoon/2007/Germany	Europe Wildlife	Procyonidae	Raccoon
JN153022.1/raccoon/2007/Germany	Europe Wildlife	Procyonidae	Raccoon
JN153025.1/redfox/2008/Germany	Europe	Procyonidae	Raccoon
JN153024.1/redfox/2008/Germany	Europe	Procyonidae	Raccoon
GQ214373.2/dog/2003/Austria	Arctic-like	Canidae	Domestic dog
GQ214374.2/badger/2006/Austria	Europe Wildlife	Mustelidae	Badger
GQ214369.2/stonemartin/2007/Austria	Europe Wildlife	Mustelidae	Stone martin
GQ214376.2/dog/2002/Austria	Europe	Canidae	Domestic dog
GQ214378.2/dog/2002/Austria	Europe	Canidae	Domestic dog
GQ214384.2/dog/2002/Austria	Europe	Canidae	Domestic dog
GQ214380.2/dog/2002/Austria	Europe	Canidae	Domestic dog
DQ226088.1/dog/2005/Italy	Arctic-like	Canidae	Domestic dog
DQ226087.1/dog/2005/Italy	Arctic-like	Canidae	Domestic dog
DQ228166.1/dog/2005/Italy	Europe Wildlife	Canidae	Domestic dog
DQ494317.1/dog/2006/Italy	Europe	Canidae	Domestic dog
DQ494319.1/dog/2006/Italy	Europe	Canidae	Domestic dog
HM120874.1/redfox/2009/Italy	Europe	Canidae	Red fox

Sample name	Lineage	Family	Species
DQ494318.1/dog/2006/Italy	Europe	Canidae	Domestic dog
GU001863.1/Iberianlynx/2005/Spain	Europe	Felidae	Iberian lynx
GU001864.1/Iberianlynx/2005/Spain	Europe	Felidae	Iberian lynx
DQ889177.1/dog/2006/Hungary	Europe	Canidae	Domestic dog
AY542312.2/racoon/2004/USA	America I	Procyonidae	Raccoon
AY445077.2/raccoon/2004/USA	America I	Procyonidae	Raccoon
AY466011.2/raccoon/2004/USA	America I	Procyonidae	Raccoon
AY526496.1/raccoon/2004/USA	America II	Procyonidae	Raccoon
AY438597.1/raccoon/2003/USA	America II	Procyonidae	Raccoon
AY498692.1/raccoon/2003/USA	America II	Procyonidae	Raccoon
AY465925.1/raccoon/2003/USA	America II	Procyonidae	Raccoon
AY649446.1/raccoon/2004/USA	America II	Procyonidae	Raccoon
AY548111.1/racoon/2004/USA	America I	Procyonidae	Raccoon
AY548110.1/racoon/2004/USA	America I	Procyonidae	Raccoon
AY548109.1/racoon/2004/USA	America I	Procyonidae	Raccoon
AY964114.1/dog/2005/USA	Rockborn-like	Canidae	Domestic dog
AY964112.1/dog/2005/USA	Arctic-like	Canidae	Domestic dog
AY964108.1/dog/2005/USA	Arctic-like	Canidae	Domestic dog
AY964110.1/dog/2005/USA	South America II	Canidae	Domestic dog
FJ392652.1/dog/2003/Argentina	South America I / Europe	Canidae	Domestic dog
FJ392651.1/dog/2005/Argentina	South America II	Canidae	Domestic dog
KC257464.1/dog/2010/Argentina	South America II	Canidae	Domestic dog
FJ011005.1/dog/2005/Argentina	South America II	Canidae	Domestic dog
JN215476.1/dog/2009/Uruguay	South America I / Europe	Canidae	Domestic dog
JN215475.1/dog/2008/Uruguay	South America I / Europe	Canidae	Domestic dog
JN215473.1/dog/2007/Uruguay	South America I / Europe	Canidae	Domestic dog
JN215477.1/dog/2009/Uruguay	South America I / Europe	Canidae	Domestic dog
