UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

STANDARDIZATION AND VALIDATION OF AN IMMUNOPEROXIDASE TEST FOR AFRICAN HORSESICKNESS VIRUS USING FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUES

by

SARAH JANE CLIFT

Submitted in partial fulfilment of the requirements for the degree of

Magister Scientiae

in the Department of Paraclinical Sciences in the Faculty of Veterinary Science,
University of Pretoria

Date submitted: November 2008

DO NOT GO GENTLE INTO THAT GOOD NIGHT

by Dylan Thomas

Do not go gentle into that good night,
Old age should burn and rave at close of day;
Rage, rage against the dying of the light.

Though wise men at their end know dark is right,
Because their words had forked no lightning they
Do not go gentle into that good night.

Good men, the last wave by, crying how bright
Their frail deeds might have danced in a green bay,
Rage, rage against the dying of the light.

Wild men who caught and sang the sun in flight,
And learn, too late, they grieved it on its way,
Do not go gentle into that good night.

Grave men, near death, who see with blinding sight
Blind eyes could blaze like meteors and be gay,
Rage, rage against the dying of the light.

And you, my father, there on the sad height,
Curse, bless me now with your fierce tears, I pray.
Do not go gentle into that good night.
Rage, rage against the dying of the light.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to the following people:

- My beloved husband and best friend, Jaco, for his invaluable assistance and for being my anchor in this life.
- My darling daughter, Ruth for all of her hugs and kisses during this most trying of times.
- Professor Mark Williams for his friendship, for reminding me to laugh, and for giving me free reign with this project.
- Professor Leon Prozesky for his constant encouragement and support in the bad times and the worst of times.
- Professor John Lawrence for his insight and honesty.
- Mrs Marie Smit for her invaluable technical assistance and unwavering faith in me.
- Mrs Rephima Phaswane for her technical assistance and for just putting up with me.
- Mrs Elma Vorster for her kind assistance with the final touches.
- My other colleagues in the Pathology Section for their patience and willingness to give me the time to do this work.
- Staff in the Department of Genetics (UP) for their friendship, guidance and interest in my work.
- Last but definitely not least, Ronnie Nelson, for his endless patience and assistance with the preparation of the plates.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DECLARATION

I, Sarah Jane Clift, hereby declare that the dissertation submitted herewith for the degree MSc. (Veterinary Science) at the University of Pretoria contains my own independent work and has not been submitted for any degree at any other institution.

_____

Sarah Jane Clift

November 2008

TABLE OF CONTENTS

PAGE

PAGE

PAGE

PAGE

PAGE

PAGE

PAGE

PAGE

LIST OF FIGURES

LIST OF TABLES

PAGE

LIST OF ABBREVIATIONS

1F1	name given to the VP7-specific anti-AHSV monoclonal antibody (isotype IgG2b) donated by W. van Wyngaardt
AA	amino acid
AAVLD	American Association of Veterinary Laboratory Diagnosticians
ABC	avidin-biotin complex
AFIP	Armed Forces Institute of Pathology
AGID	agar gel immunodiffusion
AHS	African horsesickness
AHSV	African horsesickness virus
AIC	Akaike Information Criterion
AR	antigen retrieval
ASFV	African swine fever virus
BHK	baby hamster kidney
BHK-21	baby hamster kidney-21 clone 13
BSA	bovine serum albumin
BT	bluetongue
BTV	bluetongue virus
BVDV	bovine viral diarrhoea virus
CDV	canine distemper virus
CF	complement fixation
CI	confidence interval
CPE	cytopathic effect
CsCl	Cesium chloride
CSFV	classical swine fever virus
ºC	degree Celsius
D.C.	District Columbia
DIC	disseminated intravascular coagulation

DNA	deoxyribonucleic acid
DPS	Department of Paraclinical Sciences
D-SN	diagnostic sensitivity
D-SP	diagnostic specificity
dsRNA	double-stranded ribonucleic acid
EE	equine encephalosis
EEV	equine encephalosis virus
EHD	epizootic haemorrhagic disease of deer
EHDV	epizootic haemorrhagic disease of deer virus
EHV	equine herpesvirus
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ENSO	El Niño-Southern Oscillation
et al	et alia (and others)
F	female
FAT	fluorescent antibody technique
FCA	Freund's complete adjuvant
FF	formalin-fixed
FFPE	formalin-fixed, paraffin-embedded
FICA	Freund's incomplete adjuvant
Fig.	figure
FMDV	foot-and-mouth disease virus
G	gram
GIT	gastrointestinal tract
HE	haematoxylin and eosin
HIER	heat-induced epitope retrieval
IFA	immunofluorescence
IgG	immunoglobulin G
IHC	immunohistochemistry
ISH	*in situ* hybridization

ISVP	infectious sub-viral particles
KZN	Kwazulu Natal Province
M	male or molar
MALT	mucosa-associated lymphoid tissue
mAb	monoclonal antibody
µl	microliter
mg	milligram
ml	milliliter
MPS	mononuclear phagocyte system
mRNA	messenger ribonucleic acid
N	no
NS	non-structural
NZ	New Zealand
OBP	Onderstepoort Biological Products
OIE	*Office International des Epizooties* (The World Organization for Animal Health)
OVI	Onderstepoort Veterinary Research Institute
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PALS	periarterial lymphatic sheaths
PAMs	pulmonary alveolar macrophages
PBM	peripheral blood mononuclear
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	paraffin-embedded
PIMs	pulmonary intravascular macrophages
PRRS	porcine reproductive and respiratory syndrome
PRRSV	porcine reproductive and respiratory syndrome virus
R	R Development Core Team 2007 (version 2.6.1)
RNA	ribonucleic acid
RT	reverse transcription

RT-PCR	reverse transcription-polymerase chain reaction
SA	South Africa
SANDF	South African National Defence Force
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssRNA	single-stranded ribonucleic acid
TB	Thoroughbred
TEM	transmission electron microscope/microscopy
UK	United Kingdom
UP	University of Pretoria
US	United States
USA	United States of America
VIBs	virus inclusion bodies
VN	virus neutralization
VP	viral protein
WB	Western blot
WCPVL	Western Cape Provincial Veterinary Laboratory
Y	yes

SUMMARY

STANDARDIZATION AND VALIDATION OF AN IMMUNOPEROXIDASE TEST FOR AFRICAN HORSESICKNESS VIRUS USING FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUES

by

Sarah Jane Clift

Promoter: Professor M.C. Williams
Department: Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria
Degree: MSc

The aim of this study was to standardize and validate an immunohistochemical test for the routine diagnosis of African horsesickness in horses. Hamblin developed the primary anti-African horsesickness virus serum that I used and the avidin-biotin complex detection system was employed. During the standardization process I demonstrate that lung, heart and spleen samples are the most reliable. I also show that it is not necessary to take multiple samples per organ, because the AHSV-positive signal is generally widespread throughout the lung and heart, in particular. In order to validate the technique, samples from 118 negative and 128 positive horse cases, including all nine known serotypes, were immunostained. All of the positive cases were confirmed by means of virus isolation. Negative horse samples were obtained from countries where African horsesickness does not occur. None of the negative cases stained positive and all the positive cases were correctly identified. Therefore, there was 100 % concordance between immunohistochemistry (when applied to formalin-fixed, paraffin-embedded heart and/or lung and/or spleen tissues from positive horse cases that had been archived for less than 10 years) and virus isolation results. Heart and lung had consistently more positive signal than spleen. The Hamblin antiserum did not cross-react with closely-related orbiviruses (specifically equine encephalosis virus and bluetongue virus) in selected

horse and sheep tissues, respectively. Characteristic positive staining was observed in lung, heart and spleen samples from two dogs that died of African horsesickness. Positive signal was not affected by long-term storage in formaldehyde (up to 365 days). Also, specific positive staining could be detected in heart and/or lung and/or spleen samples in more than 95 % of positive horses where tissue blocks had been stored for between 10 and 83 years.

The principal target cells in the horse and dog cases were microvascular endothelial cells, intravascular monocyte-macrophages and, to a lesser extent, interstitial macrophages in lung, spleen and liver, in particular. Positive staining is intracytoplasmic with a bead/dot and/or granular character. Beads, dots or granules may occur singly or in clusters. Occasionally, linear deposits of positive signal delineate segments of capillary vessels. The veterinary pathologist must look for characteristic positive signal in target cells, because, occasionally, certain bacteria (*Rhodococcus equi* and *Helicobacter* sp.) cross-react with the Hamblin antiserum. Clearly, the test is highly sensitive, specific and robust, sufficiently so for the routine diagnosis of African horsesickness virus.

*Key words: S*tandardization; validation; immunoperoxidase; test; African horsesickness; African horse sickness; AHS; African horsesickness virus; African horse sickness virus; AHSV; orbivirus; horses; dogs; immunohistochemistry; pathology; diagnostic sensitivity; diagnostic specificity; heart; lung; spleen; microvascular endothelial cells; monocytes; pulmonary intravascular macrophages; PIMs; interstitial macrophages; erythrocytes.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

OPSOMMING

STANDARDISERING EN STAWING VAN ’N IMMUNOPEROKSIDASETOETS VIR PERDESIEKTEVIRUS MET BEHULP VAN FORMALIEN-GEFIKSEERDE WEEFSEL INGEBED IN PARAFFIENWAS

deur

Sarah Jane Clift

Promotor: Professor M.C. Williams
Departement: Seksie Patologie, Departement Parakliniese Wetenskappe, Fakulteit Veeartsenykunde, Universiteit van Pretoria
Graad: MSc

Die doel van die studie was om ‘n immunohistochemiese toets vir die roetinediagnose van perdesiekte in perde te standardiseer en te staaf. Hamblin se primêre perdesiekte-antiserum en die avidien-biotienkompleks opsporingsisteem is in die studie gebruik. Tydens die standardiseringsproses toon ek dat long-, hart- en miltmonsters die mees betroubare resultate lewer. Ek toon ook dat dit onnodig is om meervoudige monsters per orgaan te neem aangesien die perdesiektevirus oor die algemeen wydverspreid in veral die long en hart voorkom. Ten einde die tegnieke te staaf, is monsters van 118 negatiewe gevalle en 128 positiewe gevalle in perde, wat al nege bekende serotipes ingesluit het, immuungekleur. Al die positiewe gevalle is deur middel van virusiolering bevestig. Die negatiewe monsters is verkry van lande waar perdesiekte nie voorkom nie. Nie een van die negatiewe gevalle het positief gekleur nie en al die positiewe gevalle is korrek geïdentifiseer. Daar was dus ‘n honderdpersent-korrelasie tussen die immunohistochemie (van hart- en/of long- en/of miltweefsel van positiewe perde, in formalien gefikseer, in parafien ingebed en vir minder as tien jaar gestoor) en virusisoleringsresultate. Hart en long het deurgaans meer positiewe tekens as milt getoon. Die Hamblin-antiserum het nie kruisreageer met nabyverwante orbivirusse (spesifiek perde-enkefalosevirus en bloutongvirus) in uitgesoekte perde- en skaapweefsel nie. Karakteristieke

positiewe kleuring is opgemerk in long-, hart- en miltmonsters van twee honde wat dood is aan perdesiekte. Positiewe toetsing is nie deur langtermyn-storing (tot solank as 365 dae) in formaldehied beïnvloed nie. Voorts was positiewe kleuring aanwesig in meer as 95 persent van hart- en/of long- en/of miltmonsters afkomstig van postiewe perde waar die weefselblokke vir tussen 10 en 83 jaar gestoor is.

Die primêre teikenselle in perde en honde was mikrovaskulêre endoteliale selle, intravaskulêre monosiet-makrofage en, in ‘n mindere mate, interstisiële makrofage in veral long, milt en lewer. Positiewe kleuring is intrasitoplasmies met ‘n kraalagtige/kollerige en/of granulêre karakter. “Krale”, kolle of granules kan afsonderlik of in groepe voorkom. Liniêre positiewe neerslae het kapillêre segmente plek-plek omlyn. Die veterinêre patoloog moet op die uitkyk wees vir die karakteristieke positiewe tekens in teikenselle aangesien sekere bakterieë (*Rhodococcus equi* en *Helicobacter* sp.) soms met die Hamblin-antiserum kruisreageer. Dit is duidelik dat die toets uiters sensitief, spesifiek en robuust is, en as sodanig geskik vir die roetinediagnose van perdesiekte.

*Sleutelwoorde: S*tandardisering; stawing; immunoperoksidase; toets; perdesiekte; perdesiektevirus; orbivirus; perde; honde; immunohistochemie; patologie; diagnostiese sensitiwiteit; diagnostiese spesifisiteit; hart; long; milt; mikrovaskulêre endoteliale selle; monosiete; pulmonêre intravaskulêre makrofage; PIMs; interstisiële makrofage; eritrosiete.

CHAPTER 1

INTRODUCTION

African horsesickness (AHS) is an infectious, non-contagious, arthropod-borne viral disease (family Reoviridae, genus *Orbivirus*) affecting equids and occasionally dogs (Coetzer & Guthrie 2004; Mellor & Hamblin 2004). Although the disease only rarely causes significant pathology in dogs (Theiler 1910), AHS is one of the most lethal systemic illnesses of horses, particularly in sub-Saharan Africa, with mortality rates in susceptible animals of up to 95 % (Burrage & Laegreid 1994; Coetzer & Guthrie 2004; Mellor & Hamblin 2004). The acute and subacute disease in naïve horses (and in the odd dog that dies of the disease) is characterized by significant alterations in respiratory and circulatory functions (Piercy 1951; Coetzer & Guthrie 2004). Mules and donkeys are less susceptible to infection than horses and zebras are the most resistant, usually experiencing only subclinical infection (Coetzer & Guthrie 2004; Mellor & Hamblin 2004).

The disease is endemic in tropical and subtropical areas of eastern, central and southern Africa, including many areas in South Africa, where mortalities occur annually, particularly in late summer and early autumn (beginning of February to the end of May), up until the onset of the first frosts (Coetzer & Guthrie 2004). Apart from its seasonal incidence, AHS also has a cyclical incidence, with major epizootics in southern Africa occurring during the warm (El Niño) phase of the El Niño-Southern Oscillation (ENSO) (Baylis, Mellor & Meiswinkel 1999). However, the frequency, extent and severity of AHS outbreaks in southern Africa have decreased quite considerably over the last century, coinciding with the introduction of AHS vaccines and a significant decline in zebra (considered to be the natural vertebrate host and reservoir of African horsesickness virus/AHSV) and horse numbers (Mellor & Hamblin 2004).

African horsesickness virus, the causative agent of AHS, is transmitted primarily by biting midges of the genus *Culicoides*, especially the Afro-oriental species *C. imicola*, the most widely distributed of all *Culicoides* species (Du Toit 1944; Burrage & Laegreid 1994; Venter & Meiswinkel 1994;

Meiswinkel 1997; Meiswinkel, Baylis & Labuschagne 2000; Meiswinkel & Paweska 2003; Meiswinkel, Venter & Nevill 2004; Mellor & Hamblin 2004; Sánchez-Vizcaíno 2004). The closely-related *C. bolitinos* was only recently identified as a vector of AHS during a field outbreak of the disease in the eastern Free State Province of South Africa (Meiswinkel *et al.* 2000). These insects become infected with AHSV when biting an infected equid, and spread the disease when subsequently feeding on susceptible animals. Therefore, AHS may be introduced into new areas by virus-infected insects or when infected animals are moved into AHS-free areas that harbour *Culicoides* midges (Brown & Dardiri 1990). These midges also transmit equine encephalosis virus (EEV), bluetongue virus (BTV) and epizootic haemorrhagic disease virus of deer (EHDV) (Meiswinkel *et al.* 2004). All of these viruses are closely-related orbiviruses (Roy 2001). Vector control is achieved by stabling horses at night (when *Culicoides* midges are most active) and through the use of insecticides (Coetzer & Guthrie 2004).

Despite the fact that AHS is considered to be enzootic to sub-Saharan Africa, several epizootics of the disease have occurred in parts of Southwest Asia, South Asia, North Africa, as well as southern and southwestern Europe (Coetzer & Guthrie 2004; Mellor & Hamblin 2004; Sánchez-Vizcaíno 2004). These outbreaks have been associated with increased international movement of horses, and it is mainly for this reason that AHS has been included in the OIE's (*Office International des Epizooties*) "A" list of important trans-boundary diseases (Powell 1985; Mellor & Hamblin 2004). AHS is also a controlled disease in South Africa (Animal Diseases Act 35 of 1984, Regulation 254 of 6 February 1997).

Climate change has recently allowed *C. imicola* to expand northwards into many areas of Europe previously considered to be AHS-risk free (Mellor 1993; Mellor & Hamblin 2004). The so-called "baton effect" is considered to be the biggest problem associated with vector spread; the term is used to explain the phenomenon of vector transmission of disease to closely-related vectors that are better adapted to colder climes (Wittmann & Baylis 2000). Also, the recent unprecedented outbreaks of bluetongue (BT) in ruminants in Europe (Mellor & Hamblin 2004; Purse, Mellor, Rogers, Samuel, Mertens & Baylis 2005) may serve as a warning for the possible future expansion of AHS (Mellor & Hamblin 2004). The disease has also long been considered the most serious

foreign animal disease threat to the United States (US) horse industry (Brown & Dardiri 1990). A recent study of BTV, the causative agent of BT, has shown that the virus is able to persist, and therefore possibly 'overwinter' (survive from one vector season to the next), in ovine gammadelta (null) T-lymphocytes (Takamatsu, Mellor, Mertens, Kirkham, Burroughs & Parkhouse 2003). In light of these discoveries, there is clearly a growing need to improve the safety and efficacy of existing vaccines and to develop new vaccines for AHS. The recent advances in recombinant DNA technology have certainly improved the means of developing new vaccines, and a wide variety of different vaccine strategies are currently being investigated in order to accomplish this goal (van Rensburg 2004; Kretzmann 2006).

The rest of this chapter has been subdivided into seven main sections. Section 1.1 briefly reviews the molecular biology of orbiviruses and selected physico-chemical characteristics of AHSV. The next section (1.2) examines other orbivirus species that cause disease in southern Africa, and their phylogenetic relationship to AHSV. The third major section (1.3) is a mini-review of the pathology associated with AHSV in horses. Section 1.4 summarises the benefits and limitations of available diagnostic tests for AHS in horses that present for necropsy. Subsequently, section 1.5 defines relevant aspects of immunohistochemistry (IHC). In addition, a summary is given of the advantages and disadvantages of IHC as a diagnostic tool for infectious diseases. The second last section (1.6) defines standardization and validation with respect to IHC, while the final section (1.7) outlines the objectives of the present study.

1.1. Orbiviral molecular biology and selected physico-chemical properties of African horsesickness virus

African horsesickness virus is a double-stranded ribonucleic acid (dsRNA) orbivirus; the *Orbivirus* genus is one of nine genera in the *Reoviridae* family (Coetzer & Guthrie 2004; Mellor & Hamblin 2004). African horsesickness virus has nine known antigenically distinct serotypes (identified by serum neutralization tests) (Coetzer & Guthrie 2004), all of which have been implicated in outbreaks of AHS in South Africa (G.H. Gerdes, unpublished data 2008).

The orbivirus proteins, structure, assembly and replication have been comprehensively reviewed and the minireview that follows has largely been extracted from these larger reviews (Oellermann, Els & Erasmus 1970; Grubman & Lewis 1992; Roy 1992; Roy 1996; Grimes, Burroughs, Gouet, Diprose, Malby, Zientara, Mertens & Stuart 1998; Williams, Inoue, Lucus, Zanotto & Roy 1998; Roy 2001; Mertens & Diprose 2004; Roy 2008). Like BTV, the prototype orbivirus, which causes disease in sheep and cattle, the AHSV genome is composed of 10 dsRNA segments, which encode seven structural proteins (VP1-VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3A). The dsRNA segments are grouped as large, medium or small segments and are named L1-L3, M4-M6 and S7-S10 respectively. The evolution of the genome has undoubtedly been influenced by structural and functional constraints, some of which will be briefly mentioned later. Four of the seven structural proteins are major proteins (VP2, VP3, VP5 and VP7) while the other three are minor proteins (VP1, VP4 and VP6). The seven structural proteins are organized in a double-layered protein capsid. There is an outer capsid layer containing two major proteins, VP2 and VP5 (which are shed during cell entry, and are encoded by the L2 and M6 genes respectively), and an inner core containing the other five proteins as well as the dsRNA genome. Immediately after infection, parental virions are uncoated to core particles, following which a large number are converted to subcore particles during further infection (Huismans, van Dijk & Els 1987b). The core particles contain the five proteins VP1, VP3, VP4, VP6 and VP7 (encoded by the L1, L3, M4, S9 and S7 genes respectively), with VP3 and VP7 as the major components. The subcore particle is thought to form the scaffold for the assembly of the capsomeres (VP7) (Burroughs, Grimes, Mertens & Stuart 1995), and these particles consist of one major protein (VP3), the three minor proteins, and dsRNA (Huismans & van Dijk 1990). The four non-structural proteins are only found in virus-infected cells.

The surface topography of BTV and that of the underlying core particle has been determined by cryon electron microscopy (EM) and the structure of the core particle determined by X-ray crystallography. The core capsid is separated into two layers. The VP3 protein forms a thin, smooth inner shell made up of 120 triangular plates, which is made rigid by the addition of 260 trimers of VP7 (on the surface of VP3), forming an icosahedral lattice. The ring structures derived from the icosahedral form of the outer core layer gave the genus its name (orbis means ring or circle). VP3

also binds to RNA and interacts with the three minor proteins of the inner core. Both VP3 and VP7 are highly conserved among the nine AHSV serotypes. The VP2 and VP5 outer capsid proteins appear to form a continuous layer around the core, except for holes through which a few VP7 molecules are exposed on the surface of the virion. The VP2 proteins, which take the form of sail-shaped spikes, are the major constituents of the outer capsid. Because of its location and the degree to which it is exposed on the surface of the virion, VP2 is the protein in greatest contact with the immune system of the host. Immunological pressures force the virus to evolve and alter its structure (Williams *et al.* 1998). VP2 is the major determinant of serotype specificity and (with VP5) it has been shown to be involved in the generation as well as the specificity of neutralizing antibodies. The globular VP5 proteins, which are largely unexposed on the capsid surface, interact with conserved core proteins (VP7 trimers) and also with the VP2 molecules. This ability to interact with proteins that change, as well as with conserved proteins, demonstrates the flexibility of the VP5 protein.

Although VP5 is also able to induce the formation of neutralizing antibodies, immunization with VP2 alone is sufficient to induce a protective immune response (Stone-Marschat, Moss, Burrage, Barber, Roy & Laegreid 1996). This has formed the basis of much of the vaccine research in recent years (Burrage, Trevejo, Stone-Marschat & Laegreid 1993; Bentley, Fehrsen, Jordaan, Huismans & du Plessis 2000). However, it is now known that immunization with VP7 also induces a protective effect (Wade-Evans, Pullen, Hamblin, O'Hara, Burroughs & Mertens 1997, 1998). As a result, the utilization of VP7 alone, or in combination with VP2 and VP5, as a subunit vaccine has been investigated quite extensively over the past decade (Martínez-Torrecuadrada, Díaz-Laviada, Roy, Sánchez, Vela, Sánchez-Vizcaíno & Casal 1996; Stone-Marschat *et al.* 1996; Roy, Bishop, Howard, Aitchison & Erasmus 1996; van Rensburg 2004; Kretzmann 2006).

The orbivirus replication cycle, which occurs in the cytoplasm of infected cells, includes the following steps: Viral adsorption, entry and uncoating, transcription and replication, protein synthesis, assembly and lastly, viral release. VP2, the most exposed protein, is a viral haemagglutinin that appears to mediate cell attachment and membrane penetration of mammalian cells. After the virion enters the cell, the virus is enclosed in endocytotic vesicles, in which the outer

capsid is removed, which results in the release of transcriptionally active core particles into the cytoplasm. The VP5 protein seems to play a role in the destabilization of the endocytotic vesicle membrane, thereby mediating the release of virus cores into the cytoplasm. It appears that the disassembly of virion particles is critical to the induction of apoptosis in infected cells (Connolly & Dermody 2002). No doubt, the flexibility and avoidance of steric constraints of the outer capsid proteins allows them to attach and enter cells (Wu & Nemerow 2004). On the other hand, the VP3-VP7 core remains intact during virus penetration. In order to avoid an interferon response, the virion does not release any dsRNA into the cells it infects (Jacque, Triques & Stevenson 2002; Heidel, Hu, Liu, Triche & Davis 2004). The VP3 protein plays a significant structural role (the subcore scaffold that supports the VP7 layer), while VP7 is the immunodominant group-specific antigen, and it has been suggested that this protein may initiate specific binding to membrane receptors and facilitate VP2 and VP5 in virus cell entry. The three minor structural proteins have different enzymatic activities that are vital for RNA transcription: VP1 functions as an RNA-dependant RNA polymerase (Maree, Durbach & Huismans 1998), VP4 has capping and guanyltransferase activity (Martínez-Costas, Sutton, Ramadevi & Roy 1998), and VP6 has a helicase function and binds to single-stranded RNA (ssRNA) and dsRNA (de Waal & Huismans 2005). The transcribed messenger RNAs (mRNAs) formed within the core are moved into the cytoplasm, where translation occurs and all the necessary structural and non-structural proteins are formed.

Of the non-structural proteins, NS1 (encoded by the M5 gene) and NS2 (encoded by the S8 gene) are synthesized in abundance. Their synthesis coincides with the formation of two virus-specific structures, namely tubules (NS1) and granular inclusion bodies (predominantly NS2) that are characteristic features of orbivirus-infected cells. NS2 is also able to bind ssRNA (Uitenweerde, Theron, Stoltz & Huismans 1995). Virus inclusion bodies (VIBs), which are thought to be the sites of virus assembly (Kar, Bhattacharya & Roy 2007), form soon after the initiation of transcription. The tubules are associated with the VIBs and may be involved in the translocation of virus particles to the cell membrane. Virulence and cytotoxicity in infected vertebrate cells are commonly associated with the two smallest non-structural proteins, NS3 and NS3A (both encoded by the S10 gene), which mediate viral release from infected cells, via exocytosis (Stoltz, van der Merwe,

Coetzee & Huismans 1996; van Staden, Smit, Stoltz, Maree & Huismans 1998; van Niekerk, Smit, Fick, van Staden & Huismans 2001; Huismans, van Staden, Fick, van Niekerk & Meiring 2004). The virus also replicates in insect vector cells, but there is no detectable cytopathic effect in these cells (Beaton, Rodriguez, Reddy & Roy 2002); this may be, at least partly, due to differences in viral release mechanisms (E. Venter, unpublished data 2008).

The physico-chemical characteristics of orbiviruses have been reviewed (Coetzer & Guthrie 2004; Mellor & Hamblin 2004). African horsesickness virus is readily inactivated at pH values below 6.0 but the virus remains relatively stable at more alkaline pH values. The optimal pH for virus survival is 7.0-8.5. The virus is resistant to lipid solvents and ultraviolet radiation. African horsesickness virus is also relatively heat stable and it can survive in putrefied tissues.

1.2. Orbivirus species (other than African horsesickness virus) that cause disease in southern Africa and their phylogenetic relationship to African horsesickness virus

The *Orbivirus* genus is large and, at present, 19 species are recognized (Coetzer & Tustin 2004). African horsesickness virus is morphologically and biochemically similar to other species within the *Orbivirus* genus, such as BTV, EHDV and EEV (Mellor & Hamblin 2004). Furthermore, important similarities exist between the cytopathology of AHSV, BTV, EHDV and EEV (Roy 2001; Coetzer & Guthrie 2004; Howell, Guthrie & Coetzer 2004; MacLachlan & Osburn 2004; Verwoerd & Erasmus 2004). Despite their similarities, however, orbiviruses have distinct pathobiological properties and host ranges (Roy 1996).

In the southern African context AHS might be confused with equine encephalosis (EE), a closely-related orbiviral disease of horses (Paweska, Gerdes, Woods & Williams 1999; Coetzer & Guthrie 2004; Howell *et al.* 2004). Its causative agent, EEV, which has seven known serotypes (Howell *et al.* 2004), is considered to be endemic in equids in much of South Africa, Botswana, Namibia, Zimbabwe and Kenya (Barnard 1997; Paweska *et al.* 1999). Due to the fact that many of the epidemiological features of EE and AHS are similar, and because EEV has been associated with AHS-like lesions, especially swelling of the eyelids, supraorbital fossae, or the entire head (Coetzer

& Guthrie 2004; Howell *et al.* 2004), it is common practice for veterinarians to send samples to a laboratory for both AHS and EE virus isolation and serotyping. Despite the fact that AHSV is responsible for a much higher mortality rate in horses than EEV (Coetzer & Guthrie 2004; Howell *et al.* 2004), it is still important to differentiate between these two diseases as quickly as possible so that suitable control measures can be implemented.

Bluetongue virus, with 24 known serotypes, causes BT, an infectious but non-contagious insect-borne viral disease of sheep and other domestic and wild ruminants. The disease has been comprehensively reviewed by Verwoerd & Erasmus (2004), from which the following brief synopsis has been derived. Bluetongue occurs in many regions of the world and the outcome of infection ranges from inapparent or subclinical in the vast majority of infected cases to fatal in a proportion of sheep and some wild ruminants. The disease is endemic in the entire southern African region, where it commonly causes significant disease in sheep, particularly during the summer months and, above all, in wet seasons. In fatal cases, the pathology is characterized by severe congestion, hyperaemia, haemorrhage, oedema, necrosis and inflammation (all caused by vascular injury), particularly affecting the gastrointestinal and respiratory tracts, the cardiovascular system, lymphoid organs, skeletal musculature, skin, wool and hooves. Due to its economic importance and worldwide distribution, BTV remains the focus of attention among the orbiviruses.

Epizootic haemorrhagic disease virus (with nine distinct serotypes) causes outbreaks of disease in wild ruminants, particularly white-tailed deer (*Odocoileus virginianus*) in North America and in other regions of the world (MacLachlan & Osburn 2004). Rather like BT in sheep, epizootic haemorrhagic disease (EHD) in deer is largely characterized by lung oedema and/or haemorrhagic enteropathy (MacLachlan & Osburn 2004). Recently, EHDV has been implicated as the cause of a rather vague syndrome of oral ulceration and coronitis in cattle in South Africa and the United States of America/USA (Gerdes, Neser, Barnard & Larsen 1996; Barnard, Gerdes & Meiswinkel 1998; House, Shipman & Weybright 1998).

Phylogenetic studies have demonstrated a close genetic relationship between AHSV, BTV and EHDV (Roy 2001), although BTV and EHDV appear to be even more closely related to each other

than to AHSV and EEV (van Staden, Theron, Greyling, Huismans & Nel 1991; Iwata, Chuma & Roy 1992; Pritchard & Gould 1995; Pritchard, Gould, Wilson, Thompson, Mertens & Wade-Evans 1995; van Niekerk, Freeman, Paweska, Howell, Guthrie, Potgieter, van Staden & Huismans 2003). Interestingly, BTV may in fact be genetically closer to AHSV than is EEV (Quan, van Vuuren, Howell, Groenewald & Guthrie 2008).

1.3. Pathology of African horsesickness in horses

1.3.1. Characteristic macropathology

The macroscopic pathology associated with AHS in horses has been described in detail by Henning (1956), Maurer & McCully (1963), Erasmus (1973), Newsholme (1983), Coetzer & Guthrie (2004), Mellor & Hamblin (2004) and Maxie & Robinson (2007). In general, serofibrinous effusions and haemorrhages in various organs and tissues (the result of vascular injury) characterize the disease.

As early as 1921, Theiler distinguished between four clinicopathological syndromes of AHS, which are still referred to by equine clinicians and veterinary pathologists today to help them assess the clinical and macroscopic presentation of the disease. Only the first three forms of the disease cause death in horses. There is a particularly lethal peracute pulmonary ("dunkop" or "thin-head") form, where the most characteristic lesions are severe diffuse serofibrinous pulmonary oedema and/or exudative pneumonia and hydrothorax. The subacute cardiac ("dikkop" or "thick-head") form is characterized by oedema of the head (initially affecting the eyelids and temporal or supraorbital fossae, later spreading to the lips, cheeks, tongue and intermandibular space) and neck (especially prominent laterally, along the deep intermuscular fascia in close proximity to the ligamentum nuchae). At necropsy, serofibrinous hydropericardium and coalescing ecchymotic and suggilatory haemorrhages on the epi- and endocardial surfaces of the heart (especially affecting the left ventricle) tend to be most severe in this form of the disease. Mild nephrosis is also commonly associated with the cardiac form of AHS. Both the pulmonary and cardiac forms of horsesickness also exhibit severe diffuse congestion of the mucosa of the glandular stomach, swollen and

oedematous lymph nodes, mild congestive splenomegaly with prominent white pulp and variably-sized capsular haemorrhages, and mild congestive hepatomegaly with distinct lobulation. A third acute form, which is a mixture of the first two forms, has also been described, although it is generally accepted that either the pulmonary or the cardiac form tends to dominate the clinical picture. In most cases no *clear* distinction can be made between the first three forms of the disease in the post mortem hall, and therefore most necropsy cases are classified as the mixed form of AHS. Finally, there is a mild "fever" form of AHS where remittent pyrexia, which may last up to a week, is the central manifestation of the disease. This is the form of the disease that occurs in partially immune horses, or following infection with less virulent strains of virus (Mellor & Hamblin 2004). It is most often confused with EE and is not often diagnosed clinically, since these horses generally recover without incident. This is also the form that occurs with highest frequency in resistant species like the African donkey and zebra (Mellor & Hamblin 2004).

Although a number of the macroscopic lesions are very characteristic for AHS (particularly the supraorbital swellings in the cardiac and cardiac-dominated mixed forms), other equine diseases in South Africa, for example, purpura haemorrhagica, theileriosis (*Theileria equi*), snake-bite, malignant oedema (clostridial myositis), and EE can cause this and/or some of the less characteristic macrolesions that are commonly associated with AHS.

1.3.2. Histopathology

Histological changes are not considered to be helpful in the diagnosis of AHS, nor are they considered useful in understanding the pathogenesis of the disease (Erasmus 1973; Maxie & Robinson 2007). Maurer & McCully (1963) and Newsholme (1983) described the histopathology of AHS in horses in great detail. The following brief summary has been drawn largely from these comprehensive accounts. In general, there is evidence of widespread microvascular injury, especially in sections of lung and heart. This is characterized by: Adventitial serocellular to fibrinous exudation (the cells are predominantly mononuclear leukocytes and neutrophils); endothelial cell swelling/hypertrophy; occasional endothelial cell necrosis/apoptosis (Wohlsein, Pohlenz, Davidson, Salt & Hamblin 1997; Wohlsein, Pohlenz, Salt & Hamblin 1998), and

haemorrhage. In the lung these changes are accompanied by generalized fibrin-rich alveolar oedema and in the heart they may be related to foci of myocardial necrosis. Maurer & McCully (1963) also described severe myocarditis in some of their cardiac-form cases of AHS, although others have not reported this. Leukostasis (mononuclear leukocytes and neutrophils) has also been described in microvessels throughout the lung and heart, in particular.

Variable degrees of lymphoid depletion and lymphocytolysis, with relative prominence of reticuloendothelial or macrophage-like cells in the germinal centres of lymphatic nodules, have been observed in the mucosa-associated lymphoid tissues (MALT) and in the spleen and lymph nodes in the lethal forms of AHS. Severe, diffuse mucosal congestion (variably-associated with mucosal haemorrhage and inflammation) has also been described in the glandular part of the stomach and in the small intestine of horses that died of AHS, especially the cardiac and cardiac-dominated mixed forms of the disease.