JN215474.1/dog/2008/Uruguay	South America I / Europe	Canidae	Domestic dog
EU098105.1/dog/2007/Brazil	South America I / Europe	Canidae	Domestic dog
EU098103.1/dog/2007/Brazil	South America I / Europe	Canidae	Domestic dog
EU098104.1/dog/2007/Brazil	South America I / Europe	Canidae	Domestic dog
EU098102.1/dog/2007/Brazil	South America I / Europe	Canidae	Domestic dog
HQ403645.1/dog/2009/China	America I	Canidae	Domestic dog
EF445052.1/fox/2007/China	Arctic-like	Canidae	Fox
GQ332531.1/dog/2008/China	America I	Canidae	Domestic dog
JN381191.1/dog/2011/China	Asia I	Canidae	Domestic dog
EF445053.1/fox/2007/China	Asia I	Canidae	Fox

Sample name	Lineage	Family	Species
FJ405223.1/monkey/2008/China	Asia I	Primates	Monkey
FJ405224.1/monkey/2008/China	Asia I	Primates	Monkey
EU325721.1/fox/2007/China	Asia I	Canidae	Fox
HM448829.1/fox/2009/China	Asia I	Canidae	Fox
HM448831.1/fox/2009/China	Asia I	Canidae	Fox
HM448832.1/raccoondog/2009/China	Asia I	Canidae	Raccoon dog
FJ810213.1/fox/2008/China	Asia I	Canidae	Fox
EU325728.1/raccoondog/2007/China	Asia I	Canidae	Raccoon dog
EU564813.1/dog/2007/China	Asia I	Canidae	Domestic dog
EU564812.1/dog/2007/China	Asia I	Canidae	Domestic dog
EU684265.1/dog/2007/China	Asia I	Canidae	Domestic dog
DQ922630.1/fox/2006/China	Asia I	Canidae	Domestic dog
GQ332530.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ409464.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ851458.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ851452.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ848530.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ851456.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ535063.1/dog/2008/China	Asia I	Canidae	Domestic dog
GQ332535.1/dog/2008/China	Asia I	Canidae	Domestic dog
GQ332533.1/dog/2008/China	Asia I	Canidae	Domestic dog
HM623891.1/dog/2009/China	Asia I	Canidae	Domestic dog
HM623893.1/dog/2009/China	Asia I	Canidae	Domestic dog
EU325724.1/mink/2007/China	Asia I	Mustelidae	Mink
FJ851454.1/dog/2008/China	Asia I	Canidae	Domestic dog
EU325723.1/mink/2007/China	Asia I	Mustelidae	Mink
EU379560.1/mink/2007/China	Asia I	Mustelidae	Mink
EU325720.1/fox/2007/China	Asia I	Canidae	Fox
HM448834.1/fox/2009/China	Asia I	Canidae	Fox
EU934233.1/raccoondog/2006/China	Asia I	Canidae	Raccoon dog
EU325722.1/fox/2006/China	Asia I	Canidae	Domestic dog
EU325726.1/raccoondog/2006/China	Asia I	Canidae	Raccoon dog
FJ851450.1/dog/2008/China	Asia I	Canidae	Domestic dog
EF445051.1/fox/2007/China	Asia I	Canidae	Fox
EF042818.1/raccoondog/2006/China	Asia I	Canidae	Raccoon dog
EU325729.1/raccoondog/2007/China	Asia I	Canidae	Raccoon dog
FJ848536.1/dog/2008/China	Asia I	Canidae	Domestic dog
GQ332534.1/dog/2008/China	Asia I	Canidae	Domestic dog
HQ850147.1/dog/2008/China	Asia I	Canidae	Domestic dog
HM623895.1/dog/2009/China	Asia I	Canidae	Domestic dog
HM448833.1/raccoondog/2009/China	Asia I	Canidae	Raccoon dog
HM448830.1/raccoondog/2009/China	Asia I	Canidae	Raccoon dog
JF343962.1/dog/2009/China	Asia I	Canidae	Domestic dog
HM749644.1/dog/2009/China	Asia I	Canidae	Domestic dog

Sample name	Lineage	Family	Species
FJ851455.1/dog/2008/China	Asia I	Canidae	Domestic dog
EU325731.1/mink/2007/China	Asia I	Mustelidae	Mink
EU325730.1/raccoondog/2007/China	Asia I	Canidae	Raccoon dog
FJ810215.1/fox/2008/China	Asia I	Canidae	Fox
EF445054.1/raccoondog/2007/China	Asia I	Canidae	Raccoon dog
EU325727.1/raccoondog/2007/China	Asia I	Canidae	Raccoon dog
EU325725.1/mink/2006/China	Asia I	Mustelidae	Mink
HQ128601.1/raccoondog/2010/China	Asia I	Canidae	Raccoon dog
HQ128600.1/dog/2010/China	Asia I	Canidae	Domestic dog
HQ128599.1/dog/2010/China	Asia I	Canidae	Domestic dog
FJ848534.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ848535.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ848531.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ848533.1/dog/2008/China	Asia I	Canidae	Domestic dog
HQ657209.1dog/2010/China	Asia I	Canidae	Domestic dog
FJ848532.1/dog/2008/China	Asia I	Canidae	Domestic dog
GQ332532.1/dog/2008/China	Asia I	Canidae	Domestic dog
AF178038.1/gaintpanda/China	Rockborn-like	Ursidae	Gaint Panda
AF178039.1/LesserPanda/China	Rockborn-like	Ailuridae	Lesser Pada
FJ810214.1/raccoondog/2008/China	Asia I	Canidae	Raccoon dog
EU716075.1/dog/2007/SouthKorea	Asia II	Canidae	Domestic dog
EU716074.1/marten/1998/SouthKorea	Asia II	Mustelidae	Marten
EU716073.1/dog/1997/SouthKorea	Asia II	Canidae	Domestic dog
EU716072.1/dog/2007/SouthKorea	Asia I	Canidae	Domestic dog
AB025270.1/dog/1999/Japan	Asia II	Canidae	Domestic dog
AB040767/dog/2000/Japan	Asia II	Canidae	Domestic dog
AB605890.1/raccoondog/2008/Japan	Asia I	Canidae	Raccoon dog
AB619774.1/tiger/2010/Japan	Asia I	Felidae	Tiger
AB619775.1/raccoondog/2009/Japan	Asia I	Canidae	Raccoon dog
AB605891.1/raccoondog/2007/Japan	Asia I	Canidae	Raccoon dog
AB025271.2/dog/1999/Japan	Asia I	Canidae	Domestic dog
FJ851453.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ851451.1/dog/2008/China	Asia I	Canidae	Domestic dog
FJ851457.1/dog/2008/China	Asia I	Canidae	Domestic dog
EU296492.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
EU296491.1/dog/2006/Taiwan	Asia I	Canidae	Domestic dog
EU296493.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
EU296490.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
EU296485.1/dog/2006/Taiwan	Asia I	Canidae	Domestic dog
FJ705234.1/dog/2008/Taiwan	Asia I	Canidae	Domestic dog
EU296486.1dog/2006/Taiwan	Asia I	Canidae	Domestic dog
DQ191175.1/dog/2004/Taiwan	Asia I	Canidae	Domestic dog