1.3.3. Transmission electron microscopic (TEM) studies of African horsesickness virus in tissues from experimentally-infected horses

Newsholme (1983) failed to find virus particles or virus-related structures associated with AHSV replication *in vitro*, namely tubules and inclusion bodies (Lecatsas & Erasmus 1967; Breese, Ozawa & Dardiri 1969; Huismans & Els 1979) in tissues from horses that had died of natural or experimentally-induced AHS. In addition, there was no evidence of vascular injury in myocardial and pulmonary tissues, despite the presence of serofibrinous oedema and small haemorrhages in these tissues. Newsholme did observe the separation of type I pneumocytes from the alveolar wall in lung sections from infected horses. He interpreted this as a manifestation of severe alveolar interstitial oedema. Newsholme concluded that the tissue concentrations of virus in his study were lower than the threshold required to visualize virus particles *in situ*.

Some years later, a number of researchers detected the presence of circular virus-like particles and virus-related structures in the perinuclear cytoplasm of a small proportion of microvascular endothelial cells in sections of lung and heart from horses that were all experimentally-infected with

AHSV-4 (Laegreid, Burrage, Stone-Marschat & Skowronek 1992; Carrasco, Sánchez, Gómez-Villamandos, Laviada, Bautista, Martínez-Torrecuadrada, Sánchez-Vizcaíno & Sierra 1999; Gómez-Villamandos, Sánchez, Carrasco, Laviada, Bautista, Martínez-Torrecuadrada, Sánchez-Vizcaíno & Sierra 1999). Occasional fibroblasts, interstitial macrophages, smooth muscle fibres and pericytes were also seen to contain virus-like particles. Moreover, these studies demonstrated the presence of endothelial cell injury, which manifested as: Endothelial cell hypertrophy, degeneration, the appearance of cytoplasmic projections (which sometimes engulfed erythrocytes), separation of endothelial intercellular junctions, loss of endothelial cells, subendothelial and interstitial deposition of cell debris, fibrin and red blood cells, changes in vascular permeability (evidenced by increased erythrodiapedesis and micropinocytotic vesicles) and vascular repair. In most sections of myocardium and lung there was typically evidence of haemorrhages, oedema and microthromboses. Crucially, all of these studies established that injured endothelial cells did not necessarily contain virus.

Carrasco *et al.* (1999) also discovered the presence of replicating AHSV in plump, activated (evidenced by increased phagocytic activity) pulmonary intravascular macrophages (PIMs) in the lung of experimentally-infected horses. Equine PIMs have been shown to possess unique ultrastructural features (e.g. a globular surface coat, endocytic pathway structures and adhesive junctions with pulmonary endothelium) that characterize them as mature phagocytes within the pulmonary microvasculature. They closely resemble PIMs in ruminant lungs (Atwal, Minhas, Ferenczy, Jassal, Milton & Mahadevappa 1989; Atwal, Singh, Staempfli & Minhas 1992), about which much has been written (Warner, Barry & Brain 1986; Wheeldon & Hansen-Flaschen 1986; Warner, DeCamp, Molina & Brain 1988; Atwal *et al.* 1989; DeCamp, Warner, Molina & Brain 1992; Carrasco, Gómez-Villamandos, Bautista, Hervas, Pulido & Sierra 1996; Longworth, Albertine & Staub 1996; Singh, Ott, Bazer & de la Concha-Bermejillo 1998). These bone marrow-derived cells only occur in large numbers in some animal species (Brain, Molina, DeCamp & Warner 1999). They differentiate postnatally from monocytes that colonize lung capillaries during the perinatal period (Winkler & Cheville 1985; Longworth *et al.* 1996). Pulmonary intravascular macrophages are morphologically similar to other macrophages of the mononuclear phagocytic system (MPS), especially hepatic Kupffer cells (Winkler 1988). Moreover, ultrastructural studies have shown that

PIMs tend to be more numerous and smaller than pulmonary alveolar macrophages (PAMs) in some animal species (Carrasco *et al.* 1996).

1.3.4. Observations on the pathogenesis of African horsesickness

Despite the lack of virus particles, viral matrix and obvious vascular injury in tissue sections from horses infected with AHSV, Newsholme (1983), like Maurer & McCully (1963) and Erasmus (1973) was able to deduce that the oedema in AHS was probably of inflammatory origin, arising from a virus-induced increase in localized microvascular permeability. Certainly, the unusual, localized (predominantly dorsal) distribution of the highly proteinaceous subcutaneous oedema in the cardiac and cardiac-dominated mixed forms of the disease strongly supports this argument.

More than a decade later, Brown, Meyer & Grubman (1994), used *in situ* hybridization (ISH) to detect AHSV in a number of tissues from 15 ponies that were experimentally-infected with three different serotypes. They demonstrated the presence of virus in microvascular endothlelial cells in the lung and heart, and to a much lesser extent in the spleen, neck muscle and supraorbital fat, as well as in mononuclear cells in the spleen. A few years later, Wohlsein *et al.* (1997, 1998) used an immunohistochemical staining technique to detect AHSV in a variety of formalin-fixed, paraffin-embedded (FFPE) tissues from three ponies that were experimentally-infected with serotype four. They identified endothelial cells (again, predominantly lining the cardiopulmonary microvasculature) as well as monocyte-macrophages in circulation and in the interstitium, as the target cells for AHSV. Both studies also established that AHSV is widely distributed in microvessels throughout the lung, heart and lymphoid organs (in particular). The ultrastructural studies done following Newsholme's (1983) work, conclusively demonstrated the presence of virus and viral matrix in microvascular endothelial cells, as well as endothelial cell injury, in the lung and myocardium from experimentally-infected horses. Clearly therefore, the presence of necrosis and/or degeneration, oedema and haemorrhage in the hearts of horses with the cardiac and cardiac-dominated forms of AHS can be largely attributed to vascular compromise in the myocardium, and not only to the excessive release of endogenous catecholamines due to stress (as was suggested by Newsholme

1983), or to increased vascular permeability of the pericardial capillaries (as was hypothesized by Erasmus 1973).

The post-Newsholme (1983) ultrastructural studies also established that injured endothelial cells may or may not contain virus, and the extent of pathology that occurs in a particular organ (for example the lung) does not appear to be related to the quantity of AHSV that is visible in that organ. The implication is that the pathogenesis of microvascular leakage and microthrombosis in AHS is multifactorial and is likely to involve indirect as well as direct virus-mediated pathways or mechanisms (Laegreid *et al.* 1992; Carrasco *et al.* 1999; Gómez-Villamandos *et al.* 1999). Carrasco *et al.* (1999) specifically showed that activated PIMs probably play an important role in the pathogenesis of the serofibrinous lung oedema in AHS, due to their releasing vasoactive chemical inflammatory mediators. Pulmonary intravascular macrophages have also been shown to play a central role in virus-mediated acute lung injury in pigs, for example in cases of porcine reproductive and respiratory syndrome virus (PRRSV) and African swine fever (ASFV), for which PIMs are considered to be an important replication site (Thanawongnuwech, Thacker & Halbur 1997b; Thanawongnuwech, Brown, Halbur, Roth, Royer & Thacker 2000). Similarly, in ruminants, the preferential removal of endotoxin by PIMs is thought to result in acute lung injury, which manifests as oedema, clogging of capillaries with neutrophils, platelets and fibrin, as well as endothelial cell injury (Warner *et al.* 1988). These changes are similar to the microlesions that are commonly associated with the pulmonary and pulmonary-dominated forms of AHS.

The variation in clinical signs, lesions and course of the disease (associated with the different forms of AHS) have been hypothesized to be related to a) differences in the selective affinity of different AHSV strains for certain types of blood vessels (Laegreid, Stone-Marschat, Skowronek & Burrage 1991), b) variation in virus virulence (Erasmus 1973; Laegreid, Skowronek, Stone-Marschat & Burrage 1993), and c) host immune status and genetic susceptibility (Laegreid *et al.* 1993). To-date, not much has been done to investigate the pathogenesis of AHS from the perspective of host immunity and genetic susceptibility.

At odds with the first hypothesis (in a) above), Brown *et al*. (1994), established that in 13 ponies infected with the same inoculum of AHSV-4, the virus showed tropism for microvascular endothelial cells in multiple organs in all the ponies. Furthermore, these ponies showed a variety of clinical signs and pathology, pertaining to the pulmonary, cardiac *and* mixed forms of AHS. Moreover, the amount of signal in a particular organ did not appear to be correlated with any particular clinicopathological form of the disease.

In fact, multiple serotypes of the same species have been recovered from individual hosts suffering from natural orbivirus infections (Roy 2001). Recently, it has been discovered that some field isolates of AHSV, and even some of the reference strains, consist of a mixed population of RNA segments, representing different serotypes (A.C. Potgieter, unpublished data 2008). This process of reassortment (whereby viruses containing a novel assortment of genetic segments are created) happens when two similar viruses of different strains or serotypes both infect the same cell. During replication, gene segments of parental strains can reassort to form viruses containing different combinations of genome segments than the parental strains (forming so-called reassortants). Reassortment is thought to be important for the generation of genetic diversity in virus populations in nature (Roy 2001). Furthermore, laboratory research has shown that reassortment in reoviruses commonly creates mutations that increase the fitness of the new reassortants (Nibert, Margraf & Coombs 1996). Therefore, because reassortment is a fairly common occurrence in nature (Pierce, Balasuriya & MacLachlan 1998; Alam, Kobayashi, Ishino, Naik & Taniguchi 2006), it seems plausible that new, more virulent strains of AHSV might develop with time.

Interestingly, still regarding virus virulence, Erasmus (1973) demonstrated quite convincingly that the blood from a horse infected with a field strain of AHSV contained a mixed virus population (virulent and less virulent subpopulations) with regard to tissue tropism. More virulent virus particles might replicate in endothelial cells lining the microvasculature of vital organs such the lungs, which, depending on the relative proportion of pneumotropic virus particles to other particles, might result in the particularly lethal pulmonary form of AHS. Laegreid et al. (1993) endorsed Erasmus's findings 20 years later, and today it seems plausible that Erasmus's virulent and less virulent subpopulations might correspond with virulent and less virulent reassortants.

In conclusion, a central feature of AHSV pathogenesis seems to be the ability of the virus to replicate in and to injure (either directly or indirectly) microvascular endothelial cells in particular, with tropism for cells in distinct anatomic sites (Roy 2001). This phenomenon has also been demonstrated in animals infected with BTV and EHDV (Tsai & Karstad 1970; Mahrt & Osburn 1986). No doubt, endothelial cell injury and the infection and activation of monocyte-macrophages, (especially PIMs), results in a cascade of pathophysiologic events characterized by capillary leakage, haemorrhage, inflammation and disseminated intravascular coagulation/DIC (Skowronek, LaFranco, Stone-Marschat, Burrage, Rebar & Laegreid 1995).

1.4. Overview of methods commonly used to diagnose African horsesickness in horses presented for necropsy in South Africa, as well as their major advantages and limitations

In view of the serious economic implications of outbreaks of AHSV, especially in the horse-racing industry, and when these outbreaks occur in non-endemic regions, numerous sensitive, rapid and reliable laboratory techniques have been developed and/or perfected in recent years to detect AHS virus, viral antigens, nucleic acids and/or virus-specific antibodies in live and dead animals (Laegreid 1994; Rubio, Cubillo, Hooghuis, Sánchez-Vizcaíno, Díaz-Laviada, Plateau, Zientara, Cruciere & Hamblin 1998; Sailleau, Seignot, Davoust, Cardinale, Fall, Hamblin & Zientara 2000; Coetzer & Guthrie 2004; Koekemoer & van Dijk 2004; Mellor & Hamblin 2004; Sánchez-Vizcaíno 2004). It is important to note that each of the available tests has limitations, and there is no single test that is sensitive and robust enough to consistently diagnose AHS in the wide range of samples that are submitted to diagnostic laboratories. Instead, each sample that is received for the purpose of diagnosing AHS should be processed according to the tests that are best suited to that particular sample. Wherever possible a combination of tests should be performed to diagnose an outbreak of AHS (Sánchez-Vizcaíno 2004).

Virus isolation is the gold standard test for the detection of viruses. It is an essential technique for the characterization of new viruses (or evolving known viruses) and, as such, the technology is irreplaceable. The detection of AHS early in the disease, prior to the appearance of specific antibodies, has historically always depended on virus isolation in cell cultures and either suckling-

mouse brains or embryonated hens' eggs (Laegreid 1994; Sánchez-Vizcaíno 2004). Isolates are recognized by a serogroup-specific test such as complement fixation (CF), agar gel immunodiffusion (AGID), direct and indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Serotypes are routinely identified by virus neutralization (VN) tests using type-specific antisera, or, more recently, by means of a type-specific reverse transcription-polymerase chain reaction/RT-PCR technique (Coetzer & Guthrie 2004; Mellor & Hamblin 2004). African horsesickness virus is most easily isolated from the spleen, lung and lymph nodes in the dead animal (Erasmus 1973; Laegreid 1994; Coetzer & Guthrie 2004; Mellor & Hamblin 2004; Sánchez-Vizcaíno 2004). Isolation and identification of serotypes by these traditional, expensive methods requires skill, usually takes a minimum of two weeks, and requires the use of reagents that are prepared in laboratory animals (Sailleau *et al.* 2000; Koekemoer & van Dijk 2004; Sánchez-Vizcaíno 2004; G.H. Gerdes, unpublished data 2008). In addition, isolation requires viable, infectious virus and therefore cannot detect dead virus or non-infectious core-like particles (Bremer & Viljoen 1998). Consequently, formalin-fixed material should not be submitted for isolation. Finally, the toxicity of autolyzed tissue samples may render virus isolation essentially useless for virus detection in these samples (Anderson, Phillips, Waldvogel & Osburn 1989).

The speed, specificity and sensitivity of PCR has revolutionized virus testing in diagnostic laboratories worldwide. To-date, the RT-PCR test, which is used to identify AHS virus RNA, is undoubtedly the most promising of the emerging diagnostic tests (Koekemoer & van Dijk 2004; G.H. Gerdes, unpublished data 2008), although it is not yet a prescribed test for international trade (as it is for BTV) (Sánchez-Vizcaíno 2004). The sensitivity of RT-PCR for AHSV is comparable to or even greater than virus isolation (Bremer & Viljoen 1998). The test is able to detect viable and nonviable virus (Mellor & Hamblin 2004) as well as infectious and non-infectious virus particles (Bremer & Viljoen 1998). The processing time is short, confirmation of both AHSV serogroup and serotype can be achieved within 24 hours (Laegreid 1994; Sakamoto, Punyahotra, Mizukoshi, Ueda, Imagawa, Sugiura, Kamada & Fukusho 1994; Stone-Marschat, Carville, Skowronek & Laegreid 1994; Bremer & Viljoen 1998; Sailleau *et al*. 2000; Koekemoer & van Dijk 2004). The ability to identify a serotype quickly and accurately is crucial for the rapid implementation of appropriate vaccination strategies in an outbreak (Koekemoer & van Dijk 2004).

Because of the exquisite sensitivity of the technique, the risk of contamination of samples, leading to false-positive results, is a recurring problem when using PCR techniques. Therefore, wherever possible, other tests should be used to confirm PCR-positive diagnoses (Zientara, Sailleau, Moulay, Wade-Evans & Cruciere 1995). Moreover, the fact that PCR can be too specific for some aspects of diagnostics must always be taken into account. The conservation of nucleotide sequences at sites where the primers bind is crucial to PCR methods (Koekemoer, Potgieter, Paweska & van Dijk 2000). Therefore, any nucleotide mutations or deletions at the primer-binding site would necessarily preclude viral detection via PCR. Also, molecular techniques such as PCR cannot be used to track genetic changes in infective organisms or to detect novel organisms. Isolation technology is thus irreplaceable in this regard. Lastly, and especially pertinent to the diagnostic veterinary pathologist, FFPE tissues are commonly regarded as a poor source of nucleic acids due to presumed damage during the fixation and embedding processes (Jackson, Hayden & Quirke 1991). The extraction of RNA and its detection by RT-PCR is even more critical due to its delicateness, the ubiquitous presence of ribonucleases, and the requirement for reverse transcription before amplification (Rupp & Locker 1988). However, it should be noted that the technique has been able to detect viral genome in archival FFPE tissues in many diseases caused by RNA viruses, such as bovine viral diarrhoea virus/BVDV (Gruber, Greiser-Wilke, Haas, Hewicker-Trautwein & Moennig 1993), BTV (Parsonson & Mccoll 1995), canine distemper virus/CDV (Stanton, Poet, Frasca, Bienzle & Brown 2002), classical swine fever virus/CSFV and foot-and-mouth disease virus/FMDV (Hofmann, Thur, Liu, Gerber, Stettler, Moser & Bossy 2000; Singh, Sai Kumar & Paliwal 2005).

There are a number of ELISA tests that have been developed to detect AHS virus antigen, predominantly in splenic tissues (du Plessis, van Wyngaardt & Bremer 1990; Hamblin, Mertens, Mellor, Burroughs & Crowther 1991; Hamblin, Anderson, Mellor, Graham, Mertens & Burroughs 1992; Laviada, Babín, Dominguez & Sánchez-Vizcaíno 1992; Laegreid 1994). These assays are useful for the rapid, accurate confirmation of a necropsy diagnosis of AHS; results are available in only 2-4 hours (Laegreid 1994; Sánchez-Vizcaíno 2004). In some instances, antigen-ELISA tests have even offered a clear advantage over virus isolation by giving positive reactions with tissues from infected animals in the absence of demonstrable amounts of infectious virus (Hamblin *et al.*

1992). On the other hand, negative ELISA results should always be confirmed by attempted virus isolation in suckling mice and/or tissue culture (Hamblin *et al.* 1992). Antigen-ELISA tests do not work on formalin-fixed material and, more significantly (especially from the pathologist's point of view), virus isolation, PCR and ELISA tests do not provide any information regarding the type, the proportion and the histological localization of cells containing virus, viral nucleic acids or antigens in tissues.

1.5. Overview of immunohistochemistry and its major advantages and disadvantages as a diagnostic tool

Immunohistochemistry is considered to be an accurate diagnostic method that is especially advantageous as a diagnostic tool for infectious diseases in animals (Ramos-Vara, Del Piero, Kiupel, Fitzgerald, Bermudez, Johnson & Miller 2002a; Ramos-Vara, Kiupel & Miller 2002b; Ramos-Vara, Kiupel, Baszler, Bliven, Brodersen, Chelack, Czub, Del Piero, Dial, Ehrhart, Graham, Manning, Paulsen, Valli & West 2008). The practical application of IHC in veterinary medicine has been extensively reviewed in recent years (Ramos-Vara 2005; Ramos-Vara *et al.* 2008) and the brief overview that follows has been mostly derived from these reviews.

Essentially, IHC is based on the detection of a particular antigen, using derived mono- or polyclonal antibodies, in FFPE tissue sections. After binding to the target antigen, the antibodies are detected by secondary antibodies that bind a cascade of avidin-biotin, streptavidin-biotin or polymer molecules that are labelled with peroxidase or alkaline phosphatase. These enzymes cause an insoluble colour reaction-product that permits the pathologist to detect the antibody-bound antigen within tissue sections using a light microscope.

The basic technique can be divided into three steps (taken from Ramos-Vara *et al.* 2008). The first step encompasses procedures prior to incubation of the tissue sections with the primary antibody/antiserum. This includes deparaffinization and hydration of tissue sections, blocking non-specific binding sites and endogenous activities (e.g. peroxidase and biotin), followed by antigen retrieval (AR) methods (viz. enzymatic digestion and/or heat-induced epitope retrieval/HIER). The

next step includes all immunological reactions between a) the primary antibody/antiserum and tissue antigens, b) the primary and secondary (link) antibodies and c) additional chemical reactions necessary to bind the reporter molecule (enzyme) to the preformed immune complex. The last step in the technique incorporates all methods required to visualize antigen-antibody binding in the test tissue sample. This involves a chemical reaction whereby the reporter molecule (e.g. peroxidase) reacts with an enzyme substrate/chromogen to produce deposits of insoluble colour reaction-product in the tissue section. Finally, the immunolabeled sections are counterstained and coverslipped, ready for microscopic examination.

Primary antibodies raised against a specific antigen are generally designated as either polyclonal or monoclonal. Polyclonal antibodies (pAbs) are immunochemically heterogeneous as they are directed against a variety of epitopes on the antigen against which they are raised. They are produced by different B-cell clones in multiple animal species. An antiserum is blood serum from an immunized individual that contains several polyclonal antibodies against the immunizing antigen, as well as other (irrelevant) soluble serum proteins, which can be present in high concentration if not affinity purified. Both polyclonal antibodies and antisera contain more than one antibody isotype. On the other hand, monoclonal antibodies (mAbs) are immunochemically identical since they react with a single epitope on the specific antigen against which they were raised. These antibodies are derived from a single B-cell clone and are produced via the hybridoma technique; they comprise one defined immunoglobulin isotype. In general, pAbs are better at binding antigens (i.e. they are more sensitive) than mAbs, particularly across a wide range of host species. This is largely due to their ability to detect a greater number of epitopes of the target protein. However, the “immunologic promiscuity” of pAbs (Ramos-Vara 2005) can be disadvantageous due to the greater possibility of detecting similar epitopes in irrelevant proteins, and, consequently, the greater likelihood of false-positive results. Conversely, mAbs tend to be far more specific than pAbs as they are generally less likely to cross-react with similar antigens. As a result, mAbs typically result in less background staining compared to pAbs. Monoclonal antibodies generally cost more to produce than pAbs.

The IHC detection system that will be employed in the present study is the avidin-biotin complex (ABC) method. This is the most commonly used of the available IHC detection systems because it is considered to be both sensitive and economical (Hsu, Raine & Fanger 1981a; Hsu, Raine & Fanger 1981b; Boutilier, Stratis, Bailey, McGirr & Wahid 1989; Raab 2002; Ramos-Vara 2005; Ramos-Vara *et al.* 2008). In this method, the extremely strong (essentially irreversible) affinity of the glycoprotein, avidin, for a small molecular weight vitamin, biotin, is exploited. The basic method entails the sequential addition of the following reagents: Primary antibody, secondary biotinylated antibody, and a macromolecular complex of avidin mixed with biotin linked with a reporter molecule (e.g. horseradish peroxidase enzyme). The complex retains some free biotin-binding sites for binding with the biotinylated secondary antibody. The major disadvantage of the avidin-biotin method is the possibility of producing strong background staining because of endogenous biotin, particularly in organs such as liver and kidney. This may result in false-positive signalling.

Diagnostic IHC offers certain advantages over other diagnostic techniques, although it is not considered as sensitive or specific as ISH (Nuovo 1994), PCR or virus isolation with serotyping via seroneutralization. Most importantly, IHC allows co-localization of an offending antigen and the lesion it has produced, thereby increasing diagnostic accuracy and understanding of pathogenesis. Therefore, in using IHC one would know not only the tropism of an infectious organism for a particular tissue/organ, but also the exact cellular localization of the antigen. Immunostaining of paraffin sections is semi-permanent and provides excellent histologic detail when used with an appropriate counterstain. It is also a rapid diagnostic method (generally results are available within 24 hours of receipt of a sample). Immunohistochemistry can also detect both viable and nonviable organisms in routinely-fixed and processed tissues. Moreover, because formalin is the most commonly used fixative in routine tissue fixation, IHC holds great potential for retrospective diagnosis on archival material. Formalin-fixation of tissues also eliminates the risk of contamination associated with fresh clinical samples. On the other hand, it is also well known that over-fixation in formalin can be deleterious in IHC due to the ability of formalin to cross-link proteins, thereby causing certain epitopes to become undetectable (leading to false-negative results). There is also the possibility of false-positive (and -negative) results when inexperienced pathologists interpret IHC sections. In this regard, the importance of interpreting IHC staining results in the context of

available clinico-pathological findings must be emphasized. Furthermore, IHC, like PCR, is a good diagnostic tool for microorganisms, as long as these remain typical. In other words, just as PCR is not able to detect 'known' agents that are undergoing genetic drift, so IHC may fail to detect 'known' agents that are undergoing antigenic drift.

A number of studies have reported that orbiviral antigens and viral nucleic acid have been difficult to detect in tissues using IHC and/or classical ISH techniques (Dangler, de la Concha-Bermejillo, Stott & Osburn 1990; Schoepp, Blair, Roy & Beaty 1991; de la Concha-Bermejillo, Schore, Dangler, de Mattos, de Mattos & Osburn 1992), possibly due to low numbers of virus-infected cells and/or low levels of virus replication *in situ* (Dangler *et al.* 1990). To-date, only Wohlsein *et al.* (1997, 1998) have successfully applied the ABC method to various horse tissues to determine the localization of AHSV antigens. These studies investigated tissue and cell tropism in three ponies that were experimentally-infected with serotype four. The findings were compared to a single non-infected control animal.

1.6. Standardization and validation of immunohistochemistry

For more than 20 years, investigators have debated the vagaries of IHC, the need for standardization of the techniques used, as well as the necessity for careful interpretation of stained sections (Rudiger, Hofler, Kreipe, Nizze, Pfeifer & Stein 2002). However, if one considers the number of variables inherent in the procedure, standardization seems complex and almost impossible to implement (Taylor 1994; Taylor 1998; Taylor 2000; O'Leary 2001). Debate continues today about the lack of intra- and inter-laboratory reproducibility, optimal methods for AR, variations in antibody affinity, selection of antibodies, detection systems, diagnostic thresholds, proper controls, and source and quality of reagents. To this mix can be added other variables that relate to the storage of specimens, duration and type of fixation and conditions of tissue processing! For so many variables, especially those that are difficult to control, standards that all laboratories can accept and sustain seem unattainable.

In 2003, the American Association of Veterinary Laboratory Diagnosticians (AAVLD) established a subcommittee to investigate issues of standardization and validation of immunohistochemical tests in veterinary medicine. After five years of rigorous debate, the subcommittee published guidelines for the optimisation of IHC in and between veterinary laboratories (Ramos-Vara *et al.* 2008). The present overview aims to a) introduce the reader to the concepts of standardization and validation in relation to IHC, and b) briefly outline the suggested requirements for the optimisation of IHC, as presented in the subcommittee guidelines.

1.6.1. Standardization

The standardization of an IHC test has been defined as the careful selection of a set of optimal laboratory conditions and procedures to ensure the binding of a selected primary antibody with its expected antigen. Some of these conditions and procedures include: Instrumentation, AR, pre-treatments, incubation conditions (antibody dilutions and incubation times), tissue and reagent controls, buffers and detection systems. In addition, the type of tissue fixative and what constitutes adequate fixation, as well as the choice of primary antibody and even technician error (especially critical in laboratories where immunostaining is still done manually) need to be carefully considered during the standardization process. For more detail on these topics you may refer to the proposed guidelines for IHC in veterinary laboratories (Ramos-Vara *et al.* 2008).

1.6.2. Validation

Validation is essentially the process whereby the performance characteristics of a test are established. Ideally, the sensitivity and specificity of any test, including IHC, should be compared to the gold standard method of detection for the antigen under investigation. In this section, the terms diagnostic sensitivity (D-SN) and diagnostic specificity (D-SP) will be defined. Furthermore, I briefly consider sample size and how it relates to the accuracy of these estimates.

Topics covered under the section on validation in the Ramos-Vara *et al.* (2008) guidelines include: the detection of cross-reactivity of the selected primary antibody with a) unrelated antigens, b) closely-related antigens or antigens that produce similar lesions in selected target organs, and

cross-reactivity among c) different tissues and d) different host species. Other factors that were considered include: a) The effect of the duration and type of fixation on antigen conformation (conformational changes could feasibly have a deleterious effect on antibody recognition of antigen), and b) the potential effect of the length of storage of paraffin blocks on tissue antigenicity, especially in cases that have been archived for 10 or more years. For detail on each of these topics see the proposed guidelines (Ramos-Vara *et al.* 2008). Importantly, validation should be perceived as an ongoing process requiring continual intra-laboratory monitoring of test performance, the maintenance and improvement of validation criteria during routine test use, and the establishment of an inter-laboratory validation practice (Wiegers 2004; Ramos-Vara *et al.* 2008).

1.6.2.1. Definitions of diagnostic sensitivity and diagnostic specificity and the effect of sample size on the accuracy of these estimates

Diagnostic sensitivity and D-SP are obtained from test results on samples obtained from selected reference animals (Jacobson 2004). Therefore, the methods used to select the reference animals are critical to the accuracy of the estimates (Jacobson 2004). The D-SN and D-SP are, respectively, the proportion of true positives of all positive results, and the proportion of true negatives of all negative results (Jacobson 2004). Sample size is another important consideration for obtaining reasonable certainty in the estimated values of D-SN and D-SP (Jacobson 2004). Clearly, the more samples that are scored, the more accurate these estimates will be. A typical measure of the accuracy of a parameter is its 95 % confidence interval (CI). By definition, this is the interval that will contain the true parameter with a probability of 0.95. Pertaining to the measurement of D-SN and D-SP, where samples are either scored correctly or incorrectly (and therefore should have a binomial distribution), the same reasoning applies (Clopper & Pearson 1934). The confidence intervals on estimates of D-SN and D-SP can easily be calculated with the binomial test in R (binom.test command), which is freely available (version 2.6.1. R Development Core Team 2007). Tests that are highly regarded tend to have D-SN and D-SP values that approximate 100 %. For tests with 100 % estimates, Blyth (1986) gave an easy yet exact calculation of the 95% confidence limits: The upper limit is the same as the estimate of sensitivity or specificity, namely 1. The lower limit is given by $(\alpha/2)^{1/n}$, where α is the CI (for instance α = 0.05

gives the 95% CI; Fig. 1.1). If, for such a highly sensitive and specific test, one is satisfied with a CI that is 5 % (or, in terms of probabilities, 0.05 wide), then one needs to obtain at least 59 samples in order to get initial estimates of D-SN and D-SP. However, as is more often the case in practice, if a test is not perfect (i.e. when false-positive and/or -negative results occur), the CI grows rapidly and far larger sample sizes (pertaining to proved positive and negative cases with respect to the antigen under investigation) will be required (Fig. 1.1). Because tests are continually being used, it is clearly good practice to continue scoring whether a particular test worked (compared to the gold standard test for a defined antigen) and to update the estimates and the confidence intervals on a yearly basis until such time as a decision is made to leave the gold standard behind.

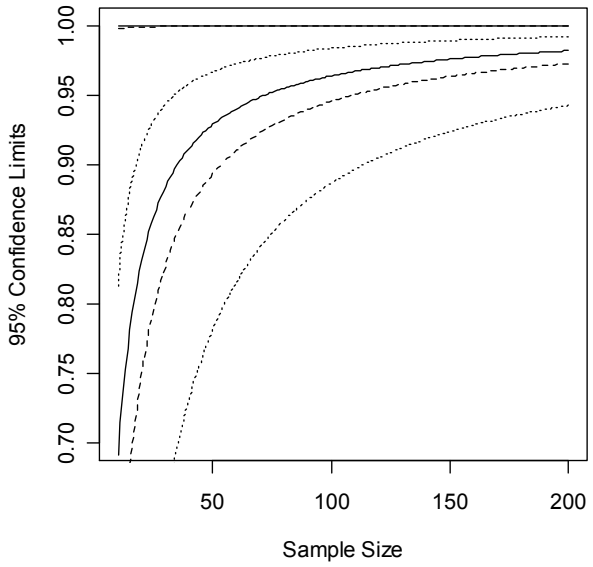

FIG. 1.1. The lower and upper 95 % confidence limits for a given sample size if all samples were scored correctly (solid lines), all but one sample was scored correctly (dashed lines), and all but five samples were scored correctly (dotted lines). Note that the upper solid line mostly occludes the upper dashed line.

1.7. Objectives of the present study

The present study will use anti-AHSV polyclonal serum and the basic ABC staining protocol as presented in the Wohlsein *et al*. studies (1997, 1998) to standardize and initiate the validation of an IHC test for AHSV in selected tissues from naturally- and experimentally-infected horses. Both the standardization and validation processes require the selection of appropriate test samples. Therefore, samples will be obtained from at least 60 AHSV-positive, and at least 60 negative horses. Positive test cases will be selected on the basis of AHS-like clinical signs and macroscopic pathology as well as confirmatory virus isolation results. Negative test cases will be obtained from countries that are AHS-free. Spleen, heart and lung specimens (not from any particular location in these organs) will be selected as the target tissues for AHSV. Target tissues from the positive and negative cases will be scored and compared in order to establish initial estimates of D-SN and D-SP for this test. In addition, multiple sections per target tissue/organ will be stained and scored in order to determine the repeatability of the staining. The target cells for AHSV and the location of viral antigen within these cells will be carefully documented per organ examined, with a view to comparing the findings with what is known regarding the cellular tropism of orbiviruses in general.

The capacity of the selected primary antiserum to detect all nine known serotypes of AHSV will be investigated, as will the cross-reactivity of the antiserum with selected serogroups in the genus *Orbivirus*. Specifically, I will investigate antiserum cross-reactivity with EEV and BTV in suitable FFPE tissues from proved positive necropsy cases (based on the presence of typical clinical signs and macroscopic pathology, with confirmatory virus isolation results). Unfortunately, no tissue samples were available from definite EHDV-positive animals in South Africa. The cross-reactivity of the antiserum with AHSV in dog spleen, lung and heart will also be investigated, although I do not anticipate a significant number of confirmed positive cases in this unusual host species. An attempt will also be made to document the target cells in this species.

A fixation kinetics study will be done on 10 tissue-culture confirmed AHSV cases, where selected target tissues will be fixed in 10 % buffered formalin and sub-samples immunostained after 2, 4, 8, and 16… up to 365 days of fixation, in order to evaluate the effect of duration of formalin fixation on

tissue immunogenicity. Finally, target (and other) tissues from AHSV-positive cases that have been archived for between 10 and 85 years will be immunostained in order to evaluate the potential effect of the length of storage of paraffin blocks on tissue antigenicity. A few of the Maurer & McCully (1963) and Newsholme (1983) cases that were used in their highly-cited reports of the pathology of AHS will be immunostained in the present study. In addition, sections of brain from horses that were intranasally inoculated with neurotropic strains of AHSV (in the 1960s) will be immunostained in the hope of identifying the target cells for neurotropic virus in the brain.

In summary, poor infrastructure in developing countries may lead to difficulties in the collection and transport of viable samples to diagnostic laboratories. Discrete outbreaks of AHS occasionally occur in far off regions of Africa and Asia and sometimes the only morbid materials available are variably-autolysed formalin-fixed materials, which are amenable only to histological and immunohistochemical examination. Indeed, in reality, a paraffin block is often the only sample available for routine diagnostics in veterinary medicine. If the present study is successful, the optimised IHC technique will be showcased as a robust alternative diagnostic test for AHSV in FFPE horse tissues. This would be a useful advance for AHS diagnostics in the African context. Furthermore, this will be the first attempt to standardize and validate an IHC test for an infectious disease according to the guidelines proposed by Ramos-Vara *et al.* (2008). Thus, the data generated from this study and the analysis thereof could contribute to the current debate on standardization and validation of IHC.