EU296481.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
EU296482.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog

Sample name	Lineage	Family	Species
EU296484.1/dog/2006/Taiwan	Asia I	Canidae	Domestic dog
EU296483.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
FJ705231.1/dog/2008/Taiwan	Asia I	Canidae	Domestic dog
FJ705232.1/dog/2008/Taiwan	Asia I	Canidae	Domestic dog
FJ705230.1/dog/2008/Taiwan	Asia I	Canidae	Domestic dog
EU296488.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
EU296489.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
EU296494.1/dog/2007/Taiwan	Asia I	Canidae	Domestic dog
EU296487.1/dog/2005/Taiwan	Asia I	Canidae	Domestic dog
FJ705233.1/dog/2008/Taiwan	Asia I	Canidae	Domestic dog
FJ705236.1/dog/2008/Taiwan	Asia I	Canidae	Domestic dog
FJ705235.1/dog/2008/Taiwan	Asia I	Canidae	Domestic dog
FJ461710.1/CDV/Canigen	America I		
FJ461709.1/CDV/NobivacPuppyDP	America I		
FJ461708.1/CDV/GalaxyDA2PPV	America I		
FJ461702.1/CDV/VanguardPlus	America I		
FJ461701.1/CDV/NobivacDHPPI	America I		
AF259552.1/CDV/SnyderHill	America I		
EU143737.1/CDV/Onderstepoort	America I		
GU266280.1/CDV/RockbornCandur	Rockborn-like		
Z35493.1/CDV/Convac	America I		
DQ903854.1/CDV/Lederle	America I		
KY971529/CDV_BUC	America I		
KY971531/CDV_OVI	America I		
KY971530/CDV_NOBI	America I		
AF164967.1/1999/Switzerland	America II		
AM903376.1/India	America I		
DQ889178.1/2006/Hungary	Arctic-like		
DQ889179.1/2006/Hungary	Arctic-like		
DQ889180.1/2006/Hungary	Arctic-like		
DQ889181.1/2006/Hungary	Arctic-like		
DQ889182.1/2006/Hungary	Arctic-like		
DQ889183.1/2006/Hungary	Arctic-like		
DQ889184.1/2006/Hungary	Arctic-like		
DQ889186.1/2006/Hungary	Arctic-like		
DQ889187.1/2006/Hungary	Europe Wildlife		
DQ889188.1/2006/Hungary	Europe Wildlife		
DQ889189.1/2006/Hungary	Europe Wildlife		

Table A2. Residues at amino acid sites of the SLAM and Nectin-4 cell binding regions on the Canine distemper virus H-protein, arranged in geographical lineages and host species (domestic dog, wild canid and non-canid). The accession number, host species, year and country of origin are indicated for each strain. South African strains isolated for this study indicated with asterisk (*). Identical amino acids are indicated with a dash (-), varying amino acids are indicated by single letter amino acid codes

	Accession number/species/year/origin	SLAM binding region			Nectin-4 binding region			
		519	530	549	478	479	537	539
	SOUTHERN AFRICA							
	Domestic dog							
1	*Z10/dog/2016/SA	R	N	Y	V	L	Y	Y
2	FJ461723.1/dog/2007/SA	-	-	-	-	-	-	-
3	FJ461698.1/dog/2007/SA	-	-	-	-	-	-	-
4	FJ461718.1/dog/2007/SA	-	-	-	-	-	-	-
5	FJ461722.1/dog/2007/SA	-	-	-	-	-	-	-
6	FJ461704.1/dog/2007/SA	-	-	-	-	-	-	-