CHAPTER 2

MATERIALS AND METHODS

2.1. Test samples

2.1.1. Selection of target tissues

The post mortem tissues routinely received during African horsesickness virus (AHSV) outbreaks usually comprise horse spleen, heart, liver, lymph node and lung (Hamblin *et al.* 1991; G.H. Gerdes, unpublished data 2003). Furthermore, based on the cellular and tissue tropism of AHSV (Laegreid *et al.* 1992; Brown *et al.* 1994; Wohlsein *et al.* 1997, 1998; Carrasco *et al.* 1999; Gómez-Villamandos *et al.* 1999; Maxie & Robinson 2007; reviewed in chapter 1), I decided to examine one spleen, heart and lung sample for each case reported in this study. African horsesickness virus does not show a preference for particular localities in the heart, lung or spleen (Brown *et al.* 1994; Wohlsein *et al.* 1997, 1998). Therefore I decided not to deliberately sample multiple different parts of these tissues per case.

For each test case (defined below) sections of spleen, lung and heart (or whichever of these was available) were combined in a single wax block. In a number of cases, other tissues were present in addition to spleen, lung and heart tissues (i.e. often wax blocks are submitted from private veterinary pathology laboratories with numerous tissues embedded in a single block). In these cases positive staining was also scored in the additional tissues. One section per block was cut and submitted to the Pathology laboratory (Department of Paraclinical Sciences/DPS, Faculty of Veterinary Science, University of Pretoria/UP) for haematoxylin and eosin (HE) staining in order to assess the level of autolysis in the sections, which was subjectively scored as mild, moderate or severe. Only the cases in which the level of autolysis in the tissues was mild or moderate were chosen for the study. This corresponds with the level of autolysis in the majority of formalin-fixed samples sent to the laboratory for immunohistochemistry (IHC) to detect infectious agents. In addition, I wanted to avoid any non-specific staining that might be associated with advanced tissue decomposition.

2.1.2. Positive test cases

To establish the diagnostic sensitivity (D-SN) of the IHC test for AHSV, positive tissues were collected from AHSV-positive horses (field cases; all nine serotypes) that were presented for necropsy at the Pathology Section, DPS, Faculty of Veterinary Science, UP. Cases that were considered positive presented with typical epidemiology, clinical signs and macroscopic pathology. More importantly however, AHSV had to have been isolated and serotyped by means of virus neutralization (VN) or polymerase chain reaction (PCR) before a case could be confirmed as positive. In addition (utilizing the same selection criteria), the recently archived (less than 10 years old, or processed from the beginning of 1997 onwards) formalin-fixed, paraffin-embedded (FFPE) tissues from AHSV-positive horses were collected from the following places in South Africa over a 5-year period:

- Section of Pathology at the Onderstepoort Veterinary Research Institute (OVI).
- Section of Pathology, DPS, Faculty of Veterinary Science, UP.
- The Western Cape Provincial Veterinary Laboratory/WCPVL (Stellenbosch).

One hundred and twenty eight suitable AHSV-positive cases (including a diverse selection of cardiac, pulmonary and predominantly mixed clinical forms of AHS) were obtained for this part of the study. Not all of the target tissues were present in each case, and in some cases additional tissues (liver, kidney, gastrointestinal tract (GIT), lymph node, thyroid and adrenal glands) were available, and these were also tested. A breakdown per tissue type is given in table 2.1.

TABLE 2.1. Summary of the number and type of positive tissue samples obtained.

Sample	Number
Lung	115
Spleen	110
Heart	76
Liver	18
Kidney	11
GIT*	8
Lymph node	4
Other	1
Total cases	128

* Gastrointestinal tract

2.1.3. Negative test cases

In order to determine the diagnostic specificity (D-SP) of the IHC test, lung, spleen and heart samples had to be obtained from known AHSV-negative horses. I decided to collect the required horse tissues from countries where a) African horsesickness (AHS) has never been reported and b) horses are not vaccinated against AHSV. One hundred and nineteen suitable negative horse cases were obtained from the United States of America (USA), United Kingdom, Australia and New Zealand (Appendix 1). The selected cases did not always contain all of the three target tissues. A summary of the negative samples is given in table 2.2.

TABLE 2.2. Summary of the number and type of negative tissue samples obtained.

Sample	Number
Total Lung	111
Total Spleen	92
Total Heart	90
Spleen, Lung & Heart	77
Lung only	15
Heart & Lung only	10
Spleen & Lung only	9
Spleen only	5
Heart only	2
Spleen & Heart only	1

2.2. Controls

2.2.1. Positive-tissue control case

A positive-tissue control case, where the presence of AHSV was confirmed via virus isolation, and where typical positive staining was observed in target cells of selected target organs (as described by Wohlsein *et al.* 1997, 1998) was chosen to establish the validity of immunostaining for the detection of AHSV in FFPE tissues, using the basic staining technique described by Wohlsein *et al.* (1997, 1998). For the positive-tissue control case, pieces of spleen, lung and heart were obtained from a fresh horse necropsy case that presented with the typical epidemiology, clinical signs and macroscopic pathology associated with AHS. The animal was a one-year-old cross-Arabian gelding that was brought to the Pathology Section, DPS, for post mortem in May 2004 (post mortem number 444.04 and histopathology accession number S1253.04). Virus isolation, done by G.H. Gerdes at the World Reference Centre for AHSV and bluetongue virus (BTV) in South Africa, confirmed the presence of AHSV-4 in the lung and spleen of this horse. One hundred reference positive control sections were stored in the form of paraffin tissue sections in closed, labelled slide boxes in the IHC laboratory,

Pathology Section, DPS. This laboratory is kept between 22 and 25 ºC throughout the year. There was no obvious variation in the staining of the positive-tissue control sections over time (i.e. no evidence of tissue section aging).

2.2.2. Reagent (antibody) control

2.2.2.1. Non-specific (irrelevant) antiserum

To assess the extent of non-specific binding by the labelling or secondary antibody, for one section from each positive and negative case, a polyclonal rabbit anti-rabies virus serum was substituted for the primary polyclonal antiserum to AHSV, keeping all other staining variables constant, including the concentration and incubation time of the primary antiserum (Haines & Chelack 1991; Taylor 1994; Taylor, Shi, Barr & Wu 2002; Ramos-Vara *et al.* 2008). In addition, to prove that the negative control antiserum could still bind to its target (rabies) antigen, one histological section of rabies-positive (fluorescent antibody technique/FAT- and IHC-positive) horse brain tissue was stained via the same staining method, using the polyclonal anti-rabies virus serum, for each batch of tissue sections submitted for immunostaining per day.

2.3. Characterization of primary antisera/antibodies and non-specific antisera

2.3.1. Anti-African horsesickness virus antibodies

2.3.1.1. Hamblin antiserum

This polyclonal rabbit anti-AHSV serum was the primary antiserum of choice for the present study. It was obtained as a gift from C. Hamblin, when he was still at the Institute for Animal Health, Pirbright Laboratory, in the United Kingdom. The same antiserum was used in an avidin-biotin complex (ABC) detection system by Wohlsein *et al*. (1997, 1998), who first demonstrated AHSV antigen in FFPE tissues from three ponies that were experimentally infected with a virulent strain of AHSV-4.

The AHSV that was used to make the antiserum was a South African isolate of serotype 9, from 2 May 1973, designated as 90/61 (MB3, BHK4), and supplied by B.J. Erasmus of the World Reference Centre for AHSV, Onderstepoort (Burroughs, O'Hara, Smale, Hamblin, Walton, Armstrong & Mertens 1994). To obtain the antiserum, rabbits were immunized (via the subcutaneous route) twice, 28 days apart, with purified AHSV particles, at first in Freund's complete adjuvant/FCA and then in Freund's incomplete adjuvant/FICA (Hamblin *et al.* 1991; Wohlsein *et al.* 1997, 1998). Rabbits were exsanguinated 10 days after the second inoculation.

The intact viral particles that were injected into the rabbits had been plaque-purified on baby-hamster kidney clone 21 (BHK-21) cells, followed by purification using a combination of detergent treatments and centrifugation on sucrose gradients, according to procedures originally described for the purification of BTV by Mertens, Burroughs & Anderson (1987, cited by Burroughs *et al.* 1994), with some differences in the centrifugation conditions and with the addition of reducing agent treatment in order to obtain a higher yield of more purified particles that were not contaminated by significant quantities of non-structural AHSV proteins (Burroughs *et al.* 1994). Ultimately the purified viral particles lacked detectable host cell BHK-21 proteins (as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis/SDS-PAGE or indirect enzyme-linked immunosorbent assay/ELISA) (Burroughs *et al.* 1994). Immunoprecipitation, followed by electrophoretic analyses and fluorography, revealed that the same viral particles lacked most of the non-structural viral proteins, but did contain the structural proteins, designated VP1 to VP7, and two additional conformational variants of VP5 (one of two major proteins forming the outer capsid), as well as nonstructural protein 2 (NS2), a virus-specific protein found in cells infected with orbiviruses, and a major component of virus inclusion bodies (VIBs) (Burroughs *et al.* 1994; Uitenweerde *et al.* 1995). The NS2 was present as a variable component of the purified intact viral particles, and probably co-purified with the particles or attached somehow to their outer surface (Burroughs *et al.* 1994). Similarly, non-structural proteins NS1 and NS2 of bluetongue virus (BTV) have both been found to be associated with BTV purified on sucrose gradients (Viljoen & Huismans 1989). Special staining of the sucrose purified AHS viral particles, followed by transmission electron microscopic (TEM) evaluation was used to confirm the basic orbivirus structure of the final viral particle product (Burroughs *et al.* 1994).

2.3.1.2. 1F1 monoclonal antibody

After all the immunostaining results (using the Hamblin antiserum) had been analysed, I briefly compared the staining results of this antiserum with those of an anti-AHSV monoclonal antibody (mAb) on tissues from a few selected cases. This was done to see if the mAb could also detect all serotypes of AHSV in horse and dog tissues, and if it would be less likely to cross-react with non-AHSV epitopes in horse and dog tissues. Cases that were selected for staining with the mAb therefore included: Nine positive horses (where tissues were less than 10 years old), one of each serotype (cases 124, 126, 127, 136, 137, 139, 141, 331 and 377; Appendix 2), all AHSV-positive and -negative dogs (Appendix 5), all the equine encephalosis virus (EEV)- and BTV-positive cases (Appendices 6 and 7 respectively), the four horses that were intranasally inoculated with neurotropic vaccine strains of AHSV (cases 286, 287, 289 and 290; Appendix 3), as well as two AHSV-negative horse cases from Australia, where the cause of death was pneumonia due to *Rhodococcus equi* (cases 54 and 72; Appendix 1).

The alternative anti-AHSV mAb (designated 1F1) was developed in a South African research laboratory, against a dominant epitope on the VP7 protein of AHSV (van Wyngaardt, du Plessis, van Wyngaardt & Verschoor 1992). This VP7-specific (therefore group-reactive) mAb (isotype IgG2b) was directed against AHSV-3. It was developed and donated as a gift to the IHC laboratory, DPS, in 2007, by W. van Wyngaardt of the OVI. The original AHSV-3 isolate was obtained from B.J. Erasmus, propagated in BHK-21 cells and purified according to methods described for BTV by Huismans, van der Walt, Cloete & Erasmus 1987a). According to van Wyngaardt *et al.* (1992), BALB/c or C57BL/6 strains of mice were immunized subcutaneously with purified AHSV, emulsified in FCA, which was repeated two weeks later. After three to five weeks the mice were further boosted via intraperitoneal inoculation, and three days after the last injection, the mice were sacrificed and splenocytes were obtained and fused with Sp2/0 myeloma cells. The specificity of the mAb for VP7 alone was determined by immunoprecipitation and the antibody was considered a promising candidate for use in a routine assay for antibodies to all AHSV serogroup members (van Wyngaardt *et al.* 1992; du Plessis, van Wyngaardt, Romito, du Plessis & Maree 1999).

2.3.2. Non-specific antisera

2.3.2.1. Equine encephalosis virus antiserum

This antiserum was a gift from C. Hamblin in 2001. It is an anti-EEV polyclonal rabbit serum directed against multiple epitopes on VP7, a conserved, serogroup-specific protein (Crafford, Guthrie, van Vuuren, Mertens, Burroughs, Howell & Hamblin 2003). The antiserum was used in the development and validation of a serogroup-specific, indirect sandwich ELISA to detect EEV antigen (Crafford *et al.* 2003). The same antiserum has subsequently been used to determine the cellular and tissue tropism of EEV in cell cultures and in FFPE tissues from experimentally-infected horses (A.D. Pardini, unpublished data 2007).

To obtain the antiserum, EEV-1 (Bryanston strain) was grown in BHK-21 cells and the harvested infectious sub-viral particles (ISVPs) and core particles were subsequently purified on discontinuous sucrose and cesium chloride/CsCl gradients (Crafford *et al.* 2003). Purified ISVP antigen was inoculated subcutaneously into rabbits twice (first in FCA, and then in FICA), 28 days apart. Ten days after the second immunization rabbit anti-EEV-ISVP serum was collected (Crafford *et al.* 2003). This antiserum was used in the antigen cross-reactivity part of the validation process (see section 2.9.1.1).

2.3.2.2. Bluetongue virus antiserum

A group-reactive polyclonal antiserum raised in rabbits against BTV-4 was obtained from the OVI in 1991. No more details regarding the development and purity of this antiserum are available. The antiserum was employed in the antigen cross-reactivity part of the validation process (see section 2.9.1.2).

2.3.2.3. Rabies virus antiserum

A polyclonal rabbit anti-rabies-and-rabies-related-viruses serum, lot number 9/2/88, was supplied by the late Dr Ken Charlton, of the Animal Diseases Research Institute in Ontario, Canada to the IHC laboratory, Section of Pathology, DPS. This antiserum was used instead of the anti-AHSV serum for negative reagent control purposes (see section 2.2.2.1).

2.3.2.4. Equine herpesvirus antiserum

This rabbit-derived polyclonal antiserum that is cross-reactive between equine herpesvirus (EHV)-1 and EHV-4 was obtained from Dr Ken Smith at the Animal Health Trust in Newmarket, England in 1993. This antiserum was applied in tandem with the AHSV, EEV and rabies virus antisera to the sections of brain from four archive cases (dating back to 1962 and 1963) where neurotropic vaccine strains of AHSV were inoculated intranasally in horses (cases 286, 287, 289 and 290; Appendix 3; see section 2.11.1.1).

2.4. Tissue fixation and processing

Wherever it was possible to control, and to ensure uniform fixation, 1-cm-thick slices from selected AHSV-positive and negative target tissues were obtained from horse (and two dog) necropsy cases. Most of these tissue samples were exposed to room air and temperature (i.e. predominantly between February and early May with peak daily temperatures ranging from 28 ºC in February down to 22 ºC in May in Pretoria) for between 2 and 5 hours before being immersed in formalin. Occasionally, samples were stored in fomalin between 18 and 24 hours after death (i.e. after overnight refrigeration of carcasses). These tissues were usually fixed in 10 % neutral buffered formalin for 24-48 hours, in accordance with approved methods (Haines & Chelack 1991; Wohlsein *et al.* 1997, 1998; Ramos-Vara 2005; Ramos-Vara *et al.* 2008). The length of formalin-fixation was sometimes unknown for the wax-embedded tissues that were 10 years and older.

The available spleen, lung and heart tissue samples from each test case (by definition less than 10 years old) were embedded in a single wax block, sectioned, and mounted according to a standard operating procedure in the Section of Pathology, DPS (Bancroft & Gamble 2002). In some cases a few extra tissues were also present in the tissue block. Initially, just one section was cut and HE-stained per positive and negative tissue block in order to assess the level of autolysis in tissues, thereby facilitating the selection of suitable test cases for the study. Once the test cases had been selected, another three sections were cut per case/tissue block (i.e. two test sections, A and B, at least 10 μm apart in the tissue block, and one section for staining with the negative control reagent) and submitted for manual immunostaining according to the ABC system detailed below. In total therefore, four 3-4 μm-thick (Wohlsein *et al*. 1997, 1998) sections were cut per test case/wax block.

2.5. Immunohistochemistry

2.5.1. Standard staining method

The ABC detection system employed in my IHC laboratory has been adapted from the basic methodology tabled by Haines & Chelack in 1991. Standardization of the present IHC test for AHSV was greatly simplified by the prior demonstration of immunoreactivity (using the same Hamblin antiserum and basic ABC detection system) in FFPE tissues from three ponies that were experimentally infected with AHSV-4 (Wohlsein *et al.* 1997, 1998). In addition, prior to the present study, a small pilot study was done on known infected horse target tissues (specifically spleen, lung and heart) to investigate optimal conditions for strong, specific positive staining. Ultimately, the best staining results were achieved by modifying the Wohlsein *et al.* (1997, 1998) staining technique only slightly to suit our standard immunostaining procedures and laboratory conditions. The Wohlsein *et al*. (1997, 1998) immunostaining method was optimized for use as follows in the IHC laboratory (Section of Pathology, DPS).

Per test case two test sections (labelled A and B) were stained according to the ABC detection system (see below), using the group-specific Hamblin antiserum. The third section was stained using the same ABC technique, but the anti-AHSV serum was substituted with an unrelated polyclonal anti-

rabies virus serum. Per batch of 20 test cases stained per day, target tissues from the positive control case were stained simultaneously to establish specific positive staining.

Sections submitted for immunostaining were mounted on positively-charged microscope slides (SuperFrost® Plus, Menzel-Glasser®) to enhance tissue adhesion for immunostaining (Haines & Chelack 1991). The sections were then dried overnight in a 58 ºC-oven to warm and melt paraffin in tissue sections and enhance tissue adhesion (Rickert & Maliniak 1989; Haines & Chelack 1991). Routine dewaxing in xylol, followed by rehydration through a graded ethanol and distilled water series took place inside a fume hood (10 minutes in xylol, and 3 minutes each in 100 %, 96 % and 70 % ethanol) (Bancroft & Gamble 2002). Sections were subsequently incubated with 3 % hydrogen peroxide in methanol (van der Lugt, Coetzer, Smit & Cilliers 1995; van Alstine, Popielarczyk & Albregts 2002) for 15 minutes in a humidified chamber at room temperature (22 to 25 ºC), to quench endogenous peroxidase activity. Sections were rinsed in distilled water, followed by 0.1 molar (M) phosphate buffered saline/PBS, pH 7,6 containing 0.1 % bovine serum albumin (BSA) for 5 minutes per rinse (Appendix 8).

The enzymatic digestion method for unmasking epitopes (as described by Wohlsein *et al.* 1997, 1998) elicited improved staining results compared to a) no antigen retrieval (AR) and b) the heat-induced epitope retrieval (HIER) method, via microwave heat in different buffer solutions, pH of 6.0-6.2 and 9 at approximately 96 ºC (Mikel 1994; Boenisch 2001; Taylor *et al*. 2002; Ramos-Vara 2005; Ramos-Vara *et al.* 2008). Accordingly, tissue sections were digested by immersion of the slides in a solution of protease XIV (catalogue no: P5147-5G, Sigma Chemical Co, St. Louis, Missouri, USA), 50 mg in 100 ml of PBS/BSA buffer solution (pH 7.6, pre-warmed to 37 ºC) for 20 minutes in a humidified chamber at 37 ºC. Sections were rinsed in distilled water and then in PBS/BSA buffer for 5 minutes.

Non-specific immunoglobulin binding was blocked by pre-incubation of slides with normal goat serum (catalogue no: G9023, Sigma Chemical Co, St. Louis, Missouri, USA), diluted 1:5 with PBS/BSA buffer, pH 7.6 for 20 minutes in a humidified chamber at room temperature. The blocking serum was drained and replaced by 100-150 µl of primary anti-AHSV serum (i.e. enough to completely cover the tissues), at a dilution of 1:1500 in PBS/BSA buffer for 45 minutes in a humidified chamber at 37 ºC

(Wohlsein *et al.* 1997, 1998). This was the optimal dilution for the Hamblin polyclonal antiserum that resulted in the best specific staining while providing the least background staining.

The biotinylated polyclonal goat-anti-rabbit secondary or link antibody (catalogue no: EO432, DakoCytomation, Glostrup, Denmark) was used at a dilution of 1:150 in PBS/BSA buffer containing 10 % normal horse serum (catalogue no: H 0146, Sigma Chemical Co, St. Louis, Missouri, USA) (Wohlsein *et al.* 1997, 1998). The link antibody was incubated with the tissue sections for 30 minutes in a humidified chamber at room temperature, followed by rinsing in distilled water and then in PBS/BSA buffer as before. Peroxidase Conjugated Avidin (diluted 1:500) (Vectastain ABC Kit, Elite PK6100 Standard, Vector Laboratories, Burlingame, California, USA) was applied and incubated on the sections for one hour in a humidified chamber at room temperature (Wohlsein *et al.* 1997, 1998). The sections were rinsed twice, as before.

For the purpose of contrasting the brownish tissue pigments, especially haemosiderin in horse spleen, the sections were immersed in a NovaRED substrate (catalogue no: SK-4800, Vector Laboratories, Burlingame, California, USA), reconstituted according to manufacturer's instructions, and using the droplet method, for approximately 3-5 minutes, in a humidified chamber, at room temperature. During this time, the positive control section with the same substrate was examined carefully for signs of positive staining at 100 X magnification, using the light microscope in the IHC laboratory, Section of Pathology, DPS. As soon as there was clear evidence of positive staining in the positive control section, all of the sections per batch of 20 cases stained per day were immediately rinsed in a distilled water bath to halt the substrate reaction. The sections were then counterstained with Mayer's haematoxylin for 3-4 minutes and rinsed under running tap water (at gentle bathing pressure) for 10 minutes to remove excess substrate. The use of a red-, as opposed to Wohlsein *et* al. (1997, 1998)'s black-coloured chromogen deposit, allowed me to differentiate easily between tissue pigments and specific positive staining. This was simply because red stands out better than black against a blue (haematoxylin-counterstained) background. Sections were routinely dehydrated through increasing alcohol concentrations and xylol, mounted and coverslipped.

2.5.2. Modifications to the standard staining method (applied to selected African horsesickness virus-positive archive horse cases and two positive dog cases)

One or more of the selected target tissues (spleen, lung and heart) from 10 of the 72 AHSV-positive archive cases that were 10 years or older failed to show any evidence of specific positive staining on the first staining attempt, despite the presence of abundant characteristic positive staining in the positive-tissue control section (cases 286, 288, 296, 307, 315, 316, 318, 325, 327 and 328; Appendix 3). For these particular cases staining was repeated at the end of the study, using a) harsher AR methods, b) more concentrated primary antiserum (1:500) and/or a longer incubation of the Hamblin antiserum with the tissue sections (90 minutes and overnight), and c) more sensitive detection systems. Tissues from the two positive dog cases (383 and 384; Appendix 5) were subjected to the same treatment in an attempt to optimize AHSV-specific positive staining in this species.

The harsher AR method involved incubating 2 mg Protease, Type XIV (as above) with 200 ml of 1X Dako Target Retrieval Solution (10X concentrate product; catalogue no: S1699, DakoCytomation, Glostrup, Denmark) for 40 minutes at 96 ºC. Tissues were also subjected to microwave heating in different buffers (without Protease), pH of 6.0-6.2 and 9 for 20, 30 and 40 minutes. More sensitive detection systems that were employed for these cases included the LSAB+ Kit, HRP (catalogue no: K0679, DakoCytomation, Glostrup, Denmark), as well as the NovoLink™ Min Polymer Detection System (catalogue no: RE7290-K, Novocastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom).

2.5.3. Modifications to the standard staining method (when the Hamblin antiserum was replaced by the 1F1 monoclonal antibody)

The slightly modified Wohlsein *et al.* (1997, 1998) technique was used as described above (section 2.5.1), except that non-specific immunoglobulin binding was blocked by incubation of slides with normal rabbit serum (catalogue no: R9133, Sigma Chemical Co, St. Louis, Missouri, USA), and the secondary antibody employed was a biotinylated rabbit-anti-mouse polyclonal antibody (catalogue no: E0354, DakoCytomation, Glostrup, Denmark).

2.5.4. Modifications to the standard staining method (when the Hamblin antiserum was replaced by equine encephalosis virus and bluetongue virus antisera)

All of the horse and sheep tissues that were stained with the primary anti-EEV serum (refer to section 2.9.1) were processed as above except that the EEV antiserum was diluted 1:6000 and incubated with the appropriate tissue sections for two hours in a humidity chamber at room temperature (between 22 and 25 ºC). The primary polyclonal BTV antiserum was also applied to horse and sheep tissue sections according to the ABC method described above, except that the antiserum was diluted 1:500 and incubated with tissues for 90 minutes in a humidity chamber at 22 to 25 ºC. The BTV and EEV positive-tissue controls were closely monitored at 100 X magnification using the light microscope for granular and/or bead-like positive staining, in order to determine the incubation time of test sections with the NovaRED substrate.

2.6. Nature of positive staining and target cells for African horsesickness virus

The interpretation of positive staining was based on the staining results as described and illustrated in Brown *et al.* (1994) and Wohlsein *et al*. (1997, 1998). Positive staining (with particular reference to the NovaRED substrate) was indicated by the presence of quite fine, bright red to fuchsin or burnt-sienna granules (i.e. dust-like particles) and/or small bead- or- dot-like deposits (usually occurring singly, but sometimes visible in small clusters) in the cytoplasm of mononuclear leukocytes (predominantly monocytes and macrophages), and endothelial cells of capillaries, small post-capillary venules and pre-capillary arterioles, particularly in sections of spleen, lung and heart (Brown *et al.* 1994; Wohlsein *et al.* 1997, 1998). For the purpose of the present study, interstitial macrophages were usually identified as such based on cell location, morphology and often, the presence of intracytoplasmic pigment granules , e.g. haemosiderin (especially in spleen, liver and lung), bile (in the liver) or carbon (in the lung).

The small, positively-staining granules or beads were best visualised under the light microscope at 400 X magnification. A case was only conclusively scored AHSV-positive if there was no ambiguous

positive staining in the accompanying irrelevant antiserum control section. An additional facet of the present study was a thorough evaluation of the cell- and tissue-tropism of natural and experimental AHSV in FFPE tissues from horses and (two) dog cases, and the correlation of our findings with those of previous studies.

2.6.1. Immunostaining scoring system

I adopted a simple, fast immunostaining scoring system (Table 2.3) that would mimic the way in which a diagnostic pathologist might evaluate any immunostained tissue.

TABLE 2.3. Grading system for positive and negative immunostaining.

Character	Grade
No AHSV-specific staining	1
Sparse positive staining (Difficult diagnosis)	2
Ample positive staining (Easy diagnosis)	3

The selected grading system was based on the staining results in 20 AHSV-positive cases selected for a pilot study in 2004. It was decided not to use intensity of positive staining as a criterion for routine scoring of positive staining, because, though the granules/beads are very small, and sometimes few in number, the pilot study demonstrated that positive staining could be scarce but still intense.

The criteria for grade 2 were as follows: The AHSV-specific positive granules and beads tended to be fine and discrete, occur singly and/or in small clusters (2-5 spots in a cluster), and in no more than two target cells per 5 or more high power (400 X) fields per tissue (on average). The criteria for grade 3 were as follows: The AHSV-positive granules and beads were usually more prominent (compared to

grade 2), and occurred singly and in conglomerates (two or more) in at least three target cells within fewer than 5 high power (400 X magnification) fields, on average. It was considered necessary to make a clear distinction between so-called 'easy' (score 3) and 'difficult' (score 2) cases so as to give the diagnostic pathologist some idea of the sensitivity of the test for AHSV.

Each immunostained test section (A or B) was evaluated by scoring the spleen, lung and heart (in that order), followed by other tissues (if present) in no particular order. A special note of the target cells per tissue examined was made for each AHSV-specific positive case

2.7. Statistical analysis

2.7.1. Estimation of the repeatability of the test

Repeatability refers to the chance of obtaining the same answer if the test is repeated on the same individual. In this study I distinguished between repeatability between and within different tissues for the 128 AHSV-positive and 119 negative test samples. This was considered an important component that would establish whether sensitivity/specificity is affected by variation within individuals or only by variation between individuals. In the former case, it was considered that sensitivity would be improved by reading multiple sections from each case. Furthermore, it would show if slides from multiple organs from each individual need to be taken, or whether some organs give erroneous results. To this purpose, two repeated measures of each tissue/organ (i.e. embedded in a single wax block per case) were evaluated for the cases that were less than 10 years old. These were called series A and B.

2.7.2. Randomization of test cases

An independent person randomized the test cases. All selected positive and negative case numbers were read into a single vector. The A series was randomly sampled from this vector without replacement. These cases were renumbered from one to 382. The sample number therefore did not contain any information as to whether the sample was negative or positive (i.e. in Appendices 1 and 2 respectively, all the negative horse cases are numbered one to 123 and the positive horse cases are

numbered 124 to 382). Due to constraints on the number of slides that could be manually immunostained and scored by the primary researcher at one time, batches of 20 blocks were sectioned, stained and scored per day from March to May 2007. An independent person selected the tissue blocks to be incorporated into each staining batch. Per block/test case, three sections were submitted for immunostaining (i.e. the A and B test series that received the primary anti-AHSV serum, and the section for staining with the irrelevant anti-rabies virus serum). The independent person renumbered the resulting slides generated per case. A few days after scoring the A series, the B series was renumbered by sequentially randomizing groups of 20 from the A series, until all test samples were renumbered again. Consequently, the B slides were scored a few days after the A slides (which eliminated the possibility of slide recall). Each randomized and renumbered test slide (A or B) was examined by the principal researcher in tandem with the negative reagent control slide pertinent to that particular case (i.e. the same negative reagent control slide was examined in tandem with the A and B test slide per case, albeit at different times).

2.7.3. Statistical tests

All statistical analyses were performed in R (version 2.6.1. R Development Core Team 2007). The binomial test of R (binom.test) was used to obtain 95% confidence intervals (CI) on estimates of D-SN and D-SP. Spearman rank correlations were calculated to confirm repeatability. Differences in the staining magnitude of different tissues were tested with paired Wilcoxon rank tests. To establish the D-SN and D-SP of the ABC IHC test for AHSV in FFPE horse tissues, samples were randomized by sampling, without replacement in the sample command of R.

2.8. Serogroup specificity of the Hamblin antiserum and 1F1 monoclonal antibody

There were multiple representatives of all nine AHSV serotypes amongst the positive test cases and the archive cases. The Hamblin antiserum was applied to all cases. The 1F1 mAb was only applied to a single horse case per serotype (see section 2.3.1.2).

2.9. Cross-reactivity of the Hamblin antiserum and 1F1 monoclonal antibody

2.9.1. Antigen cross-reactivity

I decided to investigate whether there was any cross-reactivity between the Hamblin and 1F1 antibodies and other closely-related orbiviruses. For practical purposes I only selected those closely-related orbiviruses that either a) also occur in horses in South Africa, for example EEV, or b) occur commonly in South Africa, e.g. BTV, and c) for which I could readily obtain FFPE tissues. Establishing cross-reactivity to EEV was considered to be most important, since, not only does it also occur in horses and is transmitted by the same vectors as AHS, but horses infected with EE may exhibit clinical signs and pathology resembling mild AHS (Howell *et al.* 2004), and, on the odd occasion, more severe forms of AHS (A. D. Pardini, unpublished data 2007).

2.9.1.1. Equine encephalosis virus-positive cases

Eleven EEV-positive horses with FFPE tissues (and where EEV was isolated and serotyped) were selected from recently archived material (cases from 1999, 2000, 2006 and 2007) from the WCPVL and the Section of Pathology, DPS. Of the 11 cases, three were experimental cases and the rest were field cases. Eight cases were of the Bryanston serotype/serotype 1 (including the three experimental cases), one was the Kaalplaas serotype/serotype 4, and two cases were of unknown serotype. The group-reactive polyclonal rabbit-anti-EEV serum directed against all seven known serotypes of EEV was used on a selected number of formalin-fixed tissues from each case (using the basic ABC detection system detailed above), to confirm that EEV antigen was present and visible in target cells (intravascular mononuclear leukocytes, tissue macrophages, and, to a far lesser extent, endothelial cells) (A.D. Pardini, unpublished data 2007), and to identify target organs from the few organs that were submitted for histological examination (target organs were predominantly spleen, lung, liver and lymph node) (A. D. Pardini, unpublished data 2007), for recombination into a single wax block.

2.9.1.2. Bluetongue virus-positive cases

Tissues from eight sheep cases that died due to infection with BTV were selected, where the epidemiology, macro- and microscopic pathology and virus isolation results were consistent with BTV infection. Formalin-fixed, paraffin-embedded tissues were collected from archive material (1964, 1994 and 2005 cases) in the Pathology Sections at the OVI and the DPS, Faculty of Veterinary Science, UP. The serotypes for the selected cases were not identified. A group-reactive rabbit polyclonal anti-BTV serum was applied to a limited variety of tissue sections per case (according to the modified ABC staining technique detailed above) to identify positive staining in target cells (predominantly swollen or fragmented microvascular endothelial cells and, to a lesser extent, circulating mononuclear cells), and to identify target organs (from the limited number submitted), namely oedematous, degenerate or necrotic skeletal muscle, ulcerated GIT, necrotic, oedematous and/or haemorrhagic myocardium, hyperaemic coronet and interdigital skin of the foot, and to a lesser extent oedematous, congested and interstitially inflamed lung (Thomas & Neitz 1947; Moulton 1961; Erasmus 1975; Parish, Evermann, Olcott & Gay 1982; Mahrt & Osburn 1986; MacLachlan, Jagels, Rossitto, Moore & Heidner 1990; Brodie, Bardsley, Diem, Mecham, Norelius & Wilson 1998; Verwoerd & Erasmus 2004; Brown, Baker & Barker 2007), so that they could be recombined into a single wax block per case.

2.9.1.3. African horsesickness virus-positive cases

Nine AHSV-positive cases (one of each serotype) where the tissues were less than 10 years old and that scored strongly positive via the ABC staining technique (with at least two target tissues present per case) were selected for the purpose of checking antigen cross-reactivity (cases 124, 126, 127, 136, 137, 139, 141, 331 and 377; Appendix 2).

2.9.1.4. Immunostaining strategy and data collection

The EEV-, BTV- and AHSV-positive cases with heart, lung and spleen tissues recombined in a single wax block per case were submitted for immunostaining with the AHSV, EEV, and BTV antisera and the 1F1 mAb (i.e. all the antisera and the 1F1 mAb were applied to all these cases). Therefore, four

sections were cut and immunostained per case. The stained sections were subsequently randomised and re-numbered by an independent person. The level of immunostaining was then graded (see section 2.6.1) per case per antiserum/antibody application and a brief note was made of the nature of positive staining.

2.9.2. Cross-reactivity in the dog, an unusual host species for African horsesickness virus

2.9.2.1. Positive dog cases

Due to the fact that AHS is primarily a disease of horses (and to a lesser extent mules) in South Africa (Coetzer & Guthrie 2004), it was initially not considered important to test for AHSV antigen in other species. However, in March 2006, a five-year-old male German Shepherd Dog that died suddenly and was presented for post mortem at the Section of Pathology, DPS, was found to have severe fibrin-rich pulmonary oedema and hydrothorax at necropsy. Both lung oedema and hydrothorax are recognized pathologic features pertaining to AHS in dogs (van Rensburg, DeClerk, Groenewald & Botha 1981; Maxie & Robinson 2007). The dog was diagnosed as having died from AHSV-6 (case 384, Appendix 5). Virus was isolated from the lung and spleen in this case, by G.H. Gerdes. Another adult domestic dog (four-year-old male Rottweiler) that was pyrexic and dyspneoic and died shortly after exhibiting clinical signs was presented for post mortem examination in May 2008. The only macro- and microscopic finding of any significance was the severe acute diffuse serofibrinous pneumonia (case 383, Appendix 5). Characteristic viral matrix and rare orbivirus-like particles (approximately 70 nm in diameter, with or without an electron-dense center/nucleoid and a halo of lower electron density) were identified via TEM in the FFPE section of lung from this case. Unfortunately tissues were not submitted for virus isolation in this case.

2.9.2.2. Staining strategy and data collection

Formalin-fixed pieces of spleen, lung, heart and a few other tissues (e.g. liver and kidney) were collected from the above two cases, and from 11 additional dog cases (Appendix 5), where the cause of death was not AHS (except for case 403, where AHS was a possibility). Tissues were recombined

in a single wax block per case. Four sections were cut per case. One section was HE-stained for examination with other HE-stained sections at the end of the study. The remaining three sections were submitted for immunostaining. One section was immunostained with the Hamblin antiserum, another with the 1F1 mAb and the third one with the rabies irrelevant antiserum (for negative reagent control purposes). Initially, the standard staining method was modified for the positive dog tissues to ensure optimization of specific positive staining in this species (see section 2.5.2). Ultimately however, the standard staining method described above (section 2.5.1) gave the best staining results.

The immunostained test slides (stained with the Hamblin and 1F1 antibodies) were subsequently randomised and re-numbered by an independent person. Each test slide was examined in tandem with the negative reagent control slide for that case. Staining in the test slides was graded according to the parameters described for horse tissues (see section 2.6.1). A special note was made of target tissues and target cells in the positive cases.

2.10. Effects of formalin-fixation on immunoreactivity

2.10.1. Case selection

In order to determine the stability of AHSV antigens during prolonged fixation in 10 % neutral buffered formalin, a fixation kinetics study was done whereby 1-cm-thick pieces of target tissues (spleen and/or lung and/or heart) were collected from 10 horse cases known to contain AHSV antigens (via virus isolation and positive immunostaining) that presented for necropsy at the Pathology section, DPS, during 2005 (Cases 193, 195, 196, 198, 200, 202, 203, 204, 205 and 207; Appendix 4). Only cases with tissues that were mildly autolysed were selected for the fixation kinetics study (i.e. these cases were selected after an HE-stained section was evaluated per case to determine the level of autolysis in the tissues).

Six of the nine AHSV serotypes were represented in the 10 cases selected for the fixation kinetics study. In four of the 10 cases used in this trial, formalin-fixed tissues only became available for wax embedding after four to eight days in buffered formalin. Samples from all cases were, however, kept in

formalin for the full 365 days. There were only four cases where all of the heart, lung and spleen tissues were available, and in two cases only the spleen was available. In the rest of the cases lung was available together with spleen or heart.

2.10.2. Storage of cases

All formalin-fixed material was stored together, on the same shelf in a poorly lit part of the Histopathology laboratory, Section of Pathology, Department of Paraclinical Sciences. This laboratory is routinely kept at approximately 25 ºC throughout the year.

2.10.3. Immunostaining strategy and data collection

Where more than one target tissue was collected per case, tissues were combined into a single wax block and sections were immunostained and scored for positive staining (see section 2.6.1) after 2, 4, 8, 16, 32, 64, 128, 256 and 365 days of fixation in the same glass bottle of formalin. A negative reagent control section (only one section was stained with the irrelevant rabies antiserum per case) was examined with each AHSV-immunostained section per case per interval detailed above. The quality of positive staining (pertaining to intensity and size of positively-staining granules/beads), related to duration of fixation, was subjectively described for each case at the end of the fixation kinetics study (i.e. after all cases had been immunostained after 365 days in formalin).

2.11. Immunoreactivity of paraffin blocks that were archived for 10 or more years

2.11.1. Case selection

None of the AHS-positive horse cases that were archived for 10 or more years were used to establish the D-SN and D-SP of the IHC test for AHSV in horse tissues. The 10-year or older archive material, of which there were 72 cases (the oldest dating back to 1923 and the newest to 1996), was dealt with separately to try to establish the possible effect of length of storage of wax blocks on tissue antigenicity. These wax-embedded tissue blocks were obtained from:

- Section of Pathology at the OVI (this was the primary source of material).
- Section of Pathology, DPS, Faculty of Veterinary Science, UP.
- The WCPVL.

Virus isolation results could not be traced for a number of the OVI cases that were stored specifically as AHS reference cases in the Arnold Theiler museum. For these cases the diagnosis of AHS was simply accepted. Due to the advanced age and brittleness of many of the older tissues, it was decided not to re-embed pieces of spleen, lung and heart in single wax blocks, with the result that a fairly wide variety of tissues (those accompanying heart, lung and spleen in their original wax blocks) were submitted for AHSV immunostaining, as shown in Table 2.4. However, wherever possible, sections of spleen and/or lung and/or heart were collected from these archive cases.

TABLE 2.4. Summary of sample sizes arranged by year and organ for the archived material.

		Sample by Year				
Organ	Total Samples	10-11	12-18	21-37	40-45	83
Spleen	60	18	11	7	23	1
Lung	62	16	15	8	23	
Heart	60	15	15	9	21	
Liver	13		1	3	9	
Lymph node	8		1	2	5	
Brain	6			2	4	
Stomach	8	1	2		5	
Small intestine	4		1		3	
Skeletal muscle	5			2	3	
Bladder	1				1	
Prostate	1				1	
Testes	3				3	
Adrenal	4		1	2	1	
Thyroid	1				1	
Kidney	7		2	1	4	
Thymus	1			1		

In a few OVI experimental cases, splenic suspensions of known viscerotropic AHSV strains were inoculated intravenously into horses, and wax-embedded target and other tissues were also collected from these cases. In addition, target and other tissues were obtained from some of the horse cases used by Maurer & McCully (1963) (cases 323, 324, 325, 326, and 327) and Newsholme (1983) (case 312) to describe the pathology of some of the earliest natural and experimental AHS cases (covering a variety of fatal clinical forms of the disease).

2.11.1.1. Horses that were intranasally inoculated with neurotropic vaccine strains of African horsesickness virus

Four of the archive cases obtained specifically from the AHS reference collection in the Arnold Theiler museum at the OVI (cases 286, 287, 289 and 290; Appendix 3) were experimental cases where neurotropic vaccine strains of AHSV were inoculated intranasally in horses, resulting in multiple foci of moderate to severe non-suppurative (predominantly lymphocytic) meningoencephalitis in numerous histological sections of brain. In case 286, the A501 strain (serotype 1) that was used was a neurotropic mouse brain-attenuated strain of AHSV (McIntosh 1958; Erasmus 1963, 1966; Polson & Deeks 1963). In case 287 a neurotropic serotype 9 vaccine strain was used (strain 7/60) (Erasmus 1966), and for cases 289 and 290 the neurotropic Karen strain (serotype 7) (Erasmus 1966) was instilled intranasally. Old records show that virus was isolated from the brains of all the intranasally infected horses after death. Per case, as many sections of brain as were available, were selected for immunostaining using the Hamblin and 1F1 anti-AHSV antibodies as well as the anti-rabies, anti-EEV and anti-EHV sera. Spleen and/or lung and/or heart samples were also collected from these cases for immunostaining.

2.11.2. Staining strategy and data collection

Per archive case (often with multiple wax tissue blocks per case), three sections were cut and stained per tissue block per case. An HE-stained section per case was put aside for examination at the end of the study. Two sections per wax block per case were submitted for immunostaining (one with the Hamblin antiserum and the other with the rabies antiserum). In all cases immunostaining was graded according to the scoring system detailed above (see section 2.6.1). In addition, the quality (intensity and size) of AHSV-specific positive staining was very briefly and subjectively described for these cases.

2.11.3. Statistical analysis

Taking all three of the selected tissues in turn (spleen, lung, heart), an ordinal logistic regression explaining the three levels of staining magnitude as a function of time of storage of archive samples was performed using the polr function with proportional odds of R's MASS library (Faraway 2006). To see if the chances of making a correct diagnosis decreased over time, the data was recoded into either a positive or negative diagnosis (i.e. scoring categories 2 and 3 were merged). A generalized linear model with binomial error structure (logistic regression) was fitted to the data (Fig. 3.84).

2.12. Histopathology associated with African horsesickness virus

2.12.1. Rationale

My decision to evaluate the HE sections for AHSV-associated lesions was based on the existence of (a) detailed descriptions of the histopathology associated with AHS (Maurer & McCully 1963; Newsholme 1983) and (b) the more recent claim that histological lesions are not useful in the diagnosis or understanding of the pathogenesis of AHS (Maxie & Robinson 2007). In addition, because a thorough knowledge of the histopathology of a disease is often critical for the correct interpretation of IHC, I decided to investigate whether whether histopathology alone might help to differentiate between positive and negative cases.

2.12.2. Method

Once all immunostaining results had been analysed at the end of the study, HE-stained sections from the positive and negative dog and horse cases (including the horse archive cases that were 10 years or older) were examined and the histology compared between positive and negative cases (pertaining only to heart, lung and spleen samples). Histological lesions were not graded since the histopathology of AHS was not the purpose of this study, but cognisance was taken of the presence/absence of any of the described lesions in heart, lung and spleen that have been associated with AHSV (see Chapter 1, section 1.3.2). The HE sections were randomised and renumbered by an independent observer. I

then tried to differentiate between positive and negative cases (i.e. as would be expected of the diagnostic pathologist). For each case that was put into the positive category, a note was made of all the histological lesions considered to be indicative of AHSV infection in that case.

CHAPTER 3

RESULTS

3.1. Nature of African horsesickness virus-specific positive staining (using the Hamblin antiserum)

Characteristic bright red to burnt sienna-coloured, single or clustered to dust-like, granular and bead-like positive staining was observed in (or, to a lesser extent, in close proximity to) target cells in selected target organs (see below). The positive beads and granules were either in a peri-nuclear location (Figs. 3.1, 3.2), or some distance away from cell nuclei, especially pertaining to endothelial cells (Fig. 3.3). Not infrequently, positive dots or granules aligned in endothelial cell cytoplasm, bordering capillary lumens (Fig. 3.4), occasionally demarcating segments of capillary vessel (Fig. 3.5).

3.2. Target tissues and cells for African horsesickness virus in horses

3.2.1. Target cells in selected target tissues (heart, lung and spleen)

The lung and heart in particular, and to a lesser extent the spleen, proved to be excellent target tissues for the detection of African horsesickness virus (AHSV) in horses, using the Hamblin antiserum and the avidin-biotin complex (ABC) detection system.

3.2.1.1. Heart

In the heart, positive staining occurred predominantly within the cytoplasm of normal, swollen, or, to a far lesser extent, shrunken and fragmented (apoptotic/necrotic) endothelial cells lining capillaries in the loose interstitial connective tissue between cardiac muscle cells (Figs. 3.6, 3.7). To a far smaller degree, positive granular to beaded or dot-like intracytoplasmic positive

staining was also observed in normal, swollen, or, less frequently, apoptotic/necrotic circulating mononuclear (monocyte-like) leukocytes within blood vessels in the interstitium (Fig. 3.8). Rarely, dot-like positive staining appeared to be located on the surface of erythrocytes in interstitial capillaries (Fig. 3.9), but, in many cases, this positive staining could also have been in endothelial cell cytoplasm, some distance away from the endothelial cell nucleus, immediately adjacent to intravascular erythrocytes (Fig. 3.10). Occasional red blood cells appeared to stain diffusely positive (Fig. 3.10).

When haematoxylin and eosin/HE-stained sections of the target organs were assessed for evidence of AHSV-specific histopathology at the end of the study, and the results compared with immunostaining results, it appeared that the amount of positive staining (which determined the score allocated) did not correlate with the extent or degree of haemorrhage, necrosis or inflammation in the heart of positive animals. This was especially evident in the histological sections of heart from archive case 325 (Appendix 3), from a horse in the Middle East with the cardiac form of AHS, described by Maurer & McCully in their 1963 article as having foci of haemorrhage, oedema, myocarditis, myocardial degeneration and scarring. Despite these lesions indeed being present, no AHSV-specific positive staining could be detected in the sections of heart (although strong positive staining was observed in sections of spleen and lung from the same case). This was despite repeated staining (done at the end of the study, after results had been analysed), using harsher antigen retrieval (AR) methods, increasing the concentration of the Hamblin antiserum and/or increasing the Hamblin antiserum incubation time, and even the use of more sensitive detection systems (see section 2.5). On the other hand, in case 323, a horse that was experimentally infected with AHS in 1961, that developed the mixed form of AHS with evidence of oedema, haemorrhage and necrosis in histological sections of myocardium (Maurer & McCully 1963; pers. obs.), there was ample AHSV-specific positive staining throughout the heart on the first (and only) staining attempt (Fig. 3.11).

3.2.1.2. Lung

In sections of lung, positive staining occurred a) in the cytoplasm of intravascular mononuclear leukocytes (with monocyte-macrophage-like morphology) (Figs. 3.12, 3.13), b) in the tapered cytoplasm of normal, swollen, or, more rarely, shrunken and fragmented (apoptotic/necrotic) endothelial cells lining pulmonary microvessels (Figs. 3.4, 3.14), and c) only rarely in the cytoplasm of tissue macrophages in the pulmonary interstitium. Usually positive staining was observed in both pulmonary intravascular mononuclear leukocytes and microvascular endothelial cells (Fig. 3.15). From a subjective viewpoint it seemed that if there were a predominance of positive staining in intravascular monocyte-macrophages in the lung, there would be fairly numerous positively-staining circulating mononuclear leukocytes (with monocyte-like morphology) in multiple organs. On the other hand, if endothelial cells were strongly positive in the lung, they would tend to be relatively more prominent (in terms of positive staining) in other organs too. There was negligible positive staining associated with erythrocytes in the lung. After the histopathology of positive and negative cases had been described at the end of the study, and these results compared with the immunostaining results, it was noted that the amount of positive staining did not appear to correlate with the extent of oedema, haemorrhage and/or inflammation in the lung.

3.2.1.3. Spleen

In the spleen, positive granules and beads were observed most commonly in the cytoplasm of a) monocyte-like cells (Fig. 3.2) and/or large tissue macrophages throughout the red pulp and marginal zone; macrophages often contained clumped, globular haemosiderin pigment in addition to AHSV-specific positive granules (Fig. 3.16), b) endothelial cells lining precapillary arterioles, capillaries and postcapillary venules in the red pulp and to a lesser extent lining vascular channels in the marginal zone at the periphery of the white pulp (Fig. 3.17), and c) occasional, plump reticular-like cells (with stellate cytoplasm) in the red pulp and marginal zone (Fig. 3.18). Rarely, positive staining was seen in large macrophage- and/or dendritic-like cells and in the odd endothelial cell lining precapillary arterioles and capillaries within the

depleted white pulp (Fig. 3.19). Again, the odd positive dot appeared to be associated with the surface of occasional red blood cells in the red pulp, but, in general, there was only scant positive staining of erythrocytes in the spleen.

3.2.2. Target cells in other tissues (liver, kidney, cervical muscle, gastrointestinal tract and lymph node)

3.2.2.1. Liver

In a few AHSV-positive cases there was obvious specific positive staining in the liver, in the form of granules, beads and dots in the cytoplasm of: a) Intravascular monocyte-like cells (Fig. 3.20), b) swollen, shrunken or necrotic/apoptotic Kupffer and/or endothelial cells lining the sinusoids (i.e. it was often impossible to differentiate between these cells due to their misshapen appearance, unless they were also bile- or haemosiderin-laden, in which case they were identified as Kupffer cells) (Fig. 3.21), and c) very rarely in endothelial cells lining venules and precapillary arterioles in portal tracts (Fig. 3.22).

3.2.2.2. Kidney

In a few sections of kidney, scant positive staining occurred, again, in the form of granules and small beads in the cytoplasm of a) intravascular monocyte-like cells in interstitial microvessels (Fig. 3.23) and glomerular capillaries (Fig. 3.24), and (rarely) b) endothelial cells of glomerular capillaries and/or interstitial microvessels.

3.2.2.3. Cervical muscle, gastrointestinal tract and lymph node

In the few sections of cervical muscle that were examined, positive beads and granules were seen within the cytoplasm of endothelial cells lining interstitial capillaries (Fig. 3.25) and, to a lesser extent, in the cytoplasm of circulating monocyte-like cells (Fig. 3.26). In sections of gastrointestinal tract (GIT), positive granules were scarce, but were most commonly observed

in the cytoplasm of microvascular endothelial cells in the mucosa, submucosa, muscular tunic (in particular) (Fig. 3.27) and serosa. Occasionally, dot-like positive staining was also observed in the cytoplasm of circulating mononuclear leukocytes. Positive staining was generally very scarce in all examined lymph node sections with most of the positive beads and/or granules occurring in microvascular endothelial cells in the cortex (Fig. 3.28) or medulla or, more commonly in monocyte-macrophage-like cells within cortical and/or medullary sinuses.

3.3. General pattern of positive staining in target organs

In sections of heart positive staining tended to be intense and more beaded than granular (Figs. 3.1, 3.3, 3.6, 3.8, 3.10). The variably-sized beads (which often appeared larger and more prominent than in other organs) usually occurred singly (with a multifocal distribution throughout the tissue) and to a far lesser extent in thin, elongated clusters (i.e. linear arrangements of up to 10 or more dots per cluster) dispersed multifocally between myocardial fibres (Fig. 3.3). In sections of lung, positive staining generally occurred as a mixture of variably-sized single beads (Fig. 3.13) and clustered to dust-like granules/beads (Fig. 3.29) scattered throughout the tissue. In the spleen, again, positive staining was both beaded and granular, occurring singly and in clusters, although a multifocal clustered to dust-like pattern tended to be more common in this organ (i.e. clumped or dust-like signal was often present in many target cells within a localized area of the spleen) (Fig. 3.17). No such staining was observed in tissues from AHSV-negative cases (Figs. 3.30-3.32), or in sections stained with the irrelevant rabies antiserum instead of the Hamblin antiserum (Figs. 3.33-3.35).

3.4. Cell tropism in the brain of three horses that were intranasally inoculated with neurotropic vaccine strains of African horsesickness virus

In three out of four archive cases (287, 289 and 290, but not 286; Appendix 3) from 1962 and 1963, where various neurotropic strains of AHSV (serotypes 1, 7 and 9) were inoculated intranasally into horses, there was clear AHSV-specific positive staining (using both the

Hamblin and 1F1 antibodies) within patchy foci of lymphocytic encephalitis in the brain. Specific positive staining took the form of variably-sized beads (some were quite large) through to fine dust-like granules in the cytoplasm of injured neurons (Figs. 3.36, 3.37), microglia and occasional astrocytes (Fig. 3.38). Application of the rabies virus, equine encephalosis virus (EEV) and equine herpesvirus (EHV) antisera in the place of the Hamblin antiserum failed to reveal positive staining in the same sections of brain (Fig. 3.39). Despite the presence of obvious foci of non-suppurative encephalitis (and neuronal injury) in the sections of brain in case 286 (Appendix 3), there was no AHSV-specific positive staining in any of the brain sections from this case (Fig. 3.40). This was despite repeated staining attempts (done at the end of the study) where a) heat-induced epitope retrieval (HIER) was applied in conjunction with enzymatic retrieval, b) the Hamblin antiserum dilution was decreased and/or the primary antiserum incubation time increased, c) more sensitive detection systems were used (see section 2.5.2). No specific positive staining was observed in any of the target organs (heart, lung or spleen) from these four cases.

Once the HE-stained sections of brain from these cases were examined at the end of the study, it was noted that, in all cases (including case 286), the character of the encephalitis was the same. Generally, gliosis, accompanied by numerous infiltrating lymphocytes, was marked (and extensive) in multifocal areas of grey matter in sections of cerebrum (Fig. 3.41). Within these lesions (and occasionally also in overlying leptomeninges), there was frequently a severe diffuse vascular (predominantly adventitial) cellular infiltrate, characterized by lymphocytes, plasma cells and fewer macrophages (Fig. 3.42). Neuronal injury (including necrosis) was also occasionally observed within these foci of polioencephalitis (Fig. 3.43).

3.5. Technical problems

3.5.1. Background staining

In AHSV-positive and -negative cases stained with the Hamblin antiserum, there was mild through to strong, diffuse, non-specific positive staining of collagen (Figs. 3.14, 3.44, 3.45)

and of smooth muscle and elastic fibres in the walls of alveolar ducts (Fig. 3.46) of lung specimens. This did occasionally hamper scoring (by increasing the time taken to allocate a score) in lung tissue specifically. There was also some non-specific background staining of blood plasma and platelets in the sections of spleen, but not to the extent that scoring was hampered in this organ (Fig. 3.47). There was usually minimal non-specific positive staining of interstitial connective tissues in the sections of heart.

The intensity of non-specific background staining tended to vary (sometimes quite markedly) from batch to batch of cases that were stained at different times. Background staining was always negligible after repeated staining, which could have been due to a different person cutting the sections, and/or some change in the immunostaining procedure. There was no background staining of normal tissue constituents in sections from the few cases that were stained with the 1F1 monoclonal antibody (mAb) (Fig. 3.48). In addition, background staining was present, but usually far less intense, in all sections stained with the irrelevant rabies antiserum.

Connective tissues in all examined dog tissues as well as renal tubular epithelium (more specifically distal convoluted tubule and collecting duct epithelium) and hepatocytes, stained weakly and diffusely positive when the Hamblin antiserum was applied (Figs. 3.49, 3.50). On the other hand, there was minimal non-specific positive staining in all sheep tissues stained with the Hamblin antiserum (refer to section 3.9.1).

In conclusion, due to the general diffuseness and pallor of background staining in sections stained with the Hamblin antiserum (compared to the crisp granular and bead-like nature of AHSV-specific positive staining), it was not considered important to completely eradicate background staining for the purpose of the present study. Therefore, our troubleshooting approach to this problem was kept simple (i.e. via strict adherence to the described tissue processing and immunostaining protocols), and background staining, although present in the majority of cases, was largely kept to a minimum.

3.5.2. Non-specific staining and pigments

It should be noted that in the eight horse cases (case 95, Appendix 1; cases 229, 233, 335, 346, 368 and 373, Appendix 2; and archive case 295; Appendix 3), where artificial black to brown amorphous or microcrystalline acid haematin pigment granules were present, AHSV-specific positive staining was generally scarce in the heavily-pigmented areas and tended to be confined to areas with little or no acid haematin. As a result, it was often more difficult (and therefore it usually took longer) to ascertain whether these cases were positive or negative for AHSV (Fig. 3.51). Similarly, in a few cases, non-specific staining occurred due to undissolved precipitates of the NovaRED chromogen-substrate (Fig. 3.45). These precipitates, particularly if they were very small, and despite the fact that they occurred on top of the tissues, tended to increase the time taken to make a judgment.

As a rule, the crisp reddish colour of the positive granules and beads was readily distinguished from endogenous pigments in cells e.g. haemosiderin pigment granules in splenic macrophages in particular (Fig. 3.16), peri-nuclear lipofuscin granular pigment in myocardial cells, and bile or haemosiderin pigment granules in peri-sinusoidal Kupffer cells in the liver (Figs. 3.21, 3.50, 3.52).

3.6. Comparison of African horsesickness virus-specific positive staining using the 1F1 monoclonal antibody

The 1F1 mAb was applied to: A few positive horse tissues, all positive dog tissues, sections of brain from horses that were intranasally inoculated with neurotropic vaccine strains of AHSV, and tissues from selected AHSV-negative horse and dog cases. In all positive cases, typical beaded and granular intracytoplasmic positive staining was observed predominantly in microvascular endothelial cells, pulmonary intravascular monocyte-macrophages, circulating mononuclear cells and occasional interstitial macrophages in the spleen, lung and/or heart samples in particular. There was generally ample specific positive staining in the target tissues that were present per case, but staining with the Hamblin antiserum undoubtedly

elicited a greater amount of positive staining in the same tissues. The nature of the positive staining, although similar for both antibodies, tended to be less intense, more discrete, and in the form of larger beads (versus clusters of granules) in the tissues stained with the 1F1 antibody, compared to the Hamblin antiserum (Figs. 3.48, 3.53). However, due to the small number of cases tested with the 1F1 mAb, these conclusions should be considered preliminary and therefore treated with caution.

3.7. A brief comparison with equine encephalosis virus- and bluetongue virus-specific positive staining

Equine encephalosis virus-specific positive staining generally occurred in the form of diffuse cytoplasmic staining or single to clustered intracytoplasmic granules. Target cells were predominantly intravascular mononuclear (monocyte-like) cells, interstitial macrophages and, rarely, microvascular endothelial cells in the sections of lung (Fig. 3.54), lymph node (Fig. 3.55), spleen (Fig. 3.56) and liver (Fig. 3.57) in particular (of the tissues submitted).

Bluetongue virus (BTV)-specific positive staining tended to occur in the form of small, intensely staining granules that were clustered in the cytoplasm of microvascular endothelial cells in a) the dermis of hyperaemic coronary band and interdigital skin (in particular) (Figs. 3.58, 3.59), b) the interstitium of normal or injured (oedematous, necrotic or degenerate) skeletal muscle (Fig. 3.60) and myocardium, c) the propria-submucosa bordering erosions and ulcers throughout the GIT and, to a far lesser extent, d) acutely inflamed lung. The result (particularly in sections of skin and GIT) was often a densely granular ring bordering the lumen of microvessels (Fig. 3.59). Occasionally, intravascular mononuclear leukocytes were seen with positively-staining intracytoplasmic dots or granules.

3.8. Serogroup specificity of the Hamblin antiserum and 1F1 monoclonal antibody

Both the Hamblin antiserum and the 1F1 mAb were able to detect all nine AHSV serotypes in formalin-fixed, paraffin-embedded (FFPE) horse tissues via the ABC staining method. In the

case of the Hamblin antiserum there were multiple representatives of all nine serotypes (from positive cases with tissues that were younger and older than 10 years), as shown in table 3.1.

TABLE 3.1. Number of positively-staining cases of each AHSV serotype (Hamblin antiserum).

Serotype	Number of samples
1	18
2	42
3	5
4	17
5	23
6	12
7	48
8	6
9	8

3.9. Cross-reactivity of the Hamblin antiserum and 1F1 monoclonal antibody

3.9.1. Cross-reactivity with equine encephalosis virus and bluetongue virus in tissues

Using the Hamblin antiserum, there was no cross-reactivity in tissues between the selected closely-related orbiviruses (Table 3.2), although not all the serotypes for EEV and BTV were tested. The 1F1 mAb also failed to cross-react with EEV and BTV in tissues (not reflected in the table below).

TABLE 3.2. Number of cases that stained positive with antisera against AHSV, BTV and EEV, given the cases that were AHSV-, BTV- or EEV-positive.

	Virus infection		
Antiserum applied	AHSV	BTV	EEV
AHSV	9	0	0
BTV	0	8	0
EEV	0	0	11

3.9.2. Cross-reactivity in tissues from two dogs with African horsesickness

There was ample specific positive staining (i.e. as for horse tissues) in the cytoplasm of target cells (predominantly microvascular endothelial cells, intravascular mononuclear leukocytes and interstitial macrophages) in the spleen (Fig. 3.61), lung (Fig. 3.62), heart (Fig. 3.63), and to a lesser extent the liver (Fig. 3.64), kidney (Fig. 3.65) and small intestine of the two adult male dogs that were diagnosed with AHS (cases 383 and 384; Appendix 5). Specific positive staining was detected in the tissues using both the Hamblin and 1F1 antibodies. No positive staining was observed in the sections from these two cases that were stained with the rabies antiserum in the place of the primary anti-AHSV serum. The 10 dog cases (where the cause of death was definitely not AHS) failed to reveal specific positive staining in any of the target organs when treated with the Hamblin antiserum or 1F1 mAb according to the described ABC staining method. There was also no AHSV-specific positive staining in case 403 (Appendix 5), where the cause of death was considered to be septicaemia or viraemia (i.e. where infection with AHSV was a possibility, but no virus isolation was done).

3.9.3. Cross-reactivity with unrelated antigens in horse and dog tissues

3.9.3.1. Horse lung

Cases 54, 72 and 91 (Appendix 1) were three horse cases from Western Australia that died of *Rhodococcus equi* pneumonia. In all these cases, *R. equi* was cultured from the lungs. In

case 91, no lung tissue was submitted for immunostaining. However, when lung tissues from the other two cases were immunostained (using the Hamblin antiserum), a number of macrophages and giant cells within foci of granulomatous to pyogranulomatous pneumonia were seen to contain single or clusters of variably-stained, slightly blurred, reddish intracytoplasmic dots (Figs. 3.66, 3.67). This dot-like positive staining was not considered to be AHSV-specific positive staining in either case, because a) the positively-staining cells were atypical in both cases (being activated macrophages and giant cells), and b), the positive staining, although dot-like, tended to be fuzzy and the dots mainly occurred in the cytoplasm of the majority of macrophages within (pyo)granulomatous foci (i.e. definitely not the nature and pattern of AHSV-specific positive staining). There was only very slight positive staining in macrophages and giant cells when the irrelevant rabies antiserum was used instead of the Hamblin antiserum. No positive staining was observed in sections stained with the IF1 mAb (Fig. 3.68).

The HE-stained sections of lung from cases 54 and 72 (Appendix 1) were examined with all the other HE sections at the end of the study. Examination of the HE-stained lung section in case 54 confirmed the presence of focally-extensive granulomatous pneumonia, characterized by numerous activated macrophages (Fig. 3.69) and fewer multinucleated giant cells with scant lymphocytes. Histology of the lung from case 72 also confirmed the presence of widespread purulent to pyogranulomatous pneumonia, characterized by activated macrophages and numerous degenerate neutrophils. Subsequently, a Gram-Twort stain was done on additional sections of lung from cases 54 and 72 (according to the staining method described by Swisher, 2002). This stain revealed the presence of a few blue (gram positive) coccoid bacteria in the cytoplasm of activated macrophages and occasional giant cells within the foci of pneumonia (Fig. 3.70).

In the acute *Actinobacillus* spp. pneumonia case (case 100, Appendix 1), only a few medium-sized bacterial rods stained very slightly positive with the Hamblin antiserum. These bacteria did not stain at all when the irrelevant rabies antiserum and the 1F1 mAb were applied instead of the Hamblin antiserum. However, inhaled foreign material in the same section of

lung stained quite strongly positive in some instances, more so with the Hamblin than the rabies antiserum (Fig. 3.71).

3.9.3.2. Dog stomach

In cases 396 and 403 (Appendix 5), occasional parietal, and, to a lesser extent, chief and mucous neck cells in the stomach mucosa contained intracytoplasmic orange-red, dot-like and/or coiled rod-like positive staining. Similar positive staining was observed in gastric pit and gland lumina, and in parietal cell intracellular canaliculi within the stomach mucosa of these cases (Figs. 3.72, 3.73). When the irrelevant rabies antiserum and the 1F1 mAb were applied instead of the Hamblin primary antiserum to the same sections of stomach, no such positive staining was observed, implying that the Hamblin antiserum cross-reacts with coiled *Helicobacter*-like organisms in dog stomach. When the corresponding HE-stained sections of dog stomach were examined at the end of the study, numerous pale-staining *Helicobacter*-like bacteria were observed in similar locations within the gastric mucosa (Fig. 3.74). These stomach sections were subsequently stained with Warthin-Starry (according to the method described by Swisher, 2002), which permitted the clear identification of numerous *Helicobacter*-like organisms in surface mucous, gastric pits and glands (Fig. 3.75).

3.10. Diagnostic sensitivity and specificity of the avidin-biotin complex detection system for African horsesickness virus in selected horse tissues using the Hamblin antiserum

For the 119 negative cases stained with the Hamblin antiserum, all of the samples in both the A and B test series were scored correctly as negative, making the diagnostic specificity (D-SP) of the test 100 %. In addition, given the large number of AHSV-negative cases in the study, the 95% confidence interval (CI) is at least above 96 % (Table 3.3).

TABLE 3.3. The number of individuals that obtained a specific score for negative samples, broken down per tissue. The specificity is the percentage of negative cases correctly identified as such and the 95 % confidence interval (CI) for specificity is given. The final entry was scored as positive if any of the tissues per case stained positive.

	Scores for series A				
Sample	1	2	3	Specificity	95% CI
Spleen	92	0	0	100.0	96.1 – 100.0
Lung	111	0	0	100.0	96.7 – 100.0
Heart	90	0	0	100.0	96.0 – 100.0
All tissues	119	0	0	100.0	96.9 – 100.0

The scoring of lung and heart samples was 100 % sensitive, and both, together with the spleen, tended to score 3 (Table 3.4). Importantly, a number of AHSV-positive tissues were scored negative in the immunostained sections, giving false negative results (Table 3.4). The spleen rarely gave false negative results (incidence of only 1.8 %), but false negatives became increasingly more common for liver, kidney and lymph node (the anatomical location of lymph nodes was usually unknown), with respective false negative rates of 6, 73 and 100 % respectively (Table 3.4).

TABLE 3.4. The number of individuals that obtained a specific score for positive samples, broken down per tissue. The sensitivity is the percentage of positive cases directly identified as such and the 95 % confidence interval (CI) for sensitivity is given. Where the selected three target tissues were present in a case, the case was scored as positive if any of the tissues per case stained positive.

	Scores for series A				
Sample	1	2	3	Sensitivity	95% CI
Spleen	2	14	94	98.2	93.6 – 99.8
Lung	0	9	106	100	96.8 – 100
Heart	0	1	75	100	95.3 – 100
Liver	1	4	13	94.4	72.7 – 99.9
Lymph node	4	0	0	0	0 – 60.2
GIT*	0	6	2	100	63 – 100
Kidney	3	7	1	27.7	39 – 94
Other	0	1	0	100	2.5 – 100
All tissues	0		128	100	97.2 – 100

* Gastrointestinal tract

3.10.1. Estimation of the repeatability of the test

3.10.1.1. Comparison of staining within target tissues

Taking into consideration all positive and negative cases, the A and B samples of spleen, lung and heart were highly correlated (0.99 and higher; Table 3.5). For these tissues the only disagreements between assignments occurred in two cases of spleen and five cases of lung that were scored 2 in one series and 3 in the other. The other tissues were only present in the positive cases and they occurred in much smaller sample sizes. However, the liver and GIT samples were well correlated between the A and B series, but kidney samples were not (Table 3.5).

TABLE 3.5. Spearman's rank correlation coefficients between the A and B samples and a list of the differences in scoring between the A and B series. Apart from the kidney, which was not significant, all other tissues' significance was below 0.0001.

		Number of confusions		
Sample	correlation coefficient	1 & 2	1 & 3	2 & 3
Spleen	0.99	0	0	2
Lung	0.99	0	0	5
Heart	1.00	0	0	0
Liver	1.00	0	0	0
GIT	1.00	0	0	0
Kidney	0.47	2	0	1

3.10.1.2. Comparison of staining between target tissues

For the combined positive and negative cases, there was a high correlation between the scoring of spleen, lung and heart (Table 3.6; note the difference in sample size, which occurred because cases did not always have all the tissues). All the negative cases corresponded with one another, whereas only a small fraction of positive cases differed from each other (Table 3.7). Looking at the positive cases only (Table 3.7), lung and heart did not differ significantly in their staining scores (Wilcoxon signed rank test; V = 5.5, P = 0.1483), whereas spleen was significantly less likely to score 3 than lung and heart (Wilcoxon signed rank tests: Spleen-Lung: V = 14, P = 0.008; Spleen-Heart: V = 0, P = 0.006).