7	FJ461706.1/dog/2007/SA	-	-	-	-	-	-	-
8	FJ461721.1/dog/2007/SA	-	-	-	-	-	-	-
9	FJ461695.1/dog/2007/SA	-	-	-	-	-	-	-
10	FJ461697.1/dog/2007/SA	-	-	-	-	-	-	-
11	FJ461693.1/dog/2007/SA	-	-	-	-	-	-	-
12	FJ461703.1/dog/2007/SA	-	-	-	-	-	-	-
13	FJ461715.1/dog/2007/SA	-	-	-	-	-	-	-
14	FJ461714.1/dog/2007/SA	-	-	-	-	-	-	-
15	FJ461699.1/dog/2007/SA	-	-	-	-	-	-	-
16	FJ461716.1/dog/2007/SA	-	-	-	-	-	-	-
17	FJ461719.1/dog/2007/SA	-	-	-	-	-	-	-
18	FJ461720.1/dog/2007/SA	-	-	-	-	-	-	-
19	FJ461713.1/dog/2007/SA	-	-	-	-	-	-	-
20	FJ461705.1/dog/2007/SA	-	-	-	-	-	-	-
21	FJ461696.1/dog/2007/SA	-	-	-	-	-	-	-
22	FJ461724.1/dog/2007/SA	-	-	-	-	-	-	-
23	FJ461707.1/dog/2007/SA	-	-	-	-	-	-	-
24	FJ461711.1/dog/2007/SA	-	-	-	-	-	-	-
25	FJ461694.1/dog/2007/SA	-	-	-	-	-	-	-
26	FJ461700.1/dog/2007/SA	-	-	-	-	-	-	-
27	FJ461717.1/dog/2007/SA	-	-	-	-	-	-	-
28	FJ461712.1/dog/2007/SA	-	-	-	-	-	-	-
	Wild canid							
29	*Z15/African wild dog/2016/SA	R	N	Y	V	L	Y	Y
30	*Z9/African wild dog /2016/SA	-	-	-	-	-	-	-
31	*Z2/African wild dog /2016/SA	-	-	-	-	-	-	-
32	*Z13/African wild dog /2016/SA	-	-	-	-	S	-	-
33	*Z1/African wild dog /2016/SA	I	-	N	-	-	-	-
34	*Z11/African wild dog /2016/SA	-	-	N	-	S	-	-
35	*WT01/African wild dog /2016/SA	-	-	-	-	-	-	-

	Non-canid							
36	*WT02/SpottedHyena/2016/SA	I	N	H	V	L	Y	Y
37	*Z4/BrownHyena/2016/SA	-	-	-	-	-	-	-
38	*Z6/Lion/2015/SA	-	-	-	-	-	-	-
39	*Z7/Lion/2015/SA	-	-	-	-	-	-	-
	EAST AFRICA							
	Domestic dog							
40	JN812976.1/dog/1994/Tanzania	R	D	Y	V	L	Y	Y
	Wild canid							
41	KC916716.1/bat-earedfox/1994/Tanzania	R	D	H	V	L	Y	Y
42	KC916715.1/African wild dog/2007/Tanzania	-	-	-	-	-	-	-
43	KC916714.1/goldenjackal/2011/Tanzania	-	-	Y	-	-	-	-
	Non-canid							
44	JN812975.1/lion/1994/Tanzania	I	D	H	V	L	Y	Y
45	KC916717.1/spottedhyena/1994/Tanzania	-	-	-	-	-	-	-
	AMERICA I							
46	**Domestic dog**							
47	HQ403645.1/dog/2009/China	R	N	H	V	L	Y	Y
48	GQ332531.1/dog/2008/China	-	-	-	-	-	-	-
	Non-canid							
49	AY542312.2/racoon/2004/USA	R	N	Y	V	L	Y	Y
50	AY445077.2/raccoon/2004/USA	-	-	-	-	-	-	-
51	AY466011.2/raccoon/2004/USA	-	-	-	-	-	-	-
52	AY548111.1/racoon/2004/USA	-	-	-	-	-	-	-
53	AY548110.1/racoon/2004/USA	-	-	-	-	-	-	-
54	AY548109.1/racoon/2004/USA	-	-	-	-	-	-	-
	Vaccine							
55	FJ461710.1/CDV/Canigen	R	S	H	V	L	Y	Y
56	FJ461709.1/CDV/NobivacPuppyDP	-	-	-	-	-	-	-
57	FJ461708.1/CDV/GalaxyDA2PPV	-	-	-	-	-	-	-
58	FJ461702.1/CDV/VanguardPlus	-	D	Y	-	-	-	-
59	FJ461701.1/CDV/NobivacDHPPI	-	-	-	-	-	-	-
60	AF259552.1/CDV/SnyderHill	-	N	Y	-	-	-	-
61	EU143737.1/CDV/Onderstepoort	-	-	-	-	-	-	-
62	Z35493.1/CDV/Convac	-	-	-	-	-	-	-
63	DQ903854.1/CDV/Lederle	-	-	-	-	-	-	-
	AMERICA II							
	Domestic dog							
64	Z47762.1/dog/1995/Denmark	R	G	H	V	L	Y	Y
	Non-canid							
65	Z47763.1/blackleopard/1995/Denmark	R	G	H	V	L	Y	Y