TABLE 3.6. Spearman's rank correlation of staining between tissues, combining the positive and negative cases. ρ is the correlation coefficient and n the total sample size.

	Spleen	Lung
Lung	ρ = 0.955	
	S = 49463.02	
	P < 0.001	
	n = 186	
Heart	ρ = 0.966	ρ = 0.982
	S = 16908.22	S = 11768.74
	P < 0.001	*P* < 0.001
	n = 142	n = 158

TABLE 3.7. Summary of the number of identical scores and specific differences between the three target tissues (heart, lung and spleen).

Comparison	Identical scores (%)	First tissue score versus second tissue score			
		1 vs. 2	1 vs. 3	2 vs. 3	3 vs. 2
Spleen – Lung	87 (86)	1	1	10	2
Spleen – Heart	56 (86)	0	2	7	0
Heart – Lung	66 (92)	0	0	1	5

3.11. Formalin-fixation kinetics

Over the time frame measured, there was no clear and systematic loss in staining quantity (Fig. 3.76). Because in all cases the samples were processed from at least day 8 onwards (Fig. 3.76), we compared the staining scores on day 8 with those on day 365 for each tissue per case. There was no significant difference in any of the tissues (Wilcoxon signed rank tests: Spleen: V = 4, *P* = 0.77; Lung: V = 4, *P* = 0.77; Heart: V = 1, *P* = 1; note that at least five samples must drop in score from 3 to 2 to obtain a significant result). Staining scores

tended to remain at 3 throughout, bar a few exceptions: Case 204's (Appendix 4) staining score on day 365 consistently dropped from 3 to 2 in all the target tissues suggesting a preparation or staining effect on that day. The same is true for case 203's (Appendix 4) staining scores on day 8 for both lung and spleen. The spleen sample in case 202 (Appendix 4) and the lung sample in case 198 (Appendix 4) fluctuated between 2 and 3, suggesting intermediate staining quantity and quality with some variation, rather than a steady drop due to formalin fixation.

A purely subjective assessment of the overall staining quality of the cases at the end of the fixation kinetics study revealed that, although there was little difference in the scoring of the cases over the year-long-period, the size and intensity of positive beads and granules generally appeared to decrease slightly with time (Figs. 3.77-3.80). This change was particularly apparent in heart tissue compared to lung and spleen.

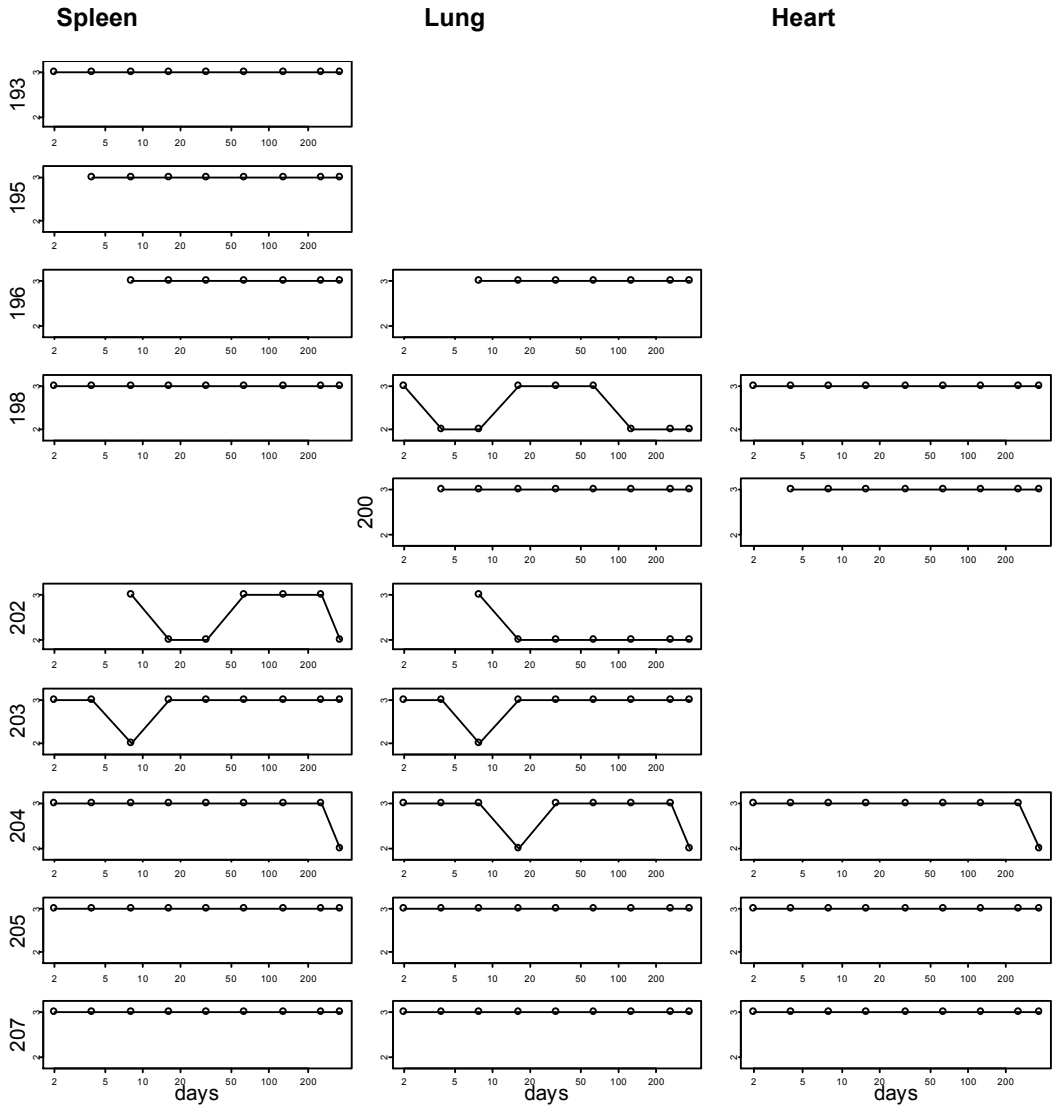

FIG. 3.76. The scoring of positive cases (either 2 or 3) on the y axis versus the number of days stored in 10 % neutral buffered formalin before embedding in wax. Note that the numbers on the left refer to case numbers and that the graphs per row all belong to that particular case. Columns from left to right correspond with spleen, lung and heart tissues respectively. Open slots are where the relevant tissues were not collected. Places where the line is not continuous are where samples were not transferred to wax on that specific day.

Note that the x-axis is on a log scale with the final sample collected on day 365.

3.12. Immunoreactivity of African horsesickness virus-positive paraffin tissue blocks that were at least 10 years old

It was interesting and exciting to note that the archive cases pertaining to some of the original work done on the pathology of AHS by Maurer & McCully (1963) (cases 323, 324, 325, 326, and 327; Appendix 3) and Newsholme (1983) (case 312; Appendix 3) showed obvious positive staining (score 3) in at least two of the selected target tissues, despite the advanced age of the wax tissue blocks (Figs. 3.8, 3.11, 3.81, 3.82). The oldest case (285; Appendix 3), which dated back to 1923, also revealed typical, strong positive staining (score 3) in the spleen, which was the only target organ in this case (Fig. 3.83).

Five of the 72 AHSV-positive archive cases failed to reveal viral antigen via IHC in any of the submitted tissues (cases 286, 288, 307, 315, and 328; Appendix 3). This was despite repeated staining (done at the end of the study, after the immunostaining results had been analysed), using harsher AR methods, increasing the concentration of the Hamblin antiserum, increasing the incubation time with the Hamblin antiserum, and the use of more sensitive detection systems (see section 2.5.2). For all of these cases at least one target tissue was present. In four of the five cases, positive virus isolation results were available (cases 286, 288, 315 and 328). Two cases were from 1962 and 1963 (cases 286 and 288), one was from 1976 (case 307), one was from 1988 (case 315) and one was from 1991 (case 328). Case 286 was one of four horses that were inoculated intranasally with neurotropic AHSV (see section 3.4). It was the only case without positive staining in multiple sections of brain, despite the presence of typical brain lesions.

For all the archive cases, the target tissues were well represented with in excess of 60 samples each. There were far fewer samples of other tissues (Table 3.8). An inspection of Table 3.8 shows that lung stained most reliably, followed by heart and then spleen. Paired Wilcoxon rank tests with continuity correction suggest that lung stained better than heart (n = 55, V = 28, P = 0.015). Note that most paired (lung-heart) samples stained equally well, but seven lung samples stained better than heart, compared to none *vice versa*. In turn, heart

stained more often than spleen, but not significantly so (n = 50, V = 36, *P* = 0.082). Again, most paired heart-spleen samples stained equally well, but 12 heart samples stained better than spleen, compared to only four *vice versa*. Not surprisingly, lung stained better than spleen (n = 53, V = 6, *P* = 0.003), although again, most paired samples stained equally well but 12 lung samples stained more than spleen, as opposed to only one *vice versa*. Too few samples of other organs were obtained to come to any conclusions. However, in most other organs staining was not reliable, with liver, GIT and cervical muscle performing better than the others.

TABLE 3.8. Average score of samples arranged by year and organ for the archive study.

		Sample by Year				
Organ	All years	10-11	12-18	21-37	40-45	83
Spleen	2.52	2.83	2.46	2.57	2.26	3
Lung	2.73	2.94	2.87	2.75	2.48	
Heart	2.58	2.87	2.60	2.56	2.38	
Liver	1.77		3.00	2.33	1.44	
Lymph node	1.13		1.00	1.00	1.20	
Brain	1.78			1.00	2.18	
Stomach	1.75	3.00	1.00		1.80	
Small intestine	1.50		1.00		1.67	
Skeletal muscle	2.00			1.50	2.33	
Bladder	1.00				1.00	
Prostate	1.00				1.00	
Testes	1.00				1.00	
Adrenal	1.50		1.00	2.00	1.00	
Thyroid	1.00				1.00	
Kidney	1.14		1.00	1.00	1.25	
Thymus	1.00			1.00		

Table 3.8 shows that there seems to be a steady decrease in scoring with elapsed time for spleen, heart and lung. For the spleen: The ordinal logistic model using the Akaike Information Criterion (AIC) for model selection, suggested that the odds of observing staining scores 3 versus 2 and 2 versus 1 did not decrease significantly with the age of the sample with a likelihood ratio test (χ^2 = 1.863, *P* = 0.17). However, the more sensitive logistic regression suggested that the chance of making a positive diagnosis decreases with time (model: chances of positive = $(e^{2.82-0.04*age})/(1 + e^{2.82-0.04*age})$; χ^2 = 3.839, *P* = 0.050; solid line in Fig. 3.84).

For the heart: The ordinal logistic model using AIC for model selection, suggested that with each year the odds of observing staining intensity 3 versus 2 and 2 versus 1 decreases by 4.2 %. Even so, this effect of age of the sample was significant with a likelihood ratio test (χ^2 = 3.96, *P* = 0.047). The logistic regression suggested that the chance of making a positive diagnosis decreases with time (model: chances of positive = $(e^{3.88-0.07*age})/(1 + e^{3.88-0.07*age})$; χ^2 = 6.799, *P* = 0.009; dashed line in Fig. 3.84).

For the lung: The ordinal logistic model using AIC for model selection, suggested that with each year the odds of observing staining intensity 3 versus 2 and 2 versus 1 decreases by 6.7%. This effect of age of the sample was significant with a likelihood ratio test (χ^2 = 6.645, *P* = 0.010). The logistic regression suggested that the chance of making a positive diagnosis decreases with time (model: chances of positive = $(e^{4.54-0.08*age})/(1 + e^{4.54-0.08*age})$; χ^2 = 6.218, *P* = 0.013; dotted line in Fig. 3.84).

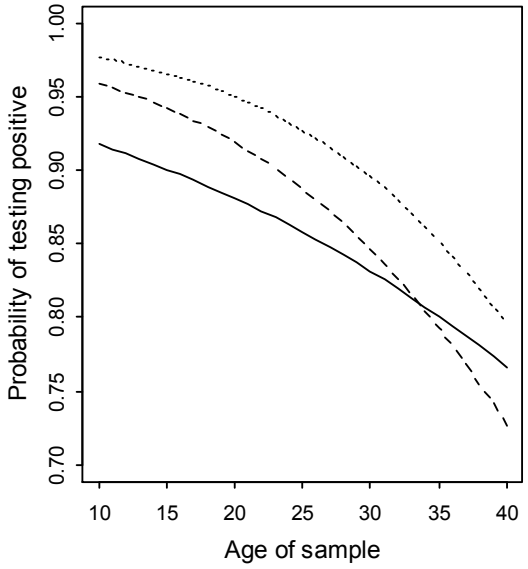

FIG. 3.84. The probability that AHSV-positive wax-embedded tissues stain positively given protracted storage of wax blocks. Based on the logistic regression fitted to the archive cases. Solid line = spleen, dotted line = lung, dashed line = heart.

A purely subjective assessment of the staining quality for the archive wax-embedded tissues, especially those dating back to the 1960s and earlier, revealed that the size and intensity of AHSV-specific positive granules and beads generally decreased only slightly over time in target tissues (Figs. 3.8, 3.81, 3.82, 3.83, 3.85).

3.13. Noteworthy histopathology in spleen, lung and heart tissues from African horsesickness virus-positive horses and two dogs

At the end of the study, the HE-stained sections of spleen, lung and heart from the positive and negative horse (younger and older than 10 years) and dog cases were examined. In my experience, the presence/absence of the following histological changes (tabulated roughly in order of importance) enabled me to differentiate between positive and negative cases approximately 70 % of the time (provided the specimens were not too autolysed and were properly fixed):

a) Hypertrophy and/or apoptosis/necrosis of microvascular endothelial cells and/or intravascular (predominantly mononuclear) leukocytes (Figs. 3.86, 3.87).

b) Serofibrinous pulmonary oedema (Figs. 3.87-3.89), which often contained variable numbers of inflammatory cells and/or alveolar macrophages. Frequently, there were also increased numbers of leukocytes (predominantly monocyte-macrophages) within alveolar septa in the lung (Figs. 3.87-3.89). Occasionally, fibrin thrombi were evident within pulmonary blood vessels.

c) Severe depletion of both nodular and diffuse lymphoid tissue (with or without lymphocytolysis) was generally observed in the spleens from positive horses (Fig. 3.90). In one of the dog cases (383; Appendix 5) there was distinct, widespread necrosis (represented by karyorrhectic debris) of splenic lymphocytes, while in the other case (384; Appendix 5), moderate lymphoid hyperplasia was evident throughout the section.

d) There were occasionally mildly increased circulating and interstitial leukocytes in the sections of heart (Fig. 3.91). Even more common, was the occurrence of myocardial oedema and multifocal or focally extensive sub-epicardial, myocardial and/or sub-endocardial haemorrhage (Fig. 3.92). Foci of myocardial necrosis and/or scarring were rare in the present study.

CHAPTER 4

DISCUSSION

This study has fulfilled many of the requirements for intra-laboratory standardization and validation of an immunohistochemistry (IHC) test for an infectious disease (in this case African horsesickness virus/AHSV), as laid down in guidelines by Ramos-Vara *et al.* (2008). The Hamblin anti-AHSV serum was successfully used in an avidin-biotin complex (ABC) detection system, very similar to that previously described (Wohlsein *et al.* 1997, 1998). The test was able to detect all nine serotypes of AHSV in formalin-fixed, paraffin-embedded (FFPE) samples of heart, lung and spleen from naturally- and experimentally-infected horses. The test was rapid, highly specific (diagnostic specificity/D-SP = 100 %) and sensitive (diagnostic sensitivity/D-SN = 100 %) in heart and lung tissues especially, in the hands of the primary researcher. Positive staining in selected target tissues from 128 positive horses appeared to be minimally affected by the clinicopathological form of African horsesickness (AHS), the stage of infection of the host, sample location, the number of sections per target organ (per case), and virus load. In the present study, as has been found in other studies where AHSV was demonstrated in horse tissues (Brown *et al.* 1994; Wohlsein *et al.* 1997, 1998), positively-staining cells were predominantly microvascular endothelial cells, pulmonary intravascular monocyte-macrophages, circulating monocyte-like cells and, to a lesser extent, interstitial macrophages in spleen and liver in particular. The same tissues and cells were targeted in two dogs that died of AHS. The Hamblin antiserum did not cross-react with closely-related orbiviruses (specifically equine encephalosis virus/EEV and bluetongue virus/BTV) in horse and sheep tissues respectively. Unfortunately, the Hamblin antiserum did cross-react with *Rhodococcus equi* in AHSV-negative horse lung samples. However, the indistinct and clustered nature of the non-specific positive staining, and the (pyo)granulomatous nature of the pneumonia associated with *R. equi* prohibited confusion with AHSV-specific positive staining. The Hamblin antiserum also cross-reacted strongly with coiled *Helicobacter*-like organisms in the gastric mucosa of two dogs. Again, the nature of

positive staining (loosely-coiled spirals) and the site of staining largely prevented confusion with AHSV-specific positive staining. African horsesickness viral antigen could be successfully detected in target tissues (with only a slight subjective decrease in the quality of staining) in up to 83-year-old wax-embedded tissues, and also after fixation for up to a year in 10 % neutral buffered formalin. Furthermore, results indicate that there is no necessity for prompt formalin-fixation of specimens. Positive staining was still observed in tissues that were immersed in formalin between two and five hours after death, and even up to 24 hours after death (see section 2.4). Consequently, we were able to confirm the robustness of AHS viral antigen, even in FFPE tissues.

This chapter has been divided into nine major subsections. In section 4.1, I attempt to explain the granular, bead and dot-like intra- to juxta-cytoplasmic nature of AHSV-specific positive staining. Section 4.2 explores AHSV-specific tissue and cell tropism in horses and two dogs, in the context of similar work that has been done on AHSV, BTV and epizootic haemorrhagic disease virus (EHDV). In the next section (4.3), I briefly examine the cellular tropism of AHSV in the brain of horses that were intranasally inoculated with known neurotropic strains. Section 4.4 examines the specificity of the Hamblin and 1F1 antibodies. I start by briefly comparing positive staining between the Hamblin antiserum and the 1F1 anti-AHSV monoclonal antibody (mAb). Then I examine the serogroup specificity of the Hamblin antiserum and the 1F1 mAb. This leads directly into a discussion on the cross-reactivity of the Hamblin antiserum and 1F1 mAb with a) selected closely-related orbiviruses, namely EEV and BTV in appropriate tissues, b) AHSV in dog tissues, and c) unrelated (bacterial) antigens in horse and dog tissues. Section 4.5 examines the effect(s) of prolonged tissue fixation in 10 % neutral buffered formalin on AHS viral antigen. I compare my results to what is known for a few other infectious disease agents, and briefly investigate why AHS viral antigens appear to be so stable in formalin-fixed tissues. The effect of prolonged storage on AHS viral antigen in paraffin blocks is the subject of section 4.6, while the next section (4.7), comments on the interpretation and reporting of IHC staining results, with particular reference to AHSV. The penultimate section (4.8) summarizes what I consider to be significant histopathology in spleen, lung and heart tissues from orbivirus-infected animals. The final section (4.9) is the

conclusion to the chapter. Some of the issues that are relevant to diagnostic IHC in general will be addressed in this final section.

4.1. Explaining the nature of African horsesickness virus-specific positive staining

In the present study, AHSV-specific positive staining occurred in the form of fine beads, dots, granules and/or dust-like deposits in, or immediately adjacent to, target cells in target organs. This was exactly as Wohlsein *et al.* (1997, 1998) characterized positive staining in tissues from three ponies. Staining was most obviously cytoplasmic, as would be expected with an RNA virus having a cytoplasmic replication cycle (Brodie *et al.* 1998). In 1974, Davies and Lund, using fluorescent antibody techniques (FATs) to detect AHSV in infected baby hamster kidney (BHK) cell cultures, reported the granular nature of specific staining. This is in agreement with the granular, beaded, dust-like and intracytoplasmic immunofluorescent staining of the closely-related BTV both *in vivo* and *in vitro* (Ruckerbauer, Gray, Girard, Bannister & Boulanger, 1967).

In the present study, it was not always possible to establish with certainty whether positively-staining granules and beads were in fact intracytoplasmic (versus extracellular). Mostly, positive staining was located in the perinuclear region of cells (therefore clearly cytoplasmic). However, quite often, beads and granules were also located some distance away from cell nuclei (especially pertaining to endothelial cells), and sometimes adjacent to the cell membrane. This makes sense if one considers the viral replication cycle. There are times when virus will be close to the cell periphery (i.e. at the beginning and end of cell infection). Interestingly, in most vertebrate cell cultures, a high proportion of BTV remains cell-associated, although some virus may be found in the culture medium after cell death and subsequent lysis (Roy 2001; Mortola, Noad & Roy 2004). It seems that released orbiviral virions, as well as those remaining on the cell surface, tend to retain an association with an underlying cortical layer of the cell cytoskeleton (Roy 2001).

Numerous *in vivo* and *in vitro* ultrastructural studies of AHSV, BTV, EHDV and EEV have demonstrated the presence of intracytoplasmic virus inclusion bodies (VIBs), tubular structures and viral particles associated with orbiviral replication in infected cells (Lecatsas & Erasmus 1967; Breese, Ozawa & Dardiri 1969; Tsai & Karstad 1970; Lecatsas, Erasmus & Els 1973; Tsai & Karstad 1973; Mahrt & Osburn 1986; Laegreid *et al.* 1992; Uitenweerde *et al.*1995; Gómez-Villamandos *et al.*1999; Howell *et al.* 2004). Both the granular, roughly circular or filamentous VIBs and paracrystalline tubular structures seem to form aggregations with mature viral particles in the cytoplasm of infected cells and both structures are considered to be characteristic for orbivirus-infected cells (Lecatsas 1968; Oellermann *et al.* 1970; Murphy, Borden, Shope & Harrison 1971; Tsai & Karstad 1973; Huismans 1979; Huismans & Els 1979; Mahrt & Osburn 1986; Eaton, Hyatt & White 1987; Howerth & Tyler 1988; Whetter, MacLachlan, Gebhard, Heidner & Moore 1989; Laegreid *et al.* 1992; Brewer & MacLachlan 1994; Carrasco *et al.* 1999; Howell *et al.* 2004). These structures can be demonstrated within the cytoplasm of infected cells within two to four hours post-infection, and they persist during the whole of the infection cycle (Huismans & Els 1979). Furthermore, orbiviral inclusion bodies are found throughout the cytoplasm of infected cells, but are principally located in the perinuclear and Golgi regions (Tsai & Karstad 1970; Lewanczuk & Yamamoto 1982; Laegreid *et al.* 1992; Roy 1996, 2001; Howell *et al.* 2004). These VIBs are thought to be involved in virion assembly and morphogenesis (Uitenweerde *et al.* 1995; Roy 1996). They consist largely of NS2 proteins and, to a lesser extent, most of the structural proteins that are necessary for the assembly of progeny virions (Uitenweerde *et al.* 1995; Roy 1996, 2001). The Hamblin antiserum probably binds with these VIBs and associated viral proteins in the cytoplasm of target cells because, as has already been established, the antiserum was obtained by immunizing rabbits with purified AHS viral particles known to contain VP1 up to VP7 as well as co-purified NS2 protein (Burroughs *et al.* 1994). Since NS1 was not seen to co-purify with these viral particles (Burroughs *et al.* 1994), it is highly unlikely that the Hamblin antiserum binds with tubules in the cytoplasm of infected cells (because tubules consist of NS1 protein).

4.2. Target tissues and cells for the routine diagnosis of African horsesickness virus

For the purpose of IHC standardization and validation, it is critical that the target tissues and cells pertinent to the infectious agent under investigation be well-characterized, and that the manner or pattern of positive staining be properly described and made available to veterinary diagnostic pathologists.

4.2.1. Tissue tropism in horses

4.2.1.1. Target tissues (heart, lung and spleen)

In this study, similar to Brown *et al.* (1994), Wohlsein *et al.* (1997, 1998) and Gómez-Villamandos *et al.* (1999), but based on a much larger sample size than exists in the literature, we found that lung and heart samples, in particular, were highly sensitive and specific tissues for AHSV localization, followed closely by the spleen. Diagnostic sensitivity scores in the present study were 100 %, 100 % and 98.2 % for lung, heart and spleen, respectively, while D-SP scores were 100 % for all three tissues. At this point, it should be noted that although the researcher made every effort to give independent scores to each tissue, due to financial constraints, the tissues were all on the same slide and some non-independence was therefore inevitable. In contrast to our results, an enzyme-linked immunosorbent assay (ELISA) developed by Hamblin *et al.* (1992) to detect AHSV antigen, detected the most antigen in the spleen, although they also found that lung, heart (and liver) contained significant amounts of antigen. Hamblin *et al.* (1992) also found that AHS viral antigens persisted for the longest period of time in splenic tissue. These contradictory results may be explained by the observation that positive staining in the spleen tended to be more localized, or, in the words of Brown *et al.* (1994), “segmentally diffuse”, compared to heart and lung tissue.

Importantly, for 22 of 128, or nearly 20 % of AHSV-positive horses in the present study, where tissues were less than 10 years old, one (usually the spleen) and sometimes two of the target tissues scored 2. This meant that positive staining was scarce in those organs, making the

diagnosis of AHSV in these cases difficult, especially if the only available target tissues for that case scored 2 (as was the case for five of 128 or 4 % of positive cases). In two of 128 positive cases, the spleen scored 1, giving a false negative result. Clearly, lung and heart should be routinely submitted for the diagnosis of AHSV via IHC, while the spleen can be misleading on occasion, and should preferably be submitted with lung and/or heart samples. It is reassuring that BTV and EHDV, two closely related orbiviruses, have also been shown (usually via methods other than IHC) to target lung and heart, as well as lymphoid tissues, including the spleen, in ruminants (Tsai & Karstad 1973; Pini 1976; Mahrt & Osburn 1986; Howerth & Tyler 1988; MacLachlan *et al.* 1990; Brodie *et al.* 1998; MacLachlan & Osburn 2004; Verwoerd & Erasmus 2004).

The present study also demonstrated that specific positive staining usually occurred throughout AHSV-positive lung and heart samples, irrespective of a) the location of the sample (i.e. left or right ventricle, anteroventral or caudal lung lobe, etc), b) the number of sections examined per sample, and c) the number of samples examined per organ. This finding, in numerous cases, largely concurs with the small amount of data obtained from a few experimentally-infected horses in the Brown *et al.* (1994) and Wohlsein *et al.* (1997, 1998) studies. Such a generally widespread distribution of viral antigen in selected target tissues is not always the case. I have already mentioned that, in the spleen, AHSV-specific positive staining tended to be more localized than in the heart or lung. Also consider, for example, porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the lung of pigs. According to a study on the kinetics of PRRSV infection in the lung of experimentally-infected pigs, antigen-specific positive staining tends to be most pronounced between seven and 21 days post-infection, and it occurs predominantly in association with areas of acute interstitial pneumonia (Beyer, Fichtner, Schirrmeier, Polster, Weiland & Wege 2000). Only a few loosely-scattered antigen-positive cells could be observed in non-pneumonic areas of lung and in foci of chronic interstitial pneumonia. Therefore, for PRRSV, the sensitivity of IHC on lung tissues tends to increase quite dramatically (from 40 % to > 95 %) if multiple samples are taken from obviously pneumonic areas of lung (especially anteroventral lung), particularly during the second and third weeks post-infection (Halbur, Paul, Frey, Landgraf, Eernisse, Meng,

Andrews, Lum & Rathje 1996; van Alstine *et al.* 2002). In contrast, there are very few constraints for AHSV IHC, especially if lung and heart are used.

4.2.1.2. Other tissues

4.2.1.2.1. Lymph node

In stark contrast to Wohlsein *et al.* (1997, 1998), we found minimal positive staining in lymph nodes from 11 naturally- and experimentally-infected horses. Admittedly, in our study, the sample number was small, and five of the 11 cases were wax-embedded tissues that were 40-45 years old (although this should not have been a significant factor, see later).

African horsesickness virus is known to occur in high concentration in lymph nodes and to replicate in them early on during the course of infection (Erasmus 1973; Coetzer & Guthrie 2004). Similarly, lymphoid tissues have been shown to be a primary site for BTV localization (Verwoerd & Erasmus 2004) and the virus can be isolated from a variety of lymph nodes (as well as the tonsils and spleen) by four days after subcutaneous inoculation in sheep (Pini 1976). Furthermore, Pini, Coackley & Ohder (1965) observed that spleen and mesenteric lymph nodes were the richest source of BTV for virus isolation in dead sheep. Bremer & Viljoen (1998) detected viral RNA via reverse transcription polymerase chain reaction (RT-PCR) in mesenteric, bronchial and cervical lymph nodes from an AHSV-infected horse. However, most amplicons were obtained from RNA extracted from the heart, lung, spleen and liver, with fewer amplicons being obtained from the kidney and cervical lymph node, and least amplicons from the bronchial and mesenteric lymph nodes (which are probably the lymph nodes that are most commonly sampled by veterinarians and veterinary pathologists).

Widespread depletion of lymphoid elements was a fairly routine finding in histological sections of spleen (and lymph node) from AHSV-positive horses in this study. Moreover, lymphopaenia is routinely seen in orbivirus infections (Karstad, Winter & Trainer 1961; Howerth, Greene & Prestwood 1988; Skowronek *et al.* 1995). Therefore, the high virus concentrations measured

in lymphoid tissues in AHS cases (Erasmus 1973; G.H. Gerdes, unpublished data 2007) implies that lymphoid depletion must be caused by direct or indirect (via host cell production of cytokines) viral injury to lymphocytes (Newsholme 1983). On the other hand, severe stress in the advanced stages of AHS may well contribute to lymphoid depletion due to excessive release of lymphocytolytic endogenous corticosteroids (Newsholme 1983). Not surprisingly, it seems as if, by the time most lymphocytes have been destroyed (and removed) from lymphoid tissues, the amount of virus left in association with these cells falls below the concentration necessary for detection via the described IHC method.

The ponies utilized by Wohlsein *et al.* (1997, 1998) were euthanased on humane grounds eight or 10 days post-infection. Similarly, most of the naturally-infected horses used in the present study died or were euthanased within a week of the onset of clinical signs. Perhaps the differences in histopathology and immunostaining results pertaining to lymph nodes (and the white pulp of the spleen) between the Wohlsein *et al.* (1997, 1998) studies and the present study can be, at least partially, ascribed to differences in host-animal immunity (Wohlsein *et al.* 1997, 1998 used naïve ponies) and the experimental versus natural mode of infection (i.e. Wohlsein *et al.* 1997, 1998 experimentally infected ponies, whereas positive horses in the present study were naturally-infected).

Suffice to say that the demonstration of AHSV in histological sections of lymph node from horses that die of AHS requires further investigation. At this stage, given the apparent contradictory nature of the immunostaining results, it would seem that lymph nodes are not a reliable sample for the detection of AHSV via IHC.

4.2.1.2.2. Liver, gastrointestinal tract, kidney and oedematous cervical muscle (adjacent to the nuchal ligament)

Based on only a few samples, and similar to the findings of Wohlsein *et al.* (1997, 1998), we found that liver (18 cases) and gastrointestinal tract (GIT) samples (eight cases) from AHSV-positive horses (where the tissues were less than 10 years old) appeared to be reasonable

samples for virus localization (provided the investigator was aware of the target cells in these organs). Scoring of the liver gave a false negative rate of 6 %, compared to no false negatives for far fewer GIT samples that were examined. However, due to the comparative sparseness of specific positive staining in these samples, we suggest that they should not be submitted without accompanying lung and heart samples for the routine diagnosis of AHSV via IHC. Again, similar to Wohlsein *et al.* (1997, 1998), we found only scant positive staining of individual cells in the kidney of a few infected animals (i.e. the kidney gave a false negative rate of 73 % in the present study). Kidney is therefore not a useful sample to submit for the routine diagnosis of AHSV via IHC.

Amongst the archive cases that were 10 years and older, there were five cases for which oedematous neck muscle samples were available. African horsesickness virus-specific positive staining scored 2 on average over all the cervical muscle samples submitted, all of which were more than 30 years old. My results largely concur with those of Brown *et al.* (1994), where *in situ* hybridization (ISH) was used to localize AHSV in neck muscle. The preliminary data on a very small number of samples therefore indicates that oedematous neck muscle may be a reasonable sample to submit (preferably together with heart and lung samples) for the diagnosis of AHSV in FFPE tissues.

4.2.2. Target cells in horse tissues

4.2.2.1. Microvascular endothelial cells

I was able to establish beyond doubt that AHSV targets microvascular endothelial cells in myocardium, lung, and spleen in particular. In our experience, these cells were usually not targeted as commonly in the lung as in the myocardium. In the spleen, endothelial cells lining microvessels were targeted predominantly in the marginal zone and red pulp, and only rarely in the white pulp. In my experience, microvascular endothelial cells were only rarely identified as target cells in samples of liver, kidney and lymph node, whereas scant AHSV-specific

positive staining tended to be quite consistently observed in endothelial cells lining capillaries in the muscular tunic of the GIT and between myofibres in oedematous cervical muscle.

In numerous other studies, and using a variety of techniques (e.g. transmission electron microscopy/TEM, ISH, immunofluorescence and IHC), AHSV and closely-related orbiviruses, namely BTV and EHDV have been shown to infect and replicate in microvascular endothelial cells in a similar selection of organs and tissues (Tsai & Karstad 1973; Mahrt & Osburn 1986; Howerth & Tyler 1988; Laegreid *et al.* 1992; Brown *et al.* 1994; Wohlsein *et al.*1997, 1998; Brodie *et al.* 1998; Gómez-Villamandos *et al.* 1999).

4.2.2.2. Monocyte-like cells

Positively-staining, generally plump, intravascular mononuclear leukocytes (with monocyte-like morphology) were observed quite commonly within hepatic sinusoids and scattered throughout the marginal zone and red pulp of the spleen in infected horses in the present study. Similar circulating mononuclear leukocytes were also observed in interstitial capillaries in the heart and oedematous neck muscle of horses, and in microvessels of the GIT, kidney (especially glomerular and interstitial capillaries) and lymph nodes. Similarly, in the lung, intravascular monocyte-like cells were often positive for AHSV, seemingly far more so than interstitial macrophages in this organ.

Increased numbers of mononuclear leukocytes have been observed in and around blood vessels in histological and ultrastructural sections of lung and heart in particular, in horses with AHS (Maurer & McCully 1963; Newsholme 1983; Laegreid *et al.* 1992; Carrasco *et al.* 1999; Skowronek *et al.* 1995). Furthermore, previous workers have shown that AHSV infects and replicates readily in monocytes *in vitro* (Stäuber, McCullough & Kihm 1992). Wohlsein *et al.* (1997, 1998), Carrasco *et al.* (1999) and Gómez-Villamandos *et al.* (1999) have also specifically mentioned the presence of AHSV in the cytoplasm of monocytes in tissues. Brown *et al.* (1994) were far less specific, referring to the presence of AHSV-positive hybridization

signal in “large mononuclear cells” in the marginal zone of the spleen. They considered these cells to be monocytes or large lymphocytes.

The tropism for peripheral blood mononuclear (PBM) cells, especially monocytes, has been extensively reported in domestic and wild ruminants infected with BTV and EHDV (Pini 1976; Barratt-Boyes, Rossitto, Stott & MacLachlan 1992; Barratt-Boyes & MacLachlan 1994, 1995; Barratt-Boyes, Rossitto, Taylor, Ellis & MacLachlan 1995; MacLachlan *et al.* 1990; Brodie *et al.* 1998). Recently, IHC has been used to investigate the cellular tropism of EEV in naturally- and experimentally-infected horses (A.D. Pardini, unpublished data 2007). Thus far, it seems that specific positive staining (crisp cytoplasmic granules through to diffuse cytoplasmic staining) has been observed predominantly within intravascular monocytes in microvessels of the spleen, with similar positively-staining monocyte-like cells evident in lung, liver and lymph node samples (A.D. Pardini, unpublished data 2007).

4.2.2.3. Pulmonary intravascular mononuclear leukocytes

In the present study, there were consistently far more positively-staining intravascular mononuclear cells in the lungs than in any other organ, which suggests a tropism for intravascular cells in the lung that are not just circulating monocytes (or large lymphocytes), but that have a monocyte-like morphology under the light microscope. An ultrastructural study of the lungs from five horses experimentally infected with AHSV, Carrasco *et al.* (1999) found that, apart from microvascular endothelial cells and a few interstitial macrophages and fibroblasts, AHSV commonly infects and replicates in so-called pulmonary intravascular macrophages (PIMs). These authors and others (Gómez-Villamandos *et al.* 1999) have suggested that the pulmonary oedema and inflammation, as well as the microvascular changes, seen at the ultrastructural level in the lungs of horses with AHS are more likely to be linked to the activation of PIMs (and their release of chemical inflammatory and vasoactive mediators), than to direct virus-induced endothelial injury. The low level of viral replication that has been observed in pulmonary endothelial cells in several ultrastructural studies (Laegreid

et al. 1992; Carrasco *et al.* 1999; Gómez-Villamandos *et al.* 1999) seems to support this hypothesis.

As was briefly discussed in the first chapter, PIMs are a resident population of mature phagocytes, which resemble mature macrophages of the mononuclear phagocyte system (MPS) in structure and function (Atwal *et al.* 1989). These cells have long been recognized as the site of substantial uptake of blood-borne particles and microbes in the lungs of a number of domestic animal species, particularly ruminants and pigs, but including horses and cats (Wheeldon & Hansen-Flaschen 1986; Winkler 1988, 1989; Warner & Brain 1990; Atwal *et al.* 1992; Brain *et al.* 1999; Molina & Brain 2007). Transmission electron microscopy is required to conclusively identify PIMs, but no TEM was done on sections of lung in the present study. Therefore, the tropism of AHSV for PIMs could not be proved in the present study. It is certainly possible that some of the positively-staining intravascular mononuclear leukocytes were actually monocytes or, much less likely, large lymphocytes. Interestingly, research on PIMs in ruminants has shown that there are no differences between the numbers of these cells in the different pulmonary lobes, indicating that a sample taken from any lobe would be representative (Warner *et al.* 1986; Carrasco *et al.* 1996). Similarly, we found that pulmonary intravascular mononuclear leukocytes were fairly consistently targeted by AHSV, irrespective of the location of the lung sample. This finding would of course also be expected if the targeted cells were in fact circulating monocytes. Evidently, further characterization of these mononuclear leukocytes (at the light microscopic level) requires the application of a panel of well-characterized mAbs to equine leukocyte differentiation antigens.

To-date, no mention has been made of PIMs in lung samples from BTV- or EHDV-infected ruminants, yet it is well known that PIMs form an important component of the MPS in ruminants (Brain *et al.* 1999). In the author's opinion, based on the findings in the present study, and the findings by Carrasco *et al.* (1999), further research into the role of PIMs in the lungs of AHSV-infected horses (and indeed all orbivirus-infected lungs), is warranted.

4.2.2.4. Interstitial macrophages

Positive AHSV signal was also observed in tissue macrophages in the red pulp and marginal zones of the spleen, and to a lesser extent, in hepatic Kupffer cells, and only very occasionally in clearly identifiable pulmonary (interstitial) macrophages. In no other organs were interstitial macrophages a prominent or common target cell for AHSV. Wohlsein *et al.* (1997, 1998) also briefly mention AHSV-specific positive staining in macrophages in spleen and lymph nodes, but in no other organ. Various ultrastructural studies on AHSV in lung and spleen samples have demonstrated structures indicative of viral infection in only a small number of interstitial macrophages in these organs (Carrasco *et al.* 1999; Gómez-Villamandos *et al.* 1999). Notably, Stäuber *et al.* (1992) found that AHSV was readily able to infect equine monocyte and young macrophage cell cultures, but that more mature equine macrophages were refractory to infection. Perhaps some of the positive staining in interstitial macrophages in the present study was derived from the phagocytosis of infected, injured cells by splenic and liver macrophages (i.e. as part of the MPS). On the other hand, it is also possible that the degradation of viral antigens within macrophages (Cheville 1975) largely precluded positive staining in these cells.

Epizootic haemorrhagic disease viral RNA has been detected in interstitial macrophages in the lung and spleen of naturally-infected wild ruminants (via RT *in situ* PCR) (Brodie *et al.* 1998). Little has been said in regard to BTV infection of interstitial macrophages, although macrophages in lymphoid organs are generally considered important in the pathogenesis of BTV infection (Barratt-Boyes *et al.* 1992).

4.2.2.5. Erythrocytes

Occasionally, we found a dot or small cluster of fine red granules that appeared to be associated with the surface of red blood cells. Even more rare was the diffuse positive staining of an occasional red blood cell. However, in some cases it was impossible to tell whether positive staining was indeed red cell-associated, or whether positive beads or dots were

actually within the elongated cytoplasm of endothelial cells adjacent to red blood cells in capillaries. Although Ozawa, Salama and Dardiri (1972) clearly demonstrated that AHSV, like BTV in ruminants (Brewer & MacLachlan 1994; Barratt-Boyes & MacLachlan 1995), is associated with erythrocytes in the blood of infected horses, Wohlsein *et al.* (1997, 1998) reported no positive staining of erythrocytes in their studies using IHC. In order to explain the lack of positive staining, Wohlsein *et al.* (1997, 1998) proposed that either a) there was too little erythrocyte-associated viral antigen for detection by IHC, or b) the AHS viral antigen is held in indentations and invaginations of the red blood cell membrane (as is the case for BTV and EHDV) (Eaton & Crameri 1989; Brewer & MacLachlan 1994; Barratt-Boyes & MacLachlan 1995; Aradaib, Brewer & Osburn 1997). In this way, viral antigen might elude detection by the primary antiserum.

Brewer and MacLachlan (1992) observed that BT viral particles associated with bovine erythrocytes often resemble core particles, which lack the outer capsid proteins (VP2 and VP5) that are required for the infection of mammalian cells. However, in 1994, using core particles and neutralizing mAbs, the same authors discovered that VP2 is necessary for attachment of BTV-10 to erythrocytes. Therefore, in theory, and because the molecular biology of BTV and AHSV is considered to be very similar (Roy 2001; Coetzer & Guthrie 2004), erythrocyte-associated AHS viral particles should contain (at least) the five core proteins (VP1, VP3, VP4, VP6 and VP7), as well as VP2. On the other hand, cells in which orbiviruses replicate are bound to contain all of the structural and nonstructural proteins associated with replicating virions (i.e. including NS2-containing VIBs, which are likely to bind with the Hamblin antiserum). Theoretically therefore, it is possible that the lack of staining of erythrocyte-associated viral particles may be due to a significant difference in the viral proteins (specifically VP5 and NS2) that are available for binding with the Hamblin antiserum.

In conclusion, it is clear that mature red blood cells are not true target cells for AHSV. They lack the necessary cellular components for virus replication. In the author's opinion therefore, one should not expect to observe noteworthy erythrocyte-associated positive staining in horses with AHS, using the Hamblin antiserum and ABC staining technique.

4.2.2.6. Other target cells

Various ultrastructural studies on AHS have also demonstrated virus in occasional fibroblasts, smooth muscle cells and pericytes (Carrasco *et al.* 1999; Gómez-Villamandos *et al.* 1999; Roy 2001). Similar findings have been reported in ultrastructural and immunofluorescent studies on the pathogenesis of BT (Stair 1968; Howerth & Tyler 1988). In the present study, none of these cells were obviously targeted by AHSV in the tissues examined. In support of my findings, AHS viral nucleic acid and antigens have not been previously reported in fibroblasts, smooth muscle cells or pericytes by using classical ISH techniques and IHC (Brown *et al.* 1994; Wohlsein *et al.* 1997, 1998). In my experience, it is often difficult to classify cells with certainty in immunostained sections (unless double immunolabeling procedures are used). This is due to the relative lack of cellular detail and contrast, compared to HE-stained sections.

4.2.3. Target tissues and cells for African horsesickness virus in two naturally-infected dogs

In two AHSV-positive dogs, where only a limited number of tissues were available, it would appear that the lung, in particular, as well as the spleen, heart and liver were good samples for the demonstration of AHSV via IHC. The cellular tropism and pattern of positive staining was very similar to that observed in horse tissues in the present study, even for non-target organs such as the liver, kidney and small intestine. In addition, my findings were similar to those reported in five pregnant bitches that were experimentally infected with BTV (Brown, Rhyan, Grubman & Wilbur 1996).

The predominant AHSV-positive cells were a) microvascular endothelial cells (which were especially prominent in the heart, lung and muscular tunic of the small intestine), b) intravascular mononuclear leukocytes in the lungs, spleen, liver, kidney and small intestine, and c) interstitial macrophages (especially prominent in the spleen, and to some extent in the liver).

Severe pulmonary oedema (with or without hydrothorax) is a well-reported and consistent finding in AHSV-infected dogs (Theiler 1910; Bevan 1911; Piercy 1951; Haigh, McIntosh, Cumming & Hempstead 1956; Dardiri & Ozawa 1969). Severe lung oedema has also been reported in pregnant bitches that were experimentally infected with BTV (Verwoerd & Erasmus 2004). Both dogs in the present study had severe diffuse serofibrinous pulmonary oedema. In both cases, antigen-specific positive staining was observed in the cytoplasm of pulmomary intravascular mononuclear leukocytes, as well as microvascular endothelial cells and the occasional interstitial macrophage in the lungs. Dogs (like rats, mice, guinea pigs, rabbits and chickens) have not been shown to rely much on PIMs for the active removal of blood-borne particulates or pathogens, unless their hepatosplenic clearance system fails (Chitko-McKown & Blecha 1992). This could explain why, in the lungs of BTV-infected bitches, most of the viral nucleic acid was found within pulmonary microvascular endothelial cells (Brown *et al.* 1996). No doubt, TEM on orbivirus-infected dog lung would make it possible to determine the nature of the infected intravascular mononuclear leukocytes in the lungs. Are these cells in fact PIMs, or are they simply mononuclear leukocytes that adhere to endothelium via the upregulation of adhesion molecules on leukocytes and activated endothelial cells?

In both positive dogs, ample, specific positive staining was observed in the cytoplasm of large macrophage-like cells and/or large lymphocytes/immunoblasts in the necrotic or hyperplastic white pulp (in addition to the red pulp) of the spleen. Similarly, in an ISH study, Brown *et al.* (1996) visualized BT viral nucleic acid in mononuclear cells of the splenic periarterial lymphatic sheaths (PALS) in two experimentally-infected pregnant bitches. Hence, lymphoid organs might be useful target tissues for the detection of AHSV in dogs; unfortunately, lymph nodes were not examined from either dog in the present study.

4.2.4. Implications for future studies on pathogenesis

In the present study, we found that AHSV consistently infected microvascular endothelial cells representing distinct anatomic sites. This occurred irrespective of the serotype and clinical form of AHS (pulmonary, cardiac or mixed) manifested by the host. Erasmus (1973)

hypothesized that horses infected with a particular serotype of AHSV have a heterogeneous mixture of highly virulent and less virulent viruses in their blood. He proposed that more virulent viruses might have a predilection for pulmonary microvascular endothelial cells, while less virulent particles might selectively multiply in lymphoid tissues or in endothelial cells lining blood and lymph vessels of the head and neck. Recent molecular research has shown that genetic reassortment occurs in BTV infections (Roy 2001) and AHSV (A.C. Potgieter, unpublished data 2008). This implies co-infection of cells with different strains of virus in a susceptible host. In theory, and largely in line with Erasmus's (1973) hypothesis, new, more virulent viral strains (reassortants) may form naturally within an infected host. Using laboratory-generated reassortants, T. Meiring (unpublished data 2008) demonstrated an increase in viral release and yield of most of the reassortants, and an increase in the cytopathic effect (CPE) of some of the reassortants. However, whether or not the degree of virulence of viral particles determines their cellular tropism (as was suggested by Erasmus 1973), does not appear to have been investigated. What is interesting in this regard is the observation that the amount of virus in the lung does not seem to correlate with the severity of oedema and inflammation in this organ (Laegreid *et al.* 1992; Carrasco *et al.* 1999; Gómez-Villamandos *et al.* 1999). The implication is that mechanims other than direct virus-induced cell injury (e.g. PIM activation and the subsequent release of cytokines) are likely to be involved in the development of the observed lesions (Laegreid *et al.* 1992; Carrasco *et al.* 1999; Gómez-Villamandos *et al.* 1999).

From our observations, using IHC, it seems feasible to investigate whether consistently-targeted populations of microvascular endothelial cells share receptor molecule(s) for AHSV (which might also be expressed on PIMs and circulating monocytes). It is well known that endothelial cells from different anatomical locations display antigenic heterogeneity (Page, Rose, Yacoub & Pigott 1992). DeMaula, Jutila, Wilson & MacLachlan (2001) discovered inherent differences in the susceptibility of specific ovine and bovine microvascular endothelial cells to BTV infection. This led them to conclude that endothelial cell phenotype is central to the pathogenesis of endotheliotropic virus infections.

In the researcher's opinion, the present study contributes significant data to support the observation that AHSV targets endothelial cells representing distinct anatomic sites. This phenomenon has also been demonstrated in animals infected with EHDV (Tsai & Karstad 1970). A thorough investigation of many more tissues from AHSV-infected dogs, and an investigation of the tissue and cell tropism of AHSV in chicken embryos, as has been done for BTV (Gleiser, Stair & McGill 1969; Wang, Kemp, Roy & Collisson 1988), and which is currently underway for AHSV (L.H. Maartens, unpublished data 2008), may provide us with more data on which to base research into host cell-receptor molecule(s) for AHSV. So far, it appears that most of the work on orbivirus entry into host cells has focused on the biochemistry of particular viral proteins (Basak, Gouet, Grimes, Roy & Stuart 1996; Hassan & Roy 1999). Nothing of significance appears to have been done with regard to viral-monocyte/macrophage and viral-endothelial cell receptors.

4.3. Tissue and cell tropism in three horses that were inoculated intranasally with neurotropic vaccine strains of African horsesickness virus

Within multiple foci of lymphocytic encephalitis in three out of four horses that were intranasally inoculated with neurotropic strains of AHSV (cases 287, 289 and 290; Appendix 3), there was obvious positive staining in the cytoplasm of injured neurons, and in smaller numbers of glial cells and astrocytes. In regard to case 286 however (Appendix 3), there was no positive staining in multiple sections of brain, despite the presence of similar (albeit less severe) histopathology. Notably, the A501 strain (which was used to inoculate horse 286; Appendix 3) is known to produce less severe neurological signs in horses, compared to the Karen/serotype seven and 7/60 or serotype nine strains (used to inoculate the other horses; Appendix 3) (Erasmus 1966). It is possible that the amount of viral antigen in the brain of case 286 (Appendix 3) was beneath the threshold necessary for detection via the immunostaining method described in the present study. It could have been that a low virus titre was inoculated in this case. Unfortunately, no information was available on the dose administered, or the virus titre in the intranasal inoculum, for any of the experimentally-infected horses used in the present study.

In none of the four intranasally-inoculated horses was any virus discernable via immunostaining in accompanying samples of spleen, lung and heart. Unfortunately, virus isolation results from these organs were not available for these cases. The lack of staining in these target organs is not unexpected, however, since the neurotropic mouse strains of AHSV apparently retained very few of their viscerotropic properties (Erasmus 1966). In addition, virus was inoculated via the intranasal, versus the intravenous route, which makes it less likely that significant quantities of virus would reach the viscera.

As far as I know, this is the first report that defines the histopathology of, as well as the specific cells targeted by, two of the neurotropic strains of AHSV (the Karen and 7/60 strains) within brain tissue, following intranasal inoculation. A similar histopathology and pattern of infection has been demonstrated in the brains of neonatal mice that were intracerebrally inoculated with various strains of reovirus (Tyler 1998). Reovirus is the prototype orthoreovirus, and, like AHSV, it also belongs in the family *Reoviridae* (Coetzer & Tustin 2004).

4.4. Specificity of the Hamblin and 1F1 antibodies

4.4.1. Comparison of staining between the Hamblin antiserum and the 1F1 monoclonal antibody

The Hamblin antiserum contains, amongst other things, the soluble product of multiple clones of activated B-lymphocytes that were programmed to recognize a range of AHS viral antigens (e.g. VP1 to VP7) via multiple epitopes on each antigen. On the other hand, the 1F1 mAb is a class IgG2b antibody that is specific for one antigenically-dominant site on VP7 alone (van Wyngaardt *et al.* 1992). The group-specific 1F1 mAb was only applied to tissues from a small number of cases in the present study (see section 2.3.1.2). Pertaining to these few cases, the Hamblin antiserum appeared to be more sensitive than the 1F1 mAb. This is undoubtedly due to the ability of the Hamblin antiserum to bind with a multiplicity of epitopes on AHSV,

compared to the 1F1 mAb, which was only able to bind with a single epitope on the VP7 core protein. On the other hand, the Hamblin antiserum did cross-react with specific horse and dog bacteria in target and other organs (see later), whereas the 1F1 mAb did not. No doubt, the ability of the antiserum to recognize multiple epitopes on a variety of AHS viral proteins, and the likely occurrence of non-specific antibodies in the antiserum, increased the likelihood of cross-reactivity with unrelated proteins.

4.4.2. Serogroup specificity of the Hamblin and 1F1 antibodies

The Hamblin antiserum, when applied according to the described ABC staining method, was able to detect all nine known serotypes of AHSV in FFPE horse tissues. This is not surprising, since Hamblin *et al.* (1991, 1992), using similar antisera raised against purified virus particles, infectious sub-viral particles (ISVPs) and core particles, have routinely detected AHSV and AHSV-specific antibodies in infected tissue cultures and field samples via established serogroup-specific ELISAs.

The virus core polypeptide VP7 is the group-specific determinant (Huismans & Erasmus 1981; Gumm & Newman 1982; Oldfield, Adachi, Urakawa, Hirasawa & Roy 1990; Chuma, Le Blois, Sánchez-Vizcaíno, Díaz-Laviada & Roy 1992). Moreover, VP7 appears to be strongly immunogenic in a variety of domestic animal species, including horses and rabbits (Erasmus 1963; Huismans *et al.* 1987a; Laviada, Arias & Sánchez-Vizcaíno 1993; Martínez-Torrecuadrada *et al.* 1996; Brodie *et al.* 1998). The immunogenicity of VP7 may be related to the presence of at least one serogroup-reactive epitope in the top (outermost) domain of VP7, which is accessible on the surface of intact virions and core particles (and therefore able to elicit a humoral response). Reactivity of the Hamblin antiserum to VP7 is undoubtedly the most feasible explanation for the serogroup-specific positive staining that was observed in horse tissues in the present study.

The 1F1 mAb was also able to detect all nine serotypes of AHSV in horse tissues. This was entirely expected because a) as has already been mentioned, VP7 is the group-specific

antigen for AHSV and BTV (Huismans & Erasmus 1981; Oldfield *et al.* 1990; Chuma *et al.* 1992), and b) the same mAb was used to detect antibodies to all nine AHSV serotypes in an inhibition ELISA (van Wyngaardt *et al.* 1992) and an indirect sandwich ELISA (du Plessis *et al.* 1999).

4.4.3. Cross-reactivity of the Hamblin antiserum and 1F1 monoclonal antibody

4.4.3.1. Cross-reactivity with equine encephalosis virus and bluetongue virus in tissues

There was no cross-reactivity whatsoever in selected tissues, using the Hamblin and 1F1 antibodies. This lack of cross-reactivity was not altogether unexpected, considering that no cross-reactivity has been demonstrated among the selected orbiviruses by means of a number of antigen- and antibody-ELISAs (Erasmus, Boshoff & Pieterse 1976; Hamblin *et al.* 1991; Williams, du Plessis & van Wyngaardt 1993; du Plessis *et al.* 1999; Crafford *et al.* 2003; Maree & Paweska 2005). Furthermore, regarding the 1F1 mAb, antisera against BTV-4 and EHDV had no significant effect on the binding of the mAb to purified viral antigen in an inhibition ELISA (van Wyngaardt *et al.* 1992).

4.4.3.2. Cross-reactivity with African horsesickness virus in dog tissues

In the present study we established that the Hamblin antiserum, the 1F1 mAb and the ABC staining method described for horse tissues could be successfully applied (without modification) to the same target tissues from two dogs infected with AHSV. For the two dogs that were examined, the pattern of specific positive staining, the cells targeted by the virus, as well as the target tissues (spleen, lung and heart), appeared to be very similar to what has been observed a) in horses, and b) via ISH in tissues from dogs that were experimentally infected with BTV (Brown *et al.* 1996).

4.4.3.3. Cross-reactivity with unrelated antigens in horse and dog tissues

There was potentially confusing non-specific positive staining of *Rhodococcus equi* bacteria in horse lung and *Helicobacter* sp. bacteria in dog stomach, when the Hamblin antiserum was used. The staining of *R. equi* was distinctly granular, as was the staining of some *Helicobacter* bacteria (presumably those bacteria in cross section), and intense (particularly the *Helicobacter* bacteria). The same bacteria were not visible at all when the 1F1 mAb was substituted for the Hamblin antiserum, and they were only slightly apparent (pale pink and blurred) when the rabies antiserum was used instead of the Hamblin antiserum. One can therefore deduce that the Hamblin antiserum contains antibodies that are able to bind with epitopes on *R. equi* and some *Helicobacter* spp. Either AHSV-specific antibodies in the antiserum are able to cross-react with epitopes on these bacteria, or the antiserum also contains irrelevant serum proteins that are able to cross-react with bacterial antigens. Whatever the case, the ability of the antiserum to bind with these, and other, bacteria needs to be further investigated and carefully documented as part of an ongoing validation process for AHSV IHC.

It should be noted that, in the hands of the primary investigator, no false positive results were recorded, despite the fact that the Hamblin antiserum was the primary antibody of choice for the present study. This is because the nature/pattern of non-specific positive staining was generally sufficiently different to AHSV-specific positive staining. Finally, if there was still any doubt, the histopathology associated with non-specific positive staining is not typical for AHSV, thus enabling the primary researcher to differentiate between positive and negative cases.

4.5. Effect of delayed and prolonged formalin-fixation on African horsesickness viral antigens

4.5.1. Deleterious effects of formalin-fixation on immunohistochemistry

It is well known that formalin induces cross-linking of reactive sites within and between tissue proteins and nucleic acids (Shi, Key & Kalra 1991; Sompuram, Vani, Messana & Bogen 2004;

Ramos-Vara 2005; Ramos-Vara *et al.* 2008). The subsequent conformational changes that occur in the tertiary structure of proteins may make it difficult, if not impossible, for antibodies to recognize epitopes on related antigens (Ramos-Vara 2005). Furthermore, the formation of these cross-links is progressive, being dependent on time and temperature (Ramos-Vara 2005; Ramos-Vara *et al.* 2008). As a result, it is generally accepted that over-fixation in formaldehyde can produce false negative results (decreased sensitivity) in IHC, simply due to the formation of too many cross-links (Ramos-Vara 2005). Tissue antigen retrieval (AR) techniques can sometimes reverse formalin-induced cross-linking of antigens, but in some cases the cross-linking appears to be irreversible (van Alstine *et al.* 2002).

Shi, Gu, Turrens, Cote & Taylor (2000) deduced that, in solution, formaldehyde is capable of binding to particular amino acids, namely: lysine (K), arginine (R), tyrosine (Y), asparagine (N), histidine (H), glutamine (Q), and serine (S). So, in theory, these particular amino acids can be altered by formalin-fixation because they can form cross-links with formalin. The same authors concluded that, during formalin-fixation, tyrosine, if present in the antibody-binding site, tends to bind covalently to arginine (R), if this particular amino acid exists in close proximity to the antibody-binding site. Moreover, this particular cross-link appears to be reversible via heat-induced epitope retrieval (HIER), probably due to the loosening or breaking of the cross-linkages induced by formalin-fixation. Although the effects of enzyme-induced epitope retrieval (as was used in the present study) were not discussed by Shi *et al.* (2000), it seems feasible that enzymes would perform a similar function to heat because they increase the rate of reactions by lowering the activation energy required.

If formalin is acidic (unbuffered), fewer cross-links should form within and between tissue proteins (Ramos-Vara 2005). Therefore, unbuffered formalin should result in improved IHC results compared to buffered formalin, provided the AR method is adjusted accordingly (Ramos-Vara 2005). In the present study, however, although most of the AHSV-positive tissues that were submitted in unbuffered formalin had ample positive staining (score 3), the positive staining tended to be confined to areas with minimal/no acid haematin pigment. It generally took me longer to find specific positive staining in tissues that were submitted in

unbuffered formalin. No doubt this was partly due to the presence of numerous acid haematin pigment granules. However, since AHSV is sensitive to acid pH values (Coetzer & Guthrie 2004), it is possible that unbuffered formalin might actually destabilize or alter the conformation of the virus to the extent that the Hamblin antiserum was not able to bind with a significant number of the epitopes on AHS viral antigens. Another feasible explanation might be that the AR method was not adjusted for the tissues in unbuffered formalin. It is possible, therefore, that the pronase digestion method described for the study was too harsh, causing the AHS viral antigens to become damaged and therefore unrecognizable to the Hamblin antiserum.

4.5.2. Effect of delayed and prolonged formalin-fixation on African horsesickness viral antigens, compared to other infectious agents

The fixation kinetics part of the present study was performed on at least one of the three target tissues (spleen, lung and/or heart) from 10 AHSV-positive horses, representing six of the nine known serotypes. In this study, there was no significant decline in staining quantity (a function of the score allocated) after a week or less in 10 % buffered formalin, compared to an entire year in buffered formalin. From a purely subjective viewpoint, however, the quality of positive staining (pertaining to the size and intensity of beads and granules) did seem to decrease slightly over time.

Clearly, the chemical structure of AHS viral antigens, and their relationships to other tissue proteins, largely determines the effect that prolonged formalin-fixation will have on the immunoreactivity of these antigens. The results of the formalin-fixation kinetics study plainly indicate that AHS viral proteins are stable in formalin. This is certainly not always the case. In regard to PRRS viral antigen in lung tissue from pigs, the amount of positive signal was reduced after 2 days, and, in some cases (especially after 4 or more days in formalin), even eliminated (van Alstine *et al*. 2002). Miller, Ramos-Vara, Kleiboeker & Larson (2005) found that the prompt fixation of samples in formalin meant that bovine viral diarrhoea virus (BVDV)

antigen could be readily detected in specimens that were stored in formalin for up to 36 days. However, positive staining was weak or absent in tissues after 176 days in formalin.

In the Wohlsein *et al.* (1997, 1998) IHC studies on tissues from AHSV-infected ponies, no mention was made of the interim or the time interval from the death of the animals until the immersion of tissues in formalin. On the other hand, Brown *et al.* (1994), in their investigation of the location of AHSV antigen in horse tissues via ISH, stipulated that the interim was one to three hours for most cases. However, for five out of 15 ponies, the interim was 18 to 24 hours. In addition, for seven out of the 15 ponies used for the Brown *et al.* (1994) study, tissues were kept in 10 % buffered formalin for two to three weeks. For all of these cases, positive hybridization signal was obtained in most of the tissues examined, with signal present in more than 50 % of high power fields per positive tissue. In my opinion, these results lend support to my findings using IHC, despite the fact that ISH is usually considered to be more sensitive than IHC (Allan, Todd, Smyth, Mackie, Burns & McNulty 1989; Allan, Smyth, Todd & McNulty 1993).

Immunohistochemistry is potentially an extremely useful tool for the detection of particular viral or other proteins that are relatively unaffected by prolonged exposure to formalin. Many of the tissues submitted to our IHC laboratory from the rest of Africa have been subjected to prolonged formalin-fixation (often up to three months, and sometimes up to six months). In the case of AHSV we now know that this does not pose a problem.

4.5.3. Stability of African horsesickness viral antigens in formalin

In view of our and Brown *et al.*'s (1994) positive staining results, despite delayed and prolonged formalin fixation, it seems appropriate to briefly investigate the stability of those AHS viral proteins that are most capable of binding with the group-specific Hamblin antiserum. A Western blot analysis was performed to identify the antibodies present in the Hamblin antiserum (D. Bolton, unpublished data 2008). To this end, semi-purified viral proteins of AHSV, serotypes four, six and nine were separated on polyacrylamide gel electrophoresis

(PAGE). Results were not conclusive, but it appears that the Hamblin antiserum contains antibodies to VP3, VP5, VP7 and NS2 (D. Bolton, unpublished data 2008).

Logically, from an understanding of the molecular biology of orbiviruses, one would expect VP2, VP5 and VP7 to elicit the strongest humoral response after the inoculation of purified intact virus particles (Laviada *et al.* 1993; Martínez-Torrecuadrada *et al.* 1996). This probably explains their use in subunit vaccines and the availability of antigenic profiles for these particular proteins (Roy, Urakawa, van Dijk & Erasmus 1990; Martínez-Torrecuadrada, Iwata, Venteo, Casal & Roy 1994). VP2 and VP5 are the outer capsid proteins, and they should therefore be most exposed to the immune system. Core protein VP7, which lies directly underneath VP2 and VP5, is also accessible to host antibodies through holes in the surface of the outer capsid (Basak *et al.* 1996; Martínez-Torrecuadrada, Langeveld, Venteo, Sanz, Dalsgaard, Hamilton, Meloen & Casal 1999; Roy 2001).

Although VP2 is the major target of the host neutralizing response (Roy 2001), it is also the most variable protein of the virion and it is the main serotype-specific antigen (Bremer, Huismans & van Dijk 1990; Roy 1996; Roy 2001). Therefore, since the Hamblin antiserum is not serotype specific, it seems unlikely that it will target VP2, except in tissues from horses infected with serotype nine and possibly serotype six (i.e. there is cross-reactivity between these two serotypes). Not unexpectedly then, in the Western blot, VP2 was barely detectable as a faint band in the AHSV-9 lane.

Due to the group specificity of the Hamblin antiserum, it is not surprising that the antiserum contains antibodies to VP7, the group-specific core protein in BTV, AHSV and other orbiviruses (Roy 2001). The VP7 structure has an extremely stable, uniquely trimeric molecular architecture (Roy 2001), which makes this core protein particularly resistant to degradation (H. Huismans, unpublished data 2008). Clearly, it makes evolutionary sense for the virus core to be robust. It must shield the host cell cytoplasm from cytotoxic double-stranded viral RNA (Kalai, van Loo, van den Berghe, Meeus, Burm, Saelens & Vandenabeele

2002), and it must protect the three minor structural proteins (VP1, VP4 and VP6), which have different enzymatic activities, all of which are essential for viral replication.

The fact that VP7 does not appear to be affected by formalin-fixation may be at least partly due to the fact that it lacks specific amino acid residues that are affected by formalin-fixation. It is not known exactly which residues form the epitopes for AHSV VP7, but at least three small hydrophilic regions are exposed on the hydrophilic top (outermost) domain of this protein, namely: Residues 144-145, 177-178 and 200-201 (Basak *et al.* 1996; Roy 1996; van Rensburg 2004). Of these three sites, site 144-145 is tyrosine-rich, and should therefore be recoverable via HIER (and possibly also enzymatic digestion). Region 177-178 does not contain any of the residues that have been reported to cross-link with formalin. There is a glutamine residue (Q) at site 200 which could possibly be affected by formalin-fixation. Hence, two of three obviously exposed sites on VP7 are either resistant to formalin, or potentially recoverable via AR methods.

For reasons already mentioned, it is not surprising that the Hamblin antiserum appears to contain antibodies to VP5. The most immunodominant region in VP5 has been located in the N-terminal half of the molecule, comprising two antigenic regions and eight antigenic sites, including two neutralizing epitopes (Martínez-Torrecuadrada *et al.* 1999). The first of the neutralizing epitopes (defined by mAb 10AE12, located at positions 85-92 in VP5; amino acid sequence PDPLSPGE) is highly conserved among the different orbiviruses (Martínez-Torrecuadrada *et al.* 1999). It is therefore probably not recognized by the Hamblin antiserum (which is group-specific for AHSV). The other epitope (defined by mAb 10AC6, located at positions 179-185 in VP5; amino acid sequence EEDLRTR) is considered an immunogenic 'hot spot' in the VP5 of orbiviruses. However, the amino acid sequence at this region is very poorly conserved between orbiviruses (Martínez-Torrecuadrada *et al.* 1999), suggesting that it might be one of the multiple epitopes recognized by the Hamblin antiserum. Notably, epitope 10AC6 has two arginines (R), which might be corrupted by formalin-fixation.

The Hamblin antiserum also seems to contain some antibodies to VP3 (D. Bolton, unpublished data 2008), the major protein of the subcore particle (Huismans & van Dijk 1990), and the scaffold onto which VP7 deposits (Roy 2001). However, since VP3 is largely masked by VP7 in intact virions, there are likely to be far fewer VP3 antibodies compared to VP7 antibodies in the Hamblin antiserum (H. Huismans, unpublished data 2008). Moreover, no information was available regarding antigenic sites in VP3. Since VP1, VP4 and VP6 are located in the inner core, it is not surprising that the Hamblin antiserum does not appear to contain antibodies to these minor structural proteins (D. Bolton, unpublished data 2008).

NS1 and NS2 are the major antigenic nonstructural proteins (Laviada *et al.* 1993). The Hamblin antiserum appears to contain some antibodies to the NS2 protein (D. Bolton, unpublished data 2008). NS2 is a multimeric, generally hydrophilic, phosphorylated protein that exists in complexes with viral RNA in infected cells (Roy 2001). This nonstructural protein is also synthesized in high abundance in infected cells (Roy 1996; Roy 2001), and, as has already been discussed, it is the major component of VIBs (Roy 2001). The fact that these VIBs are relatively sequestered complex proteinaceous structures within the cytoplasm of infected cells might at least partially account for their resistance to modification by formalin (H. Huismans, unpublished data 2008).

In this section, I have only addressed a few mechanisms that might explain the resistance of AHSV proteins to formalin-fixation. However, formalin has numerous effects on tissues (Sompuram *et al.* 2004), and we do not yet fully understand antigen stability or instability brought about by formalin-fixation. There is no doubt, however, that a better understanding of the effects of formalin-fixation will help to improve the performance and therefore the accuracy of IHC generally.