66	Z47765.1/raccoon/1995/Denmark	-	-	-	-	-	-	-
67	Z47764.1/javelina/1995/Denmark	I	-	Y	-	W	-	-

68	Z54156.1/Chineseleopard/1995/Netherlands	I	-	-	-	-	-	-
69	AY526496.1/raccoon/2004/USA	-	-	-	-	-	-	-
70	AY438597.1/raccoon/2003/USA	I	-	-	-	-	-	-
71	AY498692.1/raccoon/2003/USA	-	R	-	-	-	-	-
72	AY465925.1/raccoon/2003/USA	-	R	-	-	-	-	-
73	AY649446.1/raccoon/2004/USA	-	R	-	-	-	-	-
	ARCTIC-LIKE							
	Domestic dog							
74	Z47760.1//Greenlandic/dog/1995/Denmark	R	N	Y	V	L	Y	Y
75	GQ214373.2/dog/2003/Austria	-	-	-	-	-	-	-
76	DQ226088.1/dog/2005/Italy	-	-	-	-	-	-	-
77	DQ226087.1/dog/2005/Italy	-	-	-	-	-	-	-
78	AY964112.1/dog/2005/USA	-	-	-	-	-	-	-
79	AY964108.1/dog/2005/USA	-	-	-	-	-	-	-
	Wild canid							
80	EF445052.1/fox/2007/China	R	N	Y	V	L	Y	Y
	ASIA I							
	Domestic dog							
81	JN381191.1/dog/2011/China	R	G	Y	V	L	Y	Y
82	EU564813.1/dog/2007/China	-	-	-	-	-	D	-
83	EU564812.1/dog/2007/China	-	-	-	-	-	-	-
84	EU684265.1/dog/2007/China	-	-	-	-	-	-	-
85	DQ922630.1/fox/2006/China	-	-	-	-	-	-	-
86	GQ332530.1/dog/2008/China	-	-	-	-	-	-	-
87	FJ409464.1/dog/2008/China	-	-	-	-	-	-	-
88	FJ851458.1/dog/2008/China	-	-	-	-	-	-	-
89	FJ851452.1/dog/2008/China	-	-	-	-	-	-	-
90	FJ848530.1/dog/2008/China	-	-	-	-	-	-	-
91	FJ851456.1/dog/2008/China	-	-	-	-	-	-	-
92	FJ535063.1/dog/2008/China	-	-	-	-	-	-	-
93	GQ332535.1/dog/2008/China	-	-	-	-	-	-	-
94	GQ332533.1/dog/2008/China	-	-	-	-	-	-	-
95	HM623891.1/dog/2009/China	-	A	-	-	-	-	-
96	HM623893.1/dog/2009/China	-	A	-	-	-	-	-
97	FJ851454.1/dog/2008/China	-	-	-	-	-	-	-
98	EU325722.1/fox/2006/China	-	-	-	-	-	-	-
99	FJ851450.1/dog/2008/China	-	-	-	-	-	-	-
100	FJ848536.1/dog/2008/China	-	-	-	-	-	-	-
101	GQ332534.1/dog/2008/China	-	-	-	-	-	-	-
102	HQ850147.1/dog/2008/China	-	-	-	-	-	-	-
103	HM623895.1/dog/2009/China	-	-	-	-	-	-	-
104	JF343962.1/dog/2009/China	-	-	-	-	-	-	-
105	HM749644.1/dog/2009/China	-	-	-	-	-	-	-
106	FJ851455.1/dog/2008/China	-	-	-	-	-	-	-
107	HQ128600.1/dog/2010/China	-	-	-	-	-	-	-
108	HQ128599.1/dog/2010/China	-	-	-	-	-	-	-
109	FJ848534.1/dog/2008/China	-	-	-	-	-	-	-
110	FJ848535.1/dog/2008/China	-	-	-	-	-	-	-

111	FJ848531.1/dog/2008/China	-	-	-	-	-	-	-
112	FJ848533.1/dog/2008/China	-	-	-	-	-	-	-
113	HQ657209.1dog/2010/China	-	-	-	-	-	-	-
114	FJ848532.1/dog/2008/China	-	-	-	-	-	-	-
115	GQ332532.1/dog/2008/China	-	-	-	-	-	-	-
116	EU716072.1/dog/2007/SouthKorea	-	-	-	-	-	-	-
117	AB025271.2/dog/1999/Japan	-	-	-	-	-	-	-
118	FJ851453.1/dog/2008/China	-	A	-	-	-	-	-
119	FJ851451.1/dog/2008/China	-	A	-	-	-	-	-
120	FJ851457.1/dog/2008/China	-	-	-	-	-	-	-
121	EU296492.1/dog/2005/Taiwan	-	-	-	-	-	-	-
122	EU296491.1/dog/2006/Taiwan	-	-	-	-	-	-	-
123	EU296493.1/dog/2005/Taiwan	-	-	-	-	-	-	-
124	EU296490.1/dog/2005/Taiwan	-	-	-	-	-	-	-