4.5.4. Future research

Our laboratory is in the process of comparing staining results (for the formalin-fixation kinetics part of the study) between the Hamblin hyperimmune serum and the 1F1 mAb. Most of the

tissues submitted for staining with the 1F1 mAb had been fixed in formalin for no longer than 2 days. For a few archive cases however (cases 286, 287, 289 and 290; Appendix 3), where there was immunoreactivity with the 1F1 mAb, the length of formalin fixation was unknown. The mAb was developed to target only one epitope on the VP7 protein, and, depending on the robustness of the epitope in question, one might expect a poorer performance by this antibody compared to the Hamblin antiserum, in tissues that have spent a year in formalin. Logically, there is a far greater likelihood that some epitopes on the AHSV antigen will be resistant to formalin, whereas a single epitope on one of the viral proteins might just be susceptible to prolonged formalin-fixation. In a study evaluating the use of monoclonal primary antibodies to BVDV in FFPE tissues, only one of 32 antibodies tested was found to yield positive staining via the ABC method (Haines, Clark & Dubovi 1992). The authors suggested that most of the BVDV epitopes that are recognized by mAbs are considered to be highly conformation-dependent and are therefore susceptible to the denaturing effects of formalin-fixation. Notably, the most immunogenic epitope in VP7 also seems to be conformational (Laviada *et al.* 1992; Laviada *et al.* 1993). Therefore, it should be interesting to observe the performance of the 1F1 mAb on tissues that have definitely been formalin-fixed for more than 48 hours.

4.6. Immunogenicity of African horsesickness virus-positive paraffin blocks that were stored for 10 years or more

I was able to detect AHS viral antigens in approximately 93 % of naturally- and experimentally-infected horses (where tissue blocks were between 10 and 83 years old). For five out of 72 archive cases, no positive staining was observed in any of the tissues submitted for immunostaining, despite a) the presence of at least one target tissue in each of these cases, and b) repeated staining attempts, with modifications to the original staining method. However, for one of these five cases, no confirmatory virus isolation results were available (case 307; Appendix 3), allowing for the possibility that this case was not a true positive case. For the remaining four cases (286, 288, 315 and 328 from 1962, 1963, 1988 and 1991 respectively; Appendix 3), virus isolation results were available. Case 286 was a horse that was intranasally inoculated with a neurotropic strain of AHSV. In this particular case, no positive staining could

be detected in brain or other tissues (refer to section 4.3). Cases 288 and 315 were from the AHSV reference collection in the Arnold Theiler museum at the Onderstepoort Veterinary Research Institute (OVI), while case 328 was a horse from the Middle East (tissue blocks were stored at the Armed Forces Institute of Pathology/AFIP in Washington, D.C., United States of America/USA). In these cases, a) the level of virus may have been beneath the concentration necessary for detection via IHC, despite repeated staining using a more concentrated antiserum, a longer incubation time with the antiserum, and more sensitive detection systems, b) the test tissues may have been over- or underfixed, although modifications to the AR method did not elicit positive staining, or c), the test samples may have been subjected to excessively high (> 60 ºC) embedding and/or drying temperatures. The latter option seems unlikely pertaining to the OVI specimens, since many other AHSV-positive tissues from the same time period stained positive via the described IHC method. In summary, the results of this part of the study clearly indicate that IHC may be used to detect AHSV in wax-embedded tissues that are more than 10 years old, with the likelihood of introducing only a small margin of error.

There are numerous retrospective studies that have been done to demonstrate the presence of a variety of infectious agents in FFPE tissues, using IHC (Shoup, Swayne, Jackwood & Saif 1996; Thanawongnuwech, Halbur & Andrews 1997a; Narita, Kimura, Tanimura & Ozaki 1999; Bart, Guscetti, Zurbriggen, Pospischil & Schiller 2000; Kondo, Hikita, Ito & Kadota 2000; Mori, Sato, Akachi, Asahi, Taniguchi & Narita 2000; Rodriguez-Arrioja, Segalés, Rosell, Rovira, Pujols, Plana-Duran & Domingo 2003; Grau-Roma & Segalés 2007). However, there do not appear to be many studies that have investigated the immunogenicity of wax-embedded tissues, where the tissues were older than 20 years. More data is necessary before we can safely conclude that paraffin blocks can be stored indefinitely with only limited deleterious effects on tissue antigens (Ramos-Vara 2005). In a few years, however, we may well deduce that it is not necessary to validate IHC on these old blocks at all.

4.7. Notes on the interpretation and reporting of immunostaining results for African horsesickness virus

Immunohistochemistry results should be interpreted within the context of the disease under investigation (Ramos-Vara *et al.* 2008). Therefore, in the case of AHSV IHC, it is essential that staining results be interpreted in relation to macro- and microscopic pathology (see section 4.8).

4.7.1. Pocket guide for the correct interpretation of African horsesickness virus immunohistochemistry

Based on data from the present study, we can safely predict that AHSV will generally target microvascular endothelial cells, intravascular monocyte-macrophage-like cells and interstitial macrophages in the vast majority of lung, heart and spleen specimens from infected horses, irrespective of the location of the sample within these organs. Positive staining is intracytoplasmic and it has a dot, bead-like or granular character. These dots/beads/granules may occur singly, in clusters or in a dust-like distribution. Occasionally, dots or granules in or adjacent to the cytoplasm of adjoining endothelial cells appear to form a line, which then delimits the luminal circumference of microvessels.

Our results also show that lung and heart samples (irrespective of sample location in these organs) are most likely to give the correct diagnosis. In addition, although staining is plentiful (score 3) in most cases, it can be scant/minimal in a small proportion of positive cases.

4.7.2. Reporting immunohistochemistry results

Ideally, when reporting any IHC result, one should be able to state (and reference) the D-SN and D-SP of the test, pertaining to the target organ(s) submitted. This is in order to stress the possibility of a margin of error. It is especially important in the case of a lack of detectable antigen in submitted tissue sections. For the small proportion of cases with minimal positive

staining in target tissues, I would strongly advise the pathologist to recommend further investigation using alternative tests. In situations where no target tissues have been submitted, it is essential that the client be informed of the preferred tissues/organs pertaining to the selected antigen. Furthermore, now that data regarding the cross-reactivity of the Hamblin antiserum with related orbiviruses, e.g. EEV, is available, it is the author's opinion that this data should be reported (in one short sentence) to the client. In the same way, a short note on the stability of the chosen antigen in formalin would be of use to the client. In summary, the more suitable a test sample, the more accurate the result. Therefore, to some extent, the reliability or reproducibility of IHC depends on effective client education. In the author's opinion therefore, the IHC report, apart from stipulating positive/negative results, should also strive to educate the client regarding sample suitability for a particular test.

4.8. Significant histopathology in lung, heart and spleen tissues from African horsesickness virus-positive horses and two dogs

Regarding the microscopic pathology of AHSV, while I appreciate that there are no pathognomonic lesions for AHSV, I do not concur with Maxie & Robinson (2007), when they state that histological lesions are not useful for diagnosis or understanding of pathogenesis.

4.8.1. Hypertrophy and/or apoptosis/necrosis of circulating mononuclear leukocytes and microvascular endothelial cells

In support of my findings, Wohlsein *et al.* (1997, 1998) briefly describe swollen endothelial cells lining interalveolar capillaries in the lung, and nuclear debris between heart muscle fibres in their experimentally-infected ponies. Various *in vivo* and *in vitro* pathologic, ultrastructural, ISH and flow cytometric studies on AHSV, BTV and EHDV in cells and tissues have also observed hypertrophy and/or apoptosis of infected cells (Lecatsas & Erasmus 1967; Stair 1968; Gleiser *et al.* 1969; Tsai & Karstad 1970; Murphy *et al.* 1971; Tsai & Karstad 1973; Mahrt & Osburn 1986; Howerth & Tyler 1988; Whetter *et al.* 1989; Barratt-Boyes *et al.* 1992; Laegreid *et al.* 1992; Brodie *et al.* 1998; Carrasco *et al.* 1999; Gómez-Villamandos *et al.*

1999; McLaughlin, DeMaula, Wilson, Boyce & MacLachlan 2003; Howerth, Parlavantzas & Stallknecht 2004). Furthermore, numerous apoptotic cells were observed via TEM in the spleen from one of three ponies that were experimentally infected with EEV (A.D. Pardini, unpublished data 2007). Closely-related reoviruses, which have a capsid architecture that is strongly indicative of a common icosahedral construction (Roy 1996; Coetzer & Tustin 2004), have also been shown to induce apoptosis of infected cells both in *vivo* and in *vitro* (Oberhaus, Dermody & Tyler 1998; Connolly, Rodgers, Clarke, Ballard, Kerr, Tyler & Dermody 2000; Barton, Chappell, Connolly, Forrest & Dermody 2001; Connolly, Barton & Dermody 2001; Connolly & Dermody 2002).

In orbivirus infections, lysis of infected cells seems to represent a complex interplay between i) the host cell response to infection, via the production of inflammatory mediators (that act to limit virus replication and spread), and ii), direct virus-induced cytopathology (Howerth & Tyler 1988; Carrasco *et al.* 1999; DeMaula *et al.* 2001; Roy 2001; DeMaula, Leutenegger, Bonneau & MacLachlan 2002a; DeMaula, Leutenegger, Jutila & MacLachlan 2002b; McLaughlin *et al.* 2003; Howerth *et al.* 2004; Mortola *et al.* 2004). No doubt, in the case of AHSV, apoptosis needs to be properly characterized (and quantified), both at a tissue level and
in selected cell populations, using different methods in parallel, for example a) fluorescent microscopy, using DNA-staining fluorochromes, b) a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method for *in situ* detection of DNA strand breaks, c) IHC or Western blot analysis, using an anti-active caspase-3 antibody, and d) TEM (Malorni, Fais & Fiorentini 1997; Sur, Doster & Osorio 1998; Nieper, Teifke, Jungmann, Lohr & Muller 1999; Funakoshi, Nakayama, Uetsuka, Nishimura, Sasaki & Doi 2000; Shibahara, Sato, Ishikawa & Kadota 2000; Kommers, King, Seal, Carmichael & Brown 2002; Kommers, King, Seal & Brown 2003; Mortola *et al.* 2004).

4.8.2. Serofibrinous pulmonary oedema and inflammation (predominantly mononuclear leukocytes in alveolar septa)

The histopathology of the pulmonary lesions in AHS has been well-described in horses (Maurer & McCully 1963; Newsholme 1983), but not in dogs. However, very similar histological lesions have been described in the lungs of dogs that were experimentally infected with BTV (Brown *et al.* 1996). Similar pulmonary lesions have also been reported for experimental and naturally-occurring BTV and EHDV infection of sheep and deer (Thomas & Neitz 1947; Moulton 1961; Karstad & Trainer 1967; Erasmus 1975; Hoff & Trainer 1981; Parish *et al.* 1982; Mahrt & Osburn 1986; Brodie *et al.* 1998). In addition, similar lung lesions have been observed in sections of lung from EEV-infected horses (Howell *et al.* 2004; A.D. Pardini, unpublished data 2007).

4.8.3. Depletion of both nodular and diffuse lymphoid tissue in the spleen

Maurer & McCully (1963) and Newsholme (1983) describe the histopathology of lymphoid organs in great detail in horses. However, neither lymphoid hyperplasia nor distinct widespread necrosis of PALS, both of which have been reported by Maurer & McCully (1963), Newsholme (1983), Brown *et al.* (1994) and Wohlsein *et al.* (1997,1998), was regularly observed in horse spleens in the present study.

Necrosis of splenic PALS has been described in one pregnant bitch that was experimentally infected with BTV (Brown *et al.* 1996). Widespread depletion of lymphocytes (with or without obvious lymphocytolysis) has been observed in the lymphoid organs of deer and horses infected with EHDV and EEV respectively (Karstad *et al.* 1961; A.D. Pardini, unpublished data 2007).

4.8.4. Myocardial oedema, haemorrhage and inflammation (predominantly mononuclear leukocytes)

Maurer & McCully (1963) and Newsholme (1983) described the typical changes in the hearts of horses in great detail. Haemorrhages have previously been observed macroscopically in the hearts of dogs that died of AHS (Theiler 1910), but there are no descriptions of the histopathology associated with AHS in dogs. Similar histological lesions have been recorded in the hearts of BTV-infected sheep and EHDV-infected deer (Karstad *et al.* 1961; Moulton 1961; Erasmus 1975; Parish *et al.* 1982; Mahrt & Osburn 1986; Brodie *et al.* 1998; MacLachlan & Osburn 2004; Verwoerd & Erasmus 2004).

4.9. Concluding remarks

There are a number of *in vivo* studies that have reported difficulties in the detection of orbiviral protein antigens and viral nucleic acid via IHC and classical ISH techniques (Dangler *et al.* 1990; Schoepp *et al.* 1991; de la Concha-Bermejillo *et al.* 1995; Brodie *et al.* 1998). Yet, the present study has found IHC (using the Hamblin antiserum and the ABC detection system), to be a highly sensitive and specific diagnostic assay for AHSV in horse tissues, through comparison with conventional virus isolation methods. The IHC test for AHSV cannot be used to differentiate between the different strains of AHSV. However, it is useful for rapid virus identification in FFPE tissues.

At least some of the success of IHC in the present study can be ascribed to: Minimal antigenic variations between field viruses, the selection and combination of appropriate target tissues, the minimal effect of autolysis (up to 24 hours) prior to fixation, and the robustness of AHS viral antigens in the face of prolonged formalin-fixation. In the author's opinion, much of the success of any IHC test must also be ascribed to the quality of the primary antibody or antiserum. In this respect, the Hamblin antiserum undoubtedly contributes to the small pool of workable antibodies that are suitable for the detection of RNA viruses in FFPE tissues. Unfortunately, however, the Hamblin antiserum does cross-react with certain bacteria in tissue

sections. Therefore, an appreciation of the exact localization of AHS viral antigen and the character of positive staining in selected tissue sections is critical for the accurate interpretation of staining in tissues. In this respect, knowledge of the histopathology associated with AHSV and *R. equi* infections (especially in horse lung samples) allows for accurate diagnosis.

Perhaps the most serious drawback to the use of antisera in IHC is the limited quantity of identical antisera available (Haines *et al.* 1992; Ramos-Vara 2005; Ramos-Vara *et al.* 2008). This factor alone greatly restricts intra- and especially inter-laboratory validation, which should be an ongoing process, and therefore requires extensive testing (Ramos-Vara *et al.* 2008). Conversely, large quantities of identical/uniform quality mAbs are available indefinitely, which certainly facilitates the ongoing standardization and validation of any IHC assay (Haines *et al.* 1992; Laviada, Babín, Dominguez & Sánchez-Vizcaíno 1992; Wade-Evans, Woolhouse, O'Hara & Hamblin 1993; Ramos-Vara 2005).

Importantly, standardization and validation of IHC should include external assessment of the quality and consistency of staining (Ramos-Vara *et al.* 2008). This is an aspect of diagnostic IHC that has been neglected, even in human medicine (Ramos-Vara *et al.* 2008). Regarding AHSV, to the author's knowledge, there are no other laboratories involved in the routine diagnosis of this infectious agent via IHC. Therefore, an inter-laboratory comparison of staining results was not practical in our situation. Regarding those infectious diseases that are more routinely diagnosed via IHC, however, it is the author's contention that there is a definite need for IHC reference laboratories that are prepared to provide external quality control (on at least an annual basis) to small, isolated IHC laboratories across the globe. In a country like the USA, where there are numerous veterinary diagnostic pathology laboratories making routine use of IHC tests, regular inter-laboratory standardization and validation seems feasible. Indeed, it is surprising that this is not the situation, although Ramos-Vara *et al.* (2008) have indicated that inter-laboratory validation of IHC tests must become routine.

If it is to be taken seriously, especially in the diagnosis of infectious diseases, the application of IHC needs to be completely reviewed, as Ramos-Vara *et al.* (2008) have attempted to do. Perhaps, while we are in the process of negotiating a better future for IHC, we should at least attempt to establish the D-SN and D-SP of all routinely-used IHC tests within a laboratory, based on an adequate number of appropriate positive and negative samples per infectious disease agent under investigation. Within this context, it is the author's hope that the present study will be of use to those interested in optimizing IHC tests for infectious agents.

As it stands, the present IHC test for AHSV in horse tissues helps to increase the accuracy of viral detection, and it is especially useful in conditions where viable tissue samples are not available for virus isolation. The described test is obviously robust, and it is therefore well-suited to African conditions, where samples are often stored in formalin for weeks or months before being submitted to a laboratory for analysis.

Ultimately, however, IHC realizes its full potential when used to research the pathogenesis of a disease. As has been indicated several times during the present study, there is still much to be learnt about the pathogenesis of orbiviral infections. For example, a proper comparative study of the target tissues and cells for orbiviruses generally, across different animal species, needs to be done. Such a study might inform future research into host (and perhaps even vector) cell surface receptors for orbiviruses. In this regard, there is further scope for AHSV IHC in FFPE tissues from more dogs and horses, and possibly even reservoir hosts like zebra (although in these reservoir hosts, the amount of virus might be below the concentration required for detection via IHC), as well as inoculated chicken embryos, and even *Culicoides* vectors. The huge challenge posed by the need to standardize and validate IHC will require great effort and more research, but the benefits to diagnostic and research-orientated pathology will undoubtedly be commensurate.

REFERENCES

ALAM, M.M., KOBAYASHI, N., ISHINO, M., NAIK, T.N. & TANGIGUCHI, K. 2006. Analysis of genetic factors related to preferential selection of the NS1 gene segment observed in mixed infection and multiple passage of rotaviruses. *Archives of Virology*, 151**:** 2149-2159.

ALLAN, G.M., SMYTH, J.A., TODD, D. & MCNULTY, M.S. 1993. In situ hybridization for the detection of chicken anemia virus in formalin-fixed, paraffin-embedded sections. *Avian Diseases*, 37**:** 177-182.

ALLAN, G.M., TODD, D., SMYTH, J.A., MACKIE, D.P., BURNS, J. & MCNULTY, M.S. 1989. In situ hybridization: an optimised detection protocol for a biotinylated DNA probe renders it more sensitive than a comparable 35S-labelled probe. *Journal of Virological Methods*, 24**:** 181-190.

ANDERSON, G.A., PHILLIPS, D.L., WALDVOGEL, A.S. & OSBURN, B.I. 1989. Detection of bluetongue virus in bovine fetuses using the avidin-biotin complex immunoperoxidase method. *Journal of Veterinary Diagnostic Investigation*, 1**:** 45-49.

ARADAIB, I.E., BREWER, A.W. & OSBURN, B.I. 1997. Interaction of epizootic hemorrhagic disease virus with bovine erythrocytes in vitro: electron microscope study. *Comparative Immunology, Microbiology and Infectious Diseases,* 20**:** 281-283.

ATWAL, O.S., MINHAS, K.J., FERENCZY, B.G., JASSAL, D.S., MILTON, D. & MAHADEVAPPA, V.G. 1989. Morphology of pulmonary intravascular macrophages (PIMs) in ruminants: ultrastructural and cytochemical behavior of dense surface coat. *American Journal of Anatomy*, 186**:** 285-299.

ATWAL, O.S., SINGH, B., STAEMPFLI, H. & MINHAS, K. 1992. Presence of pulmonary intravascular macrophages in the equine lung: some structuro-functional properties. *Anatomical Record*, 234**:** 530-540.

BANCROFT, J.D. & GAMBLE, M. (Eds.). 2002. *Theory and practice of histological techniques*, 5th ed. Philadelphia: Churchill Livingstone.

BARNARD, B.J.H. 1997. Antibodies against some viruses of domestic animals in southern African wild animals. *Onderstepoort Journal of Veterinary Research*, 64**:** 95-110.

BARNARD, B.J.H., GERDES, G.H. & MEISWINKEL, R. 1998. Some epidemiological and economic aspects of a bluetongue-like disease in cattle in South Africa. *Onderstepoort Journal of Veterinary Research*, 65**:** 145-151.

BARRATT-BOYES, S.M. & MACLACHLAN, N.J. 1994. Dynamics of viral spread in bluetongue virus-infected calves. *Veterinary Microbiology*, 40**:** 361-371.

BARRATT-BOYES, S.M. & MACLACHLAN, N.J. 1995. Pathogenesis of bluetongue virus infection of cattle. *Journal of the American Veterinary Medical Association*, 206**:** 1322-1329.

BARRATT-BOYES, S.M., ROSSITTO, P.V., STOTT, J.L. & MACLACHLAN, N.J. 1992. Flow cytometric analysis of in vitro bluetongue virus infection of bovine blood mononuclear cells. *Journal of General Virology*, 73**:** 1953-1960.

BARRATT-BOYES, S.M., ROSSITTO, P.V., TAYLOR, B.C., ELLIS, J.A. & MACLACHLAN, N.J. 1995. Response of the regional lymph node to bluetongue virus infection in calves. *Veterinary Immunology and Immunopathology*, 45**:** 73-84.

BART, M., GUSCETTI, F., ZURBRIGGEN, A., POSPISCHIL, A. & SCHILLER, I. 2000. Feline infectious pneumonia: a short literature review and a retrospective immunohistological study on the involvement of *Chlamydia* spp. and distemper virus. *Veterinary Journal*, 159**:** 220-230.

BARTON, E.S., CHAPPELL, J.D., CONNOLLY, J.L., FORREST, J.C. & DERMODY, T.S. 2001. Reovirus receptors and apoptosis. *Virology*, 290**:** 173-180.

BASAK, A.K., GOUET, P., GRIMES, J., ROY, P. & STUART, D. 1996. Crystal structure of the top domain of African horse sickness virus VP7: comparisons with bluetongue virus VP7. *Journal of Virology*, 70**:** 3797-3806.

BAYLIS, M., MELLOR, P.S. & MEISWINKEL, R. 1999. Horse sickness and ENSO in South Africa. *Nature*, 397**:** 574.

BEATON, A.R., RODRIGUEZ, J., REDDY, Y.K. & ROY, P. 2002. The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. *Proceedings of the National Academy of Sciences of the United States of America*, 99**:** 13154-13159.

BENTLEY, L., FEHRSEN, J., JORDAAN, F., HUISMANS, H. & DU PLESSIS, D.H. 2000. Identification of antigenic regions on VP2 of African horsesickness virus serotype 3 by using phage-displayed epitope libraries. *Journal of General Virology*, 81**:** 993-1000.

BEVAN, E.W. 1911. The transmission of African horsesickness to the dog by feeding. *Veterinary Journal*, 67**:** 402-408.

BEYER, J., FICHTNER, D., SCHIRRMEIER, H., POLSTER, U., WEILAND, E. & WEGE, H. 2000. Porcine reproductive and respiratory syndrome virus (PRRSV): kinetics of

infection in lymphatic organs and lung. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health*, 47**:** 9-25.

BLYTH, C.R. 1986. Approximate binomial confidence limits. *Journal of the American Statistical Association*, 81**:** 843-855.

BOENISCH, T. 2001. *Handbook on immunohistochemical staining methods*, 3rd ed. Carpinteria: DAKO Corporation.

BOUTILIER, L., STRATIS, M., BAILEY, D., MCGIRR, D. & WAHID, S. 1989. A comparison of a simple two-step immunoalkaline phosphatase technique with the peroxidase-antiperoxidase (PAP) and avidin biotin complex (ABC) technique. *Journal of Histotechnology*, 12**:** 279-282.

BRAIN, J.D., MOLINA, R.M., DECAMP, M.M. & WARNER, A.E. 1999. Pulmonary intravascular macrophages: their contribution to the mononuclear phagocyte system in 13 species. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 276**:** L146-L154.

BREESE, S.S., OZAWA, Y. & DARDIRI, A.H. 1969. Electron microscopic characterization of African horse-sickness virus. *Journal of the American Veterinary Medical Association*, 155**:** 391-400.

BREMER, C.W., HUISMANS, H. & VAN DIJK, A.A. 1990. Characterization and cloning of the African horsesickness virus genome. *Journal of General Virology*, 71**:** 793-799.

BREMER, C.W. & VILJOEN, G.J. 1998. Detection of African horsesickness virus and discrimination between two equine orbivirus serogroups by reverse transcription polymerase chain reaction. *Onderstepoort Journal of Veterinary Research*, 65**:** 1-8.

BREWER, A.W. & MACLACHLAN, N.J. 1992. Ultrastructural characterization of the interaction of bluetongue virus with bovine erythrocytes in vitro. *Veterinary Pathology*, 29**:** 356-359.

BREWER, A.W. & MACLACHLAN, N.J. 1994. The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization. *Archives of Virology*, 136**:** 287-298.

BRODIE, S.J., BADSLEY, K.D., DIEM, K., MECHAM, J. O., NORELIUS, S.E. & WILSON, W.C. 1998. Epizootic hemorrhagic disease: analysis of tissues by amplification and in situ hybridization reveals widespread orbivirus infection at low copy numbers. *Journal of Virology*, 72**:** 3863-3871.

BROWN, C.C., BAKER, D.C. & BARKER, I.K. 2007. Alimentary system, in J*ubb, Kennedy and Palmer's pathology of domestic animals*, 5th ed., edited by M.G. Maxie. London: Elsevier.

BROWN, C.C. & DARDIRI, A.H. 1990. African horse sickness: a continuing menace. *Journal of the American Veterinary Medical Association*, 196**:** 2019-2021.

BROWN, C.C., MEYER, R.F. & GRUBMAN, M.J. 1994. Presence of African horse sickness virus in equine tissues, as determined by in situ hybridization. *Veterinary Pathology*, 31**:** 689-694.

BROWN, C.C., RHYAN, J.C., GRUBMAN, M.J. & WILBUR, L.A. 1996. Distribution of bluetongue virus in tissues of experimentally infected pregnant dogs as determined by in situ hybridization. *Veterinary Pathology*, 33**:** 337-340.

BURRAGE, T.G. & LAEGREID, W.W. 1994. African horsesickness: pathogenesis and immunity. *Comparative Immunology Microbiology and Infectious Diseases*, 17**:** 275-285.

BURRAGE, T.G., TREVEJO, R., STONE-MARSCHAT, M. & LAEGREID, W.W. 1993. Neutralizing epitopes of African horsesickness virus seroptype 4 are located on VP2. *Virology*, 196**:** 799-803.

BURROUGHS, J.N., GRIMES, J.M., MERTENS, P.P. & STUART, D.I. 1995. Crystallization and preliminary x-ray analysis of the core particle of bluetongue virus. *Virology*, 210**:** 217-220.

BURROUGHS, J.N., O'HARA, R.S., SMALE, C.J., HAMBLIN, C., WALTON, A., ARMSTRONG, R. & MERTENS, P.P.C. 1994. Purification and properties of virus particles, infectious subviral particles, cores and VP7 crystals of African horsesickness virus serotype 9. *Journal of General Virology*, 75**:** 1849-1857.

CARRASCO, L., GÓMEZ-VILLAMANDOS, J.C., BAUTISTA, M.J., HERVAS, J., PULIDO, B. & SIERRA, M.A. 1996. Pulmonary intravascular macrophages in deer. *Veterinary Research*, 27**:** 71-77.

CARRASCO, L., SÁNCHEZ, C., GÓMEZ-VILLAMANDOS, J.C., LAVIADA, M.D., BAUTISTA, M.J., MARTÍNEZ-TORRECUADRADA, J., SÁNCHEZ-VIZCAÍNO, J.M. & SIERRA, M.A. 1999. The role of pulmonary intravascular macrophages in the pathogenesis of African horse sickness. *Journal of Comparative Pathology*, 121**:** 25-38.

CHEVILLE, N.F. 1975. Cytopathology in viral diseases, in *Monographs in virology*, edited by J.L. Melnick. New York: S. Karger.

CHITKOMCKOWN, C.G. & BLECHA, F. 1992. Pulmonary intravascular macrophages: a review of immune properties and functions. *Annales De Recherches Veterinaires*, 23**:** 201-214.

CHUMA, T., LE BLOIS, H., SFINCHEZ-VIZCAÍNO, J.M., M., D.-L. & ROY, P. 1992. Expression of the major core antigen VP7 of African horsesickness virus by a recombinant baculovirus and its use as a group-specific diagnostic reagent. *Journal of General Virology*, 73**:** 925-993.

CLOPPER, C.J. & PEARSON, E.S. 1934. The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika*, 26**:** 404-413.

COETZER, J.A.W. & GUTHRIE, A.J. 2004. African horse sickness, in *Infectious diseases of livestock*, 2nd ed., edited by J.A.W. Coetzer & R.C. Tustin. Cape Town: Oxford University Press Southern Africa.

COETZER, J.A.W. & TUSTIN, R.C. (Eds). 2004. Reoviridae, in *Infectious diseases of livestock*, 2nd ed. Cape Town: Oxford University Press Southern Africa.

CONNOLLY, J.L., BARTON, E.S. & DERMODY, T.S. 2001. Reovirus binding to cell surface sialic acid potentiates virus-induced apoptosis. *Journal of Virology*, 75**:** 4029-4039.

CONNOLLY, J.L. & DERMODY, T.S. 2002. Virion disassembly is required for apoptosis induced by reovirus. *Journal of Virology*, 76**:** 1632-1641.

CONNOLLY, J.L., RODGERS, S.E., CLARKE, P., BALLARD, D.W., KERR, L.D., TYLER, K.L. & DERMODY, T.S. 2000. Reovirus-induced apoptosis requires activation of transcription factor NF-κB. *Journal of Virology*, 74**:** 2981-2989.

CRAFFORD, J.E., GUTHRIE, A.J., VAN VUUREN, M., MERTENS, P.P.C., BURROUGHS, J.N., HOWELL, P.G. & HAMBLIN, C. 2003. A group-specific, indirect sandwich ELISA for the detection of equine encephalosis virus antigen. *Journal of Virological Methods*, 112**:** 129-135.

DANGLER, C.A., DE LA CONCHA-BERMEJILLO, A., STOTT, J.L. & OSBURN, B.I. 1990. Limitations of in situ hybridization for the detection of bluetongue virus in blood mononuclear cells. *Journal of Veterinary Diagnostic Investigation*, 2**:** 303-307.

DARDIRI, A.H. & OZAWA, Y. 1969. Immune and serologic response of dogs to neurotropic and viscerotropic African horse-sickness virus. *Journal of the American Veterinary Medical Association*, 155**:** 400-407.

DAVIES, F.G. & LUND, L.J. 1974. The application of fluorescent antibody techniques to the virus of African horse sickness. *Research in Veterinary Science*, 17**:** 128-130.

DECAMP, M.M., WARNER, A.E., MOLINA, R.M. & BRAIN, J.D. 1992. Hepatic versus pulmonary uptake of particles injected into the portal circulation in sheep: endotoxin escapes hepatic clearance causing pulmonary inflammation. *American Review of Respiratory Disease*, 146**:** 224-231.

DE LA CONCHA-BERMEJILLO, A., SCHORE, C.E., DANGLER, C.A., DE MATTOS, C.C., DE MATTOS, C.A. & I., O.B. 1995. Comparison of slot blot nucleic acid hybridization, immunofluorescence, and virus isolation techniques to detect bluetongue virus in blood mononuclear cells from cattle with experimentally induced infection. *American Journal of Veterinary Research*, 53**:** 2245-2250.

DEMAULA, C.D., JUTILA, M.A., WILSON, D.W. & MACLACHLAN, N.J. 2001. Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary

artery and lung microvascular endothelial cells. *Journal of General Virology*, 82**:** 787-794.

DEMAULA, C.D., LEUTENEGGER, C.M., BONNEAU, K.R. & MACLACHLAN, N.J. 2002a. The role of endothelial cell-derived inflammatory and vasoactive mediators in the pathogenesis of bluetongue. *Virology*, 296**:** 330-337.

DEMAULA, C.D., LEUTENEGGER, C.M., JUTILA, M.A. & MACLACHLAN, N.J. 2002b. Bluetongue virus-induced activation of primary bovine lung microvascular endothelial cells. *Veterinary Immunology and Immunopathology*, 86**:** 147-157.

DE WAAL, P.J. & HUISMANS, H. 2005. Characterization of the nucleic acid binding activity of inner core protein VP6 of African horse sickness virus. *Archives of Virology*, 150**:** 2037-2050.

DU PLESSIS, D.H., VAN WYNGAARDT, W. & BREMER, C.W. 1990. An indirect sandwich ELISA utilizing F(ab')2 fragments for the detection of African horsesickness virus. *Journal of Virological Methods*, 29**:** 279-289.

DU PLESSIS, D.H., VAN WYNGAARDT, W., ROMITO, M., DU PLESSIS, M. & MAREE, S. 1999. The use of chicken IgY in a double antibody sandwich ELISA for detecting African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 66**:** 25-28.

DU TOIT, R.M. 1944. The transmission of bluetongue and horse sickness by *Culicoides. Onderstepoort Journal of Veterinary Science and Animal Industry*, 19**:** 7-16.

EATON, B.T. 2004. Bluetongue, in *Manual of diagnostic tests and vaccines for terrestrial animals*, 5th ed. Paris: Office International des Épizooties.

EATON, B.T. & CRAMERI, G.S. 1989. The site of bluetongue virus attachment to glycophorins from a number of animal erythrocytes. *Journal of General Virology*, 70**:** 3347-3353.

EATON, B.T., HYATT, A.D. & WHITE, J.R. 1987. Association of bluetongue virus with the cytoskeleton. *Virology*, 157**:** 107-116.

ERASMUS, B.J. 1963. Cultivation of horsesickness virus in tissue culture. *Nature*, 200**:** 716.

ERASMUS, B.J. 1966. The attenuation of horsesickness virus: problems and advantages associated with the use of different host systems. *Proceedings of the 1st International Conference on Equine Infectious Diseases*, *Stresa*: 208-213.

ERASMUS, B.J. 1973. Pathogenesis of African horsesickness. *Proceedings of the 3rd International Conference on Equine Infectious Diseases*, *Paris*: 1-11.

ERASMUS, B.J. 1975. Bluetongue in sheep and goats. *Australian Veterinary Journal*, 51**:** 165-170.

ERASMUS, B.J., BOSHOFF, S.T. & PIETERSE, L.M. 1976. The isolation and characterization of equine encephalosis and serologically-related orbiviruses from horses, in *Equine infectious diseases*, edited by J.T. Bryans & H. Gerber. Princeton: Veterinary Publications.

FARAWAY, J.J. 2006. *Extending the linear model with R.* Boca Raton: Chapman & Hall/CRC.

FUNAKOSHI, Y., NAKAYAMA, H., UETSUKA, K., NISHIMURA, R., SASAKI, N. & DOI, K. 2000. Cellular proliferative and telomerase activity in canine mammary gland tumors. *Veterinary Pathology*, 37**:** 177-183.

GERDES, G.H., NESER, J.A., BARNARD, B.J. & LARSEN, J. 1996. Stomatitis and coronitis in cattle - an insect-borne viral disease? *Journal of the South African Veterinary Association*, 67**:** 103-104.

GLEISER, C.A., STAIR, E.L. & MCGILL, L.D. 1969. Diagnosis of bluetongue in cattle by intravenous inoculation of chicken embryos and immunofluorescence. *American Journal of Veterinary Research*, 30**:** 981-986.

GÓMEZ-VILLAMANDOS, J.C., SÁNCHEZ, C., CARRASCO, L., LAVIADA, M.D., BAUTISTA, M.J., MARTÍNEZ-TORRECUADRADA, J., SÁNCHEZ-VIZCAÍNO, J.M. & SIERRA, M.A. 1999. Pathogenesis of African horse sickness: ultrastructural study of the capillaries in experimental infection. *Journal of Comparative Pathology*, 121**:** 101-116.

GRAU-ROMA, L. & SEGALÉS, J. 2007. Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. *Veterinary Microbiology*, 119**:** 144-151.

GRIMES, J.M., BURROUGHS, J.N., GOUET, P., DIPROSE, J.M., MALBY, R., ZIENTARA, S., MERTENS, P.P. & STUART, D.I. 1998. The atomic structure of the bluetongue virus core. *Nature*, 395**:** 470-478.

GRUBER, A.D., GREISER-WILKE, I.M., HAAS, L., HEWICKER-TRAUTWEIN, M. & MOENNIG, V. 1993. Detection of bovine viral diarrhea virus RNA in formalin-fixed, paraffin-embedded brain tissue by nested polymerase chain reaction. *Journal of Virological Methods*, 43**:** 309-319.