125	EU296485.1/dog/2006/Taiwan	-	-	-	-	-	-	-
126	FJ705234.1/dog/2008/Taiwan	-	-	-	-	-	-	-
127	EU296486.1/dog/2006/Taiwan	-	-	-	-	-	-	-
128	DQ191175.1/dog/2004/Taiwan	-	-	-	-	-	-	-
129	EU296481.1/dog/2005/Taiwan	-	-	H	-	-	-	-
130	EU296482.1/dog/2005/Taiwan	-	-	H	-	-	-	-
131	EU296484.1/dog/2006/Taiwan	-	-	-	-	-	-	-
132	EU296483.1/dog/2005/Taiwan	-	-	-	-	-	-	-
133	FJ705231.1/dog/2008/Taiwan	-	-	-	-	-	-	-
134	FJ705232.1/dog/2008/Taiwan	-	-	-	-	-	-	-
135	FJ705230.1/dog/2008/Taiwan	-	-	-	-	-	-	-
136	EU296488.1/dog/2005/Taiwan	-	-	-	-	-	-	-
137	EU296489.1/dog/2005/Taiwan	-	-	-	-	-	-	-
138	EU296494.1/dog/2007/Taiwan	-	-	-	-	-	-	-
139	EU296487.1/dog/2005/Taiwan	-	-	-	-	-	-	-
140	FJ705233.1/dog/2008/Taiwan	-	-	-	-	-	-	-
141	FJ705236.1/dog/2008/Taiwan	-	-	-	-	-	-	-
142	FJ705235.1/dog/2008/Taiwan	-	-	-	-	-	-	-
	Wild canid							
143	EF445053.1/fox/2007/China	R	G	Y	V	L	Y	Y
144	EU325721.1/fox/2007/China	-	-	-	-	-	-	-
145	HM448829.1/fox/2009/China	-	-	H	-	-	-	-
146	HM448831.1/fox/2009/China	-	-	-	-	-	-	-
147	HM448832.1/raccoondog/2009/China	-	-	-	-	-	-	-
148	FJ810213.1/fox/2008/China	-	-	-	-	-	-	-
149	EU325728.1/raccoondog/2007/China	-	-	-	-	-	-	-
150	EU325720.1/fox/2007/China	-	-	-	-	-	-	-
151	HM448834.1/fox/2009/China	-	-	-	-	-	-	-
152	EU934233.1/raccoondog/2006/China	-	-	-	-	-	-	-
153	EU325726.1/raccoondog/2006/China	-	-	-	-	-	-	-
154	EF445051.1/fox/2007/China	-	-	-	-	-	-	-
155	EF042818.1/raccoondog/2006/China	-	-	-	-	-	-	-
156	EU325729.1/raccoondog/2007/China	-	-	-	-	-	-	-
157	HM448833.1/raccoondog/2009/China	-	-	-	-	-	-	-
158	HM448830.1/raccoondog/2009/China	-	-	-	-	-	-	-
159	EU325730.1/raccoondog/2007/China	-	-	-	-	-	-	-
160	FJ810215.1/fox/2008/China	-	-	-	-	-	-	-

161	EF445054.1/raccoondog/2007/China	-	-	-	-	-	-	-
162	EU325727.1/raccoondog/2007/China	-	-	-	-	-	-	-
163	HQ128601.1/raccoondog/2010/China	-	-	-	-	-	-	-
164	FJ810214.1/raccoondog/2008/China	-	-	-	-	-	-	-
165	AB605890.1/raccoondog/2008/Japan	G	-	H	-	-	-	-
166	AB619775.1/raccoondog/2009/Japan	-	-	-	-	-	-	-
167	AB605891.1/raccoondog/2007/Japan	G	-	H	-	-	-	-
	Non-canid							
168	FJ405223.1/monkey/2008/China	R	G	Y	V	L	Y	Y
169	FJ405224.1/monkey/2008/China	-	-	-	-	-	-	-
170	EU325724.1/mink/2007/China	-	-	-	-	-	-	-
171	EU325723.1/mink/2007/China	-	-	-	-	-	-	-
172	EU379560.1/mink/2007/China	-	-	H	-	-	-	-
173	EU325731.1/mink/2007/China	-	-	-	-	-	-	-
174	EU325725.1/mink/2006/China	-	-	-	-	-	-	-
175	AB619774.1/tiger/2010/Japan	-	-	-	-	-	-	-
	ASIA II							
	Domestic dog							
176	EU716073.1/dog/1997/SouthKorea	R	E	Y	V	L	Y	Y
177	EU716075.1/dog/2007/SouthKorea	-	G	-	-	-	-	-
178	AB025270.1/dog/1999/Japan	G	-	-	-	-	-	-
179	AB040767/dog/2000/Japan	-	-	-	-	-	-	-
	Non-canid							
180	EU716074.1/marten/1998/SouthKorea	R	G	Y	V	L	Y	Y
	EUROPE							
	Domestic dog							
181	HM563059.1/dog/2007/Portugal	R	G	Y	V	L	Y	Y
182	GQ214376.2/dog/2002/Austria	-	-	-	-	-	-	-
183	GQ214378.2/dog/2002/Austria	-	-	-	-	-	-	-
184	GQ214384.2/dog/2002/Austria	-	-	-	-	-	-	-
185	GQ214380.2/dog/2002/Austria	-	-	-	-	-	-	-
186	DQ494317.1/dog/2006/Italy	-	-	-	-	-	-	-
187	DQ494319.1/dog/2006/Italy	-	-	-	-	-	-	-
188	DQ494318.1/dog/2006/Italy	-	-	-	-	-	-	-
189	DQ889177.1/dog/2006/Hungary	-	-	-	-	-	-	-
	Wild canid							
190	HM563057.1/wolf/1998/Portugal	R	G	Y	V	L	Y	Y
191	HM563058.1/wolf/2008/Portugal	-	-	-	-	-	-	-
192	FJ416339.1/fox/2008/Germany	-	-	H	-	-	-	-
193	FJ416337.1/fox/2008/Germany	-	-	H	-	-	-	-
194	FJ416336.1/fox/2008/Germany	-	-	H	-	-	-	-
195	HM120874.1/redfox/2009/Italy	-	-	H	-	-	-	-
	Non-canid							
196	JN153025.1/redfox/2008/Germany	R	G	Y	V	L	Y	Y
197	FJ416338.1/badger/2008/Germany	-	-	H	-	-	-	-

198	JN153024.1/redfox/2008/Germany	-	-	-	-	-	-	-
199	GU001863.1/Iberianlynx/2005/Spain	-	-	-	-	-	-	-
200	GU001864.1/Iberianlynx/2005/Spain	-	-	-	-	-	-	-
	EUROPE WILDLIFE							
	Domestic dog							
201	DQ228166.1/dog/2005/Italy	R	N	H	V	L	Y	Y
	Non-canid							
202	JN153020.1/raccoon/2007/Germany	R	D	H	V	L	Y	Y
203	JN153021.1/raccoon/2007/Germany	-	-	-	-	-	-	-
204	JN153023.1/raccoon/2007/Germany	-	-	-	-	-	-	-
205	JN153019.1/raccoon/2007/Germany	-	-	-	-	-	-	-
206	JN153022.1/raccoon/2007/Germany	-	V	-	-	-	-	-
207	GQ214374.2/badger/2006/Austria	-	-	-	-	-	-	-
208	GQ214369.2/stonemartin/2007/Austria	-	-	-	-	-	-	-
	SOUTH AMERICA I / EUROPE							
	Domestic dog							
209	FJ392652.1/dog/2003/Argentina	R	G	Y	V	L	Y	Y
210	JN215476.1/dog/2009/Uruguay	-	-	-	-	-	-	-
211	JN215475.1/dog/2008/Uruguay	-	-	-	-	-	-	-
212	JN215473.1/dog/2007/Uruguay	-	-	-	-	-	-	-
213	JN215477.1/dog/2009/Uruguay	-	-	-	-	-	-	-
214	JN215474.1/dog/2008/Uruguay	-	-	-	-	-	-	-
215	EU098105.1/dog/2007/Brazil	-	S	-	-	-	-	-
216	EU098103.1/dog/2007/Brazil	-	S	-	-	-	-	-
217	EU098104.1/dog/2007/Brazil	-	S	-	-	-	-	-
218	EU098102.1/dog/2007/Brazil	-	-	-	-	-	-	-
	SOUTH AMERICA II							
	Domestic dog							
219	FJ392651.1/dog/2005/Argentina	R	D	Y	V	L	Y	Y
220	KC257464.1/dog/2010/Argentina	-	-	-	-	-	-	-
221	FJ011005.1/dog/2005/Argentina	-	-	-	-	-	-	-

Appendix B

Table B1: PCR primers used for the amplification of five TLR genes in wild and domestic carnivores

Locus	Fragement length (bp)	Fragment length (aa)	F/R	Primer sequence 5'-3'	T_a
TLR2	166	55	F	AGACTCTACCAGATGCCTCCTTCT	58°C
			R	GCGTGAAAGACAGGAATTCACAGG	
TLR3	256	85	F	GACCTGTCAAGCCATTACCTCTGT	58°C
			R	CAAACTGCTCTGGCTGTCTGTCTA	
TLR4	208	69	F	GCTGGCAATTCTTTCCAGGACAAC	58°C
			R	TCTGGAGGGAGTGAAGAGGTTCAT	
TLR7	172	56	F	TGGTGGGTTAACCATACAGAGGTG	58°C
			R	GAGAAAGAGCCACCGATACGGAAA	
TLR8	167	55	F	GGACCGCTACCAACCTAACCATTT	55°C
			R	ACGATGCTCTTCCCTCTTTGATCC	