GRUBMAN, M.J. & LEWIS, S.A. 1992. Identification and characterization of the structural and nonstructural proteins of African horsesickness virus and determination of the genome coding assignments. *Virology*, 186**:** 444-451.

GUMM, I.D. & NEWMAN, J.F. 1982. The preparation of purified bluetongue virus group antigen for use as a diagnostic reagent. *Archives of Virology*, 72**:** 83-93.

HAIGH, D.A., MCINTOSH, B.M., CUMMING, B.B. & HEMPSTEAD, J.F.D. 1956. An outbreak of horsesickness, complicated by distemper, in a pack of foxhounds. *Journal of the South African Veterinary Association*, 27**:** 245-249.

HAINES, D.M. & CHELACK, B.J. 1991. Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology. *Journal of Veterinary Diagnostic Investigation*, 3**:** 101-112.

HAINES, D.M., CLARK, E.G. & DUBOVI, E.J. 1992. Monoclonal antibody-based immunohistochemical detection of bovine viral diarrhea virus in formalin-fixed, paraffin-embedded tissues. *Veterinary Pathology*, 29**:** 27-32.

HALBUR, P.G., PAUL, P.S., FREY, M.L., LANDGRAF, J., EERNISSE, K., MENG, X.J., ANDREWS, J.J., LUM, M.A. & RATHJE, J.A. 1996. Comparison of the antigen distribution of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Veterinary Pathology*, 33**:** 159-170.

HAMBLIN, C., ANDERSON, E.C., MELLOR, P.S., GRAHAM, S.D., MERTENS, P.P.C. & BURROUGHS, J.N. 1992. The detection of African horse sickness virus antigens and antibodies in young equidae. *Epidemiology and Infection*, 108**:** 193-201.

HAMBLIN, C., MERTENS, P.P.C., MELLOR, P.S., BURROUGHS, J.N. & CROWTHER, J.R. 1991. A serogroup specific enzyme-linked immunosorbent assay for the detection and identification of African horse sickness viruses. *Journal of Virological Methods*, 31**:** 285-292.

HASSAN, S.S. & ROY, P. 1999. Expression and functional characterization of bluetongue virus VP2 protein: Role in cell entry. *Journal of Virology*, 73**:** 9832-9842.

HEIDEL, J.D., HU, S., LIU, X.F., TRICHE, T.J. & DAVIS, M.E. 2004. Lack of interferon response in animals to naked siRNAs. *Nature Biotechnology*, 22**:** 1579-1582.

HENNING, M.W. 1956. African horsesickness, perdesiekte, pestis equorum, in *Animal diseases of South Africa*, 3rd ed. Pretoria: Central News Agency.

HOFF, G.L. & TRAINER, D.O. 1981. Hemorrhagic diseases in wild ruminants, in *Infectious diseases of wild mammals*, 2nd ed., edited by J.W. Davis & L.H. Karstad. Ames: Iowa State University Press.

HOFMANN, M.A., THUR, B., LIU, L., GERBER, M., STETTLER, P., MOSER, C. & BOSSY, S. 2000. Rescue of infectious classical swine fever and foot-and-mouth disease virus by RNA transfection and virus detection by RT-PCR after extended storage of samples in trizol. *Journal of Virological Methods*, 87**:** 29-39.

HOUSE, C., SHIPMAN, L.D. & WEYBRIGHT, G. 1998. Serological diagnosis of epizootic hemorrhagic disease in cattle in the USA with lesions suggestive of vesicular disease. *Annals of the New York Academy of Science*, 849**:** 497-500.

HOWELL, P.G., GUTHRIE, A.J. & COETZER, J.A.W. 2004. Equine encephalosis, in *Infectious diseases of livestock*, 2nd ed., edited by J.A.W. Coetzer & R.C. Tustin. Cape Town: Oxford University Press Southern Africa.

HOWERTH, E.W., GREENE, C.E. & PRESTWOOD, A.K. 1988. Experimentally induced bluetongue virus infection in white-tailed deer: coagulation, clinical pathologic, and gross pathologic changes. *American Journal of Veterinary Research*, 49**:** 1906-1913.

HOWERTH, E.W., PARLAVANTZAS, G.S. & STALLKNECHT, D.E. 2004. Replication of epizootic haemorrhagic disease and bluetongue viruses in DH82 cells. *Veterinaria Italiana*, 40**:** 520-524.

HOWERTH, E.W. & TYLER, D.E. 1988. Experimentally induced bluetongue virus infection in white-tailed deer: ultrastructural findings. *American Journal of Veterinary Research*, 49**:** 1914-1922.

HSU, S.M., RAINE, L. & FANGER, H. 1981a. A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *American Journal of Clinical Pathology*, 75**:** 734-738.

HSU, S.M., RAINE, L. & FANGER, H. 1981b. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry*, 29**:** 577-580.

HUISMANS, H. 1979. Protein synthesis in bluetongue virus-infected cells. *Virology*, 92**:** 385-396.

HUISMANS, H. & ELS, H.J. 1979. Characterization of the tubules associated with the replication of three different orbiviruses. *Virology*, 92**:** 397-406.

HUISMANS, H. & ERASMUS, B.J. 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort Journal of Veterinary Research*, 48**:** 51-58.

HUISMANS, H., VAN DER WALT, N.T., CLOETE, M. & ERASMUS, B.J. 1987a. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology*, 157**:** 172-179.

HUISMANS, H. & VAN DIJK, A.A. 1990. Bluetongue virus structural components. *Current Topics in Microbiology and Immunology*, 162**:** 21-41.

HUISMANS, H., VAN DIJK, A.A. & ELS, H.J. 1987b. Uncoating of parental bluetongue virus to core and subcore particles in infected L cells. *Virology*, 157**:** 180-188.

HUISMANS, H., VAN STADEN, V., FICK, W.C., VAN NIEKERK, M. & MEIRING, T.L. 2004. A comparison of different orbivirus proteins that could affect virulence and pathogenesis. *Veterinaria Italiana*, 40**:** 417-425.

IWATA, H., CHUMA, T. & ROY, P. 1992. Characterization of the genes encoding two of the major capsid proteins of epizootic haemorrhagic disease virus indicates a close genetic relationship to bluetongue virus. *Journal of General Virology*, 73**:** 915-924.

JACKSON, D.P., HAYDEN, J.D. & QUIRKE, P. 1991. Extraction of nucleic acids from fresh and archival material, in *PCR: a practical approach*, edited by M.J. McPherson, P. Quirke, and G.R. Taylor. New York: Oxford University Press.

JACOBSON, R. 2004. Principles of validation of diagnostic assays for infectious diseases, in *Manual of diagnostic tests and vaccines for terrestrial animals*, 5th ed. Paris: Office International des Épizooties.

JACQUE, J.-M., TRIQUES, K. & STEVENSON, M. 2002. Modulation of HIV-1 replication by RNA interference. *Nature*, 418**:** 435-438.

KALAI, M., VAN LOO, G., VANDEN BERGHE, T., MEEUS, A., BURM, W., SAELENS, X. & VANDENABEELE, P. 2002. Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA. *Cell Death and Differentiation*, 9**:** 981-994.

KAR, A.K., BHATTACHARYA, B. & ROY, P. 2007. Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. *BMC Molecular Biology*, 8**:** 4.

KARSTAD, L., WINTER, A. & TRAINER, D.O. 1961. Pathology of epizootic hemorrhagic disease of deer. *American Journal of Veterinary Research*, 22**:** 227-235.

KARSTAD, L., TRAINER, D. O. 1967. Histopathology of experimental bluetongue disease of white-tailed deer. *Canadian Veterinary Journal*, 8**:** 247-254.

KOEKEMOER, J.J.O., POTGIETER, A.C., PAWESKA, J.T. & VAN DIJK, A.A. 2000. Development of probes for typing African horsesickness virus isolates using a complete set of cloned VP2-genes. *Journal of Virological Methods*, 88**:** 135-144.

KOEKEMOER, J.J.O. & VAN DIJK, A.A. 2004. African horsesickness virus serotyping and identification of multiple co-infecting serotypes with a single genome segment 2 RT-PCR amplification and reverse line blot hybridization. *Journal of Virological Methods*, 122**:** 49-56.

KOMMERS, G.D., KING, D.J., SEAL, B.S. & BROWN, C.C. 2003. Pathogenesis of chicken-passaged Newcastle disease viruses isolated from chickens and wild and exotic birds. *BioOne*, 47**:** 319-329.

KOMMERS, G.D., KING, D.J., SEAL, B.S., CARMICHAEL, K.P. & BROWN, C.C. 2002. Pathogenesis of six pigeon-origin isolates of Newcastle disease virus for domestic chickens. *Veterinary Pathology*, 39**:** 353-362.

KONDO, H., HIKITA, M., ITO, M. & KADOTA, K. 2000. Immunohistochemical study of *Pneumocystis carinii* infection in pigs: evaluation of pneumocystis pneumonia and a retrospective investigation. *Veterinary Record*, 147**:** 544-549.

KRETZMANN, H. 2006. The characterization of African horsesickness virus VP7 particles with foreign peptides inserted into site 200 of the VP7 protein top domain. M.Sc. thesis, University of Pretoria.

LAEGREID, W.W. 1994. Diagnosis of African horsesickness. *Comparative Immunology Microbiology and Infectious Diseases*, 17**:** 297-303.

LAEGREID, W.W., BURRAGE, T.G., STONE-MARSCHAT, M. & SKOWRONEK, A. 1992. Electron microscopic evidence for endothelial infection by African horsesickness virus. *Veterinary Pathology*, 29**:** 554-556.

LAEGREID, W.W., SKOWRONEK, A., STONE-MARSCHAT, M. & BURRAGE, T. 1993. Characterization of virulence variants of African horsesickness virus. *Virology*, 195**:** 836-839.

LAEGREID, W.W., STONE-MARSCHAT, M., SKOWRONEK, A. & BURRAGE, T. 1991. Infection of endothelial cells with African horse sickness viruses. *Bluetongue, African horse sickness, and related orbiviruses: Proceedings of the 2nd International Symposium*, *Paris*: 807-814.

LAVIADA, M.D., ARIAS, M. & SÁNCHEZ-VIZCAÍNO, J.M. 1993. Characterization of African horsesickness virus serotype 4-induced polypeptides in Vero cells and their reactivity in Western immunoblotting. *Journal of General Virology*, 74**:** 81-87.

LAVIADA, M., BABÍN, M., DOMINQUEZ, J. & SÁNCHEZ-VIZCAÍNO, J.M. 1992. Detection of African horsesickness virus in infected spleens by sandwich ELISA using two monoclonal antibodies specific for VP7. *Journal of Virological Methods*, 38**:** 229-242.

LECATSAS, G. 1968. Electron microscopic study of the formation of bluetongue virus. *Onderstepoort Journal of Veterinary Research*, 35**:** 139-149.

LECATSAS, G. & ERASMUS, B.J. 1967. Electron microscopic study of the formation of African horse-sickness virus. *Archiv für die Gesamte Virusforschung*, 22**:** 442-450.

LECATSAS, G., ERASMUS, B.J. & ELS, H.J. 1973. Electron microscopic studies on equine encephalosis virus. *Onderstepoort Journal of Veterinary Research*, 40**:** 53-57.

LEWANCZUK, R. & YAMAMOTO, T. 1982. Replication and release of epizootic haemorrhagic disease virus in BHK-21 cells. *Journal of General Virology*, 61**:** 233-244.

LONGWORTH, K.E., ALBERTINE, K.H. & STAUB, N.C. 1996. Ultrastructural quantification of pulmonary intravascular macrophages in newborn and 2-week-old lambs. *Anatomical Record*, 246**:** 238-244.

MACLACHLAN, N.J., JAGELS, G., ROSSITTO, P.V., MOORE, P.F. & HEIDNER, H.W. 1990. The pathogenesis of experimental bluetongue virus infection of calves. *Veterinary Pathology*, 27**:** 223-229.

MACLACHLAN, N.J. & OSBURN, B.I. 2004. Epizootic haemorrhagic disease of deer, in *Infectious diseases of livestock*, 2nd ed., edited by J.A.W. Coetzer & R.C. Tustin. Cape Town: Oxford University Press Southern Africa.

MAHRT, C.R. & OSBURN, B.I. 1986. Experimental bluetongue virus infection of sheep; effect of vaccination: pathologic, immunofluorescent, and ultrastructural studies. *American Journal of Veterinary Research*, 47**:** 1198-1203.

MALORNI, W., FAIS, S. & FIORENTINI, C. 1997. Morphological aspects of apoptosis, in Purdue Cytometry CD-ROM Series, vol. 4., edited by J. Watson. West Lafayette: Purdue University Cytometry Laboratories.

MAREE, S., DURBACH, S. & HUISMANS, H. 1998. Intracellular production of African horsesickness virus core-like particles by expression of the two major core proteins, VP3 and VP7, in insect cells. *Journal of General Virology*, 79**:** 333-337.

MAREE, S. & PAWESKA, J.T. 2005. Preparation of recombinant African horse sickness virus VP7 antigen via a simple method and validation of a VP7-based indirect ELISA for the detection of group-specific IgG antibodies in horse sera. *Journal of Virological Methods*, 125**:** 55-65.

MARTÍNEZ-COSTAS, J., SUTTON, G., RAMADEVI, N. & ROY, P. 1998. Guanylyltransferase and RNA 5'-triphosphatase activities of the purified expressed VP4 protein of bluetongue virus. *Journal of Molecular Biology*, 280**:** 859-866.

MARTÍNEZ-TORRECUADRADA, J.L., DÍAZ-LAVIADA, M., ROY, P., SÁNCHEZ, C., VELA, C., SÁNCHEZ-VIZCAÍNO, J.M. & CASAL, J.I. 1996. Full protection against African horsesickness (AHS) in horses induced by baculovirus-derived AHS virus serotype 4 VP2, VP5 and VP7. *Journal of General Virology*, 77**:** 1211-1221.

MARTÍNEZ-TORRECUADRADA, J.L., DÍAZ-LAVIADA, M., ROY, P., SÁNCHEZ, C., VELA, C., SÁNCHEZ-VIZCAÍNO, J.M. & CASAL, J.I. 1997. Serologic markers in early stages of African horse sickness virus infection. *Journal of Clinical Microbiology*, 35**:** 531-535.

MARTÍNEZ-TORRECAUDRADA, J.L., IWATA, H., VENTEO, A., CASAL, I. & ROY, P. 1994. Expression and characterization of the two outer capsid proteins of African horsesickness virus: the role of VP2 in virus neutralization. *Virology*, 202**:** 348-359.

MARTÍNEZ-TORRECUADRADA, J.L., LANGEVELD, J.P.M., VENTEO, A., SANZ, A., DALSGAARD, K., HAMILLON, W.D.O., MELOEN, R.H. & CASAL, J.I. 1999. Antigenic profile of African horse sickness virus serotype 4 VP5 and identification of a neutralizing epitope shared with bluetongue virus and epizootic hemorrhagic disease virus. *Virology*, 257**:** 449-459.

MAURER, F.D. & MCCULLY, R.M. 1963. African horse sickness, with emphasis on pathology. *American Journal of Veterinary Research*, 24**:** 235-266.

MAXIE, M.G. & ROBINSON, W.F. 2007. Cardiovascular system, in J*ubb, Kennedy and Palmer's pathology of domestic animals*, 5th ed., edited by M.G. Maxie. London: Elsevier.

MCINTOSH, B.M. 1958. Immunological types of horse sickness and their significance in immunization. *Onderstepoort Journal of Veterinary Research*, 27**:** 465-538.

MCLAUGHLIN, B.E., DEMAULA, C.D., WILSON, W.C., BOYCE, W.M. & MACLACHLAN, N.J. 2003. Replication of bluetongue virus and epizootic hemorrhagic disease virus in pulmonary artery endothelial cells obtained from cattle, sheep, and deer. *American Journal of Veterinary Research*, 64**:** 860-865.

MEISWINKEL, R. 1997. Discovery of a *Culicoides imicola*-free zone in South Africa: preliminary notes and potential significance. *Onderstepoort Journal of Veterinary Research*, 64**:** 81-86.

MEISWINKEL, R., BAYLIS, M. & LABUSCHAGNE, K. 2000. Stabling and the protection of horses from *Culicoides bolitinos* (Diptera: Ceratopogonidae), a recently identified vector of African horse sickness. *Bulletin of Entomological Research*, 90**:** 509-515.

MEISWINKEL, R. & PAWESKA, J.T. 2003. Evidence for a new field culicoides vector of African horse sickness in South Africa. *Preventive Veterinary Medicine*, 60**:** 243-253.

MEISWINKEL, R., VENTER, G.J. & NEVILL, E.M. 2004. Vectors: *Culicoides* spp., in *Infectious diseases of livestock*, 2nd ed., edited by J.A.W. Coetzer & R.C. Tustin. Cape Town: Oxford University Press Southern Africa.

MELLOR, P.S. 1993. African horse sickness: transmission and epidemiology. *Veterinary Research*, 24**:** 199-212.

MELLOR, P.S. & HAMBLIN, C. 2004. African horse sickness. *Veterinary Research*, 35**:** 445-466.

MERTENS, P.P. & DIPROSE, J. 2004. The bluetongue virus core: a nano-scale transcription machine. *Virus Research*, 101**:** 29-43.

MIKEL, U.V. (Ed). 1994. *Advanced laboratory methods in histology and pathology.* Washington, DC: Armed Forces Institute of Pathology, American Registry of Pathology.

MILLER, M.A., RAMOS-VARA, J.A., KLEIBOEKER, S.B. & LARSON, R.L. 2005. Effects of delayed or prolonged fixation on immunohistochemical detection of bovine viral diarrhea virus type I in skin of two persistently infected calves. *Journal of Veterinary Diagnostic Investigation*, 17**:** 461-463.

MOLINA, R.M. & BRAIN, J.D. 2007. In vivo comparison of cat alveolar and pulmonary intravascular macrophages: phagocytosis, particle clearance, and cytoplasmic motility. *Experimental Lung Research*, 33**:** 53-70.

MORI, M., SATO, K., AKACHI, S., ASAHI, S., TANIGUCHI, S. & NARITA, M. 2000. Retrospective study of porcine circovirus 2 infection in Japan: seven cases in 1989. *Veterinary Pathology*, 37**:** 667-669.

MORTOLA, E., NOAD, R. & ROY, P. 2004. Bluetongue virus outer capsid proteins are sufficient to trigger apoptosis in mammalian cells. *Journal of Virology*, 78**:** 2875-2883.

MOULTON, J.E. 1961. Pathology of bluetongue of sheep in California. *Journal of the American Veterinary Medical Association*, 138**:** 493-498.

MURPHY, F.A., BORDEN, E.C., SHOPE, R.E. & HARRISON, A. 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus – a new taxonomic group. Electron microscopic studies. *Journal of General Virology*, 13**:** 273-288.

NARITA, M., KIMURA, K., TANIMURA, N. & OZAKI, H. 1999. Immunohistochemical detection of hog cholera virus antigen in paraffin wax-embedded tissues from naturally infected pigs. *Journal of Comparative Pathology*, 121**:** 283-286.

NEWSHOLME, S.J. 1983. A morphological study of the lesions of African horsesickness. *Onderstepoort Journal of Veterinary Research*, 50**:** 7-24.

NIBERT, M.L., MARGRAF, R.L. & COOMBS, K.M. 1996. Nonrandom segregation of parental alleles in reovirus reassortants. *Journal of Virology*, 70**:** 295-300.

NIEPER, H., TEIFKE, J.P., JUNGMANN, A., LOHR, C.V. & MULLER, H. 1999. Infected and apoptotic cells in the IBDV-infected bursa of Fabricius, studied by double-labelling techniques. *Avian Pathology*, 28**:** 279-285.

NUOVO, G.J. 1994. In situ hybridization, in *PCR in situ hybridization: protocols and applications*, 2nd ed., edited by G.J. Nuovo. New York: Raven Press.

OBERHAUS, S.M., DERMODY, T.S. & TYLER, K.L. 1998. Apoptosis and cytopathic effect of reovirus, in *Reoviruses II, cytogenicity and pathogenesis*, edited by K.L. Taylor & M.B.A. Oldstone. New York: Springer-Verlag.

OELLERMANN, R.A., ELS, H.J. & ERASMUS, B.J. 1970. Characterization of African horsesickness virus. *Archiv für die Gesamte Virusforschung*, 29**:** 163-174.

OLDFIELD, S., ADACHI, A., URAKAWA, T., HIRASAWA, T. & ROY, P. 1990. Purification and characterization of the major group-specific core antigen VP7 of bluetongue virus synthesized by a recombinant baculovirus. *Journal of General Virology*, 71**:** 2649-2656.

O'LEARY, T. 2001. Standardization in immunochemistry. *Applied Immunohistochemistry*, 9**:** 3-8.

OZAWA, Y., SALAMA, S.A. & DARDIRI, A.H. 1972. Methods for recovering African horsesickness virus from horse blood. *Proceedings of the 3rd International Conference on Equine Infectious Diseases*, *Paris*: 58-68.

PAGE, C., ROSE, M., YACOUB, M. & PIGOTT, R. 1992. Antigenic heterogeneity of vascular endothelium. *American Journal of Pathology*, 141**:** 673-683.

PARISH, S.M., EVERMANN, J.F., OLCOTT, B. & GAY, C. 1982. A bluetongue epizootic in northwestern United States. *Journal of the American Veterinary Medical Association*, 181**:** 589-591.

PARSONSON, I.M. & MCCOLL, K.A. 1995. Retrospective diagnosis of bluetongue virus in stored frozen and fixed tissue samples using PCR. *Veterinary Microbiology*, 46**:** 143-149.

PAWESKA, J.T., GERDES, G.H., WOODS, P.S.A. & WILLIAMS, R. 1999. Equine encephalosis in southern Africa: current situation. *Proceeding of the 8th International Conference on Equine Infectious Diseases, Dubai*: 303–305.

PIERCE, C.M., BALASURIYA, U.B.R. & MACLACHLAN, N.J. 1998. Phylogenetic analysis of the S10 gene of field and laboratory strains of bluetongue virus from the United States. *Virus Research*, 55**:** 15-27.

PIERCY, S.E. 1951. Some observations on African horse-sickness including an account of an outbreak amongst dogs. *The East African Agricultural Journal of Kenya, Tanganyika, Uganda and Zanzibar*, 17**:** 62-64.

PINI, A. 1976. A study on the pathogenesis of bluetongue: replication of the virus in the organs of infected sheep. *Onderstepoort Journal of Veterinary Research*, 43**:** 159-164.

PINI, A., COACHLEY, W. & OHDER, H. 1966. Concentration of bluetongue virus in experimentally infected sheep and virus identification by immune fluorescence technique. *Archiv für die Gesamte Virusforschung*, 18**:** 385-390.

POLSON, A. & DEEKS, D. 1963. Electron microscopy of neurotropic horsesickness virus. *Journal of Hygiene*, 61**:** 149-153.

POWELL, D.G. 1985. The international movement of horses and its influence on the spread of infectious diseases. *Revue Scientifique et Technique (International Office of Epizootics)*, 5**:** 155-161.

PRITCHARD, L.I. & GOULD, A.R. 1995. Phylogenetic comparison of the serotype-specific VP2 protein of bluetongue and related orbiviruses. *Virus Research*, 39**:** 207-220.

PRITCHARD, L.I., GOULD, A.R., WILSON, W.C., THOMPSON, L., MERTENS, P.P. & WADE-EVANS, A.M. 1995. Complete nucleotide sequence of RNA segment 3 of bluetongue virus serotype 2 (Ona-A). Phylogenetic analyses reveal the probable origin and relationship with other orbiviruses. *Virus Research*, 35**:** 247-261.

PROPHET, E.B., MILLS, B., ARRINGTON, J.B. & SOBIN, L.H. 1992. *Laboratory methods in histotechnology*. Washington, D.C: Armed Forces Institute of Pathology, American Registry of Pathology.

PURSE, B.V., MELLOR, P.S., ROGERS, D.J., SAMUEL, A.R., MERTENS, P.P.C. & BAYLIS, M. 2005. Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews Microbiology*, 3**:** 171-181.

QUAN, M., VAN VUUREN, M., HOWELL, P.G., GROENEWALD, D. & GUTHRIE, A.J. 2008. Molecular epidemiology of the African horse sickness virus S10 gene. *Journal of General Virology*, 89**:** 1159-1168.

R Development Core Team. 2007. *R: A language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

RAAB, S.S. 2002. Cost-effectiveness of immunohistochemistry, in *Dabbs diagnostic immunohistochemistry*, edited by D.J. Dabbs. New York: Churchill Livingstone.

RAMOS-VARA, J.A. 2005. Technical aspects of immunohistochemistry. *Veterinary Pathology*, 42**:** 405-426.

RAMOS-VARA, J.A., DEL PIERO, F., KIUPEL, M., FITZGERALD, S.D., BERMUDEZ, A.J., JOHNSON, G.C. & MILLER, M.A. 2002a. Diagnostic immunohistochemistry of equine and avian infectious diseases. *Journal of Histotechnology*, 25**:** 185-198.

RAMOS-VARA, J.A., KIUPEL, M., BASZLER, T., BLIVEN, L., BRODERSEN, B., CHELACK, B., CZUB, S., PIERO, F.D., DIAL, S., EHRHART, E.J., GRAHAM, T., MANNING, L., PAULSEN, D., VALLI, V.E. & WEST, K. 2008. Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. *Journal of Veterinary Diagnostic Investigation*, 20**:** 393-413.

RAMOS-VARA, J.A., KIUPEL, M. & MILLER, A. 2002b. Diagnostic immunohistochemistry of infectious diseases in dogs and cats. *Journal of Histotechnology*, 25**:** 201-212.

RICKERT, R.R. & MALINIAK, R.M. 1989. Intralaboratory quality assurance of immunohistochemical procedures. *Archives of Pathology and Laboratory Medicine*, 113**:** 673-679.

RODRIGUEZ-ARRIOJA, G.M., SEGALÉS, J., ROSELL, C., ROVIRA, A., PUJOLS, J., PLANA-DURAN, J. & DOMINGO, M. 2003. Retrospective study on porcine circovirus type 2 infection in pigs from 1985 to 1997 in Spain. *Zoonoses and Public Health*, 50**:** 99-101.

ROY, P. 1992. Bluetongue virus proteins. *Journal of General Virology*, 73**:** 3051-3064.

ROY, P. 1996. Orbivirus structure and assembly. *Virology*, 216**:** 1-11.

ROY, P. 2001. Orbiviruses, in *Fields virology*, 4th ed., edited by D.M. Knipe & P.M. Howley. Philadelphia: Lippincott Williams & Wilkins.

ROY, P. 2008. Functional mapping of bluetongue virus proteins and their interactions with host proteins during virus replication. *Cell Biochemistry and Biophysics*, 50**:** 143-157.

ROY, P., BISHOP, D.H.L., HOWARD, S., AITCHISON, H. & ERASMUS, B. 1996. Recombinant baculovirus-synthesized African horsesickness virus (AHSV) outer-capsid protein VP2 provides protection against virulent AHSV challenge. *Journal of General Virology*, 77**:** 2053-2057.

ROY, P., URAKAWA, T., VAN DIJK, A.A. & ERASMUS, B.J. 1990. Recombinant virus vaccine for bluetongue disease in sheep. *Journal of Virology*, 64**:** 1998-2003.

RUBIO, C., CUBILLO, M.A., HOOGHUIS, H., SÁNCHEZ-VIZCAÍNO, J.M., DÍAZ-LAVIADA, M., PLATEAU, E., ZIENTARA, S., CRUCIÈRE, C. & HAMBLIN, C. 1998. Validation of ELISA for the detection of African horse sickness virus antigens and antibodies. *Archives of Virology*, 14, Supplement 1**:** 311-315.

RUCKERBAUER, G.M., GRAY, D.P., GIRARD, A., BANNISTER, G.L. & BOULANGER, P. 1967. Studies on bluetongue V. Detection of the virus in infected materials by immunofluorescence. *Canadian Journal of Comparative Medicine*, 31**:** 175-181.

RÜDIGER, T., HÖFLER, H., KREIPE, H.H., NIZZE, H., PFEIFER, U., STEIN, H., DALLENBACH, F.E., FISCHER, H.P., MENGEL, M., VON WASIELEWSKI, R. & MÜLLER-HERMELINK, H.K. 2002. Quality assurance in immunohistochemistry: results of an interlaboratory trial involving 172 pathologists. *American Journal of Surgical Pathology*, 26**:** 873-882.

RUPP, G.M. & LOCKER, J. 1988. Purification and analysis of RNA from paraffin-embedded tissues. *Biotechniques*, 6**:** 56-60.

SAILLEAU, C., SEIGNOT, J., DAVOUST, B., CARDINALE, E., FALL, B., HAMBLIN, C. & ZIENTARA, S. 2000. African horse sickness in Senegal: serotype identification and nucleotide sequence determination of segment S10 by RT-PCR. *Veterinary Record*, 146**:** 107-108.

SAKAMOTO, K., PUNYAHOTRA, R., MIZUKOSHI, N., UEDA, S., IMAGAWA, H., SUGIURA, T., KAMADA, M. & FUKUSHO, A. 1994. Rapid detection of African horsesickness virus by the reverse transcriptase polymerase chain reaction (RT-PCR) using the amplimer for segment 3 (VP3 gene). *Archives of Virology*, 136**:** 87-97.

SÁNCHEZ-VIZCAÍNO, J.M. 2004. African horse sickness, in *Manual of diagnostic tests and vaccines for terrestrial animals*, 5th ed. Paris: Office International des Épizooties.

SCHOEPP, R.J., BLAIR, C.D., ROY, P. & BEATY, B.J. 1991. Detection of bluetongue virus RNA by in situ hybridization: comparison with virus isolation and antigen detection. *Journal of Veterinary Diagnostic Investigation*, 3**:** 22-28.

SHI, S.R., GU, J., TURRENS, J.F., COTE, R.J. & TAYLOR, C.R. 2000. Development of a antigen retrieval technique: philosophy and theoretical basis, in *Antigen retrieval techniques: immunohistochemistry and molecular morphology*, edited by S.R. Shi, J. Gu, and C.R. Taylor. Natick: Ethan Publishing Company.

SHI, S.R., KEY, M.E. & KALRA, K.L. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *Journal of Histochemistry and Cytochemistry*, 39**:** 741-748.

SHIBAHARA, T., SATO, K., ISHIKAWA, Y. & KADOTA, K. 2000. Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. *Journal of Veterinary Medical Science*, 62**:** 1125-1131.

SHOUP, D.I., SWAYNE, D.E., JACKWOOD, D.J. & J., S.L. 1996. Immunohistochemistry of transmissible gastroenteritis virus antigens in fixed paraffin-embedded tissues. *Journal of Veterinary Diagnostic Investigation*, 8**:** 161-167.

SINGH, B., OTT, T.L., BAZER, F.W. & DE LA CONCHA-BERMEJILLO, D. 1998. Structural responses of pulmonary intravascular macrophages in lentivirus-infected and/or recombinant ovine interferon-τ-treated lambs. *Anatomical Record*, 251**:** 472-485.

SINGH, V.K., SAI KUMAR, G. & PALIWAL, O.P. 2005. Detection of classical swine fever virus in archival formalin-fixed tissues by reverse transcription-polymerase chain reaction. *Research in Veterinary Science*, 79**:** 81-84.

SKOWRONEK, A.J., LAFRANCO, L., STONE-MARSCHAT, M.A., BURRAGE, T.G., REBAR, A.H. & LAEGREID, W.W. 1995. Clinical pathology and hemostatic abnormalities in experimental African horsesickness. *Veterinary Pathology*, 32**:** 112-121.

SOMPURAN, S.R., VANI, K., MESSANA, E. & BOGEN, S.A. 2004. A molecular mechanism of formalin fixation and antigen retrieval. *American Journal of Clinical Pathology*, 121**:** 190-199.

STAIR, E.L. 1968. The pathogenesis of bluetongue in sheep: a study by immunofluorescence and histopathology. Ph.D. thesis, Texas A & M University.

STANTON, J.B., POET, S., FRASCA, S., JR., BIENZLE, D. & BROWN, C.C. 2002. Development of a semi-nested reverse transcription polymerase chain reaction assay

for the retrospective diagnosis of canine distemper virus infection. *Journal of Veterinary Diagnostic Investigation*, 14**:** 47-52.

STÄUBER, N., MCCULLOGH, K.C. & KIHM, U. 1992. Interaction of African horsesickness viruses with leukocytes and the capacity to induce an immune response in vitro, in *Bluetongue, African horse sickness, and related orbiviruses*, edited by T.E. Walton & B.I. Osburn. Boca Raton: CRC Press.

STOLTZ, M.A., VAN DER MERWE, C.F., COETZEE, J. & HUISMANS, H. 1996. Subcellular localization of the nonstructural protein NS3 of African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 63**:** 57-61.

STONE-MARSCHAT, M., CARVILLE, A., SKOWRONEK, A. & LAEGREID, W.W. 1994. Detection of African horse sickness virus by reverse transcription-PCR. *Journal of Clinical Microbiology*, 32**:** 697-700.

STONE-MARSCHAT, M.A., MOSS, S.R., BURRAGE, T.G., BARBER, M.L., ROY, P. & LAEGREID, W.W. 1996. Immunization with VP2 is sufficient for protection against lethal challenge with African horsesickness virus type 4. *Virology*, 220**:** 219-222.

SUR, J.H., DOSTER, A.R. & OSORIO, F.A. 1998. Apoptosis induced in vivo during acute infection by porcine reproductive and respiratory syndrome virus. *Veterinary Pathology*, 35**:** 506-514.

SWISHER, B. 2002. Microorganisms, in *Theory and practice of histological techniques*, 5th ed., edited by J.D. Bancroft & M. Gamble. Edinburgh: Churchill Livingstone.

TAKAMATSU, H., MELLOR, P.S., MERTENS, P.P.C., KIRKHAM, P.A., BURROUGHS, J.N. & PARKHOUSE, R.M.E. 2003. A possible overwintering mechanism for bluetongue virus in the absence of the insect vector. *Journal of General Virology*, 84**:** 227-235.

TAYLOR, C.R. 1994. An exaltation of experts: concerted efforts in the standardization of immunochemistry. *Human Pathology*, 25**:** 2-11.

TAYLOR, C.R. 1998. Report from the biological stain commission: FDA issues final rule for classification/reclassification of immunohistochemistry (IHC) reagents and kits. *Biotechnic and Histochemistry*, 73**:** 175-177.

TAYLOR, C.R. 2000. The total test approach to standardization of immunochemistry. *Archives of Pathology & Laboratory Medicine*, 124**:** 945-951.

TAYLOR, C.R., SHI, S.-R., BARR, N.J. & WU, N. 2002. Techniques of immunohistochemistry: principles, pitfalls, and standardization, in *Dabbs diagnostic immunohistochemistry*, edited by D.J. Dabbs. New York: Churchill Livingstone.

THANAWONGNUWECH, R., BROWN, G.B., HALBUR, P.G., ROTH, J.A., ROYER, R.L. & THACKER, B.J. 2000. Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to *Streptococcus suis* infection. *Veterinary Pathology*, 37**:** 143-152.

THANAWONGNUWECH, R., HALBUR, P.G. & ANDREWS, J.J. 1997a. Immunohistochemical detection of porcine reproductive and respiratory syndrome virus antigen in neurovascular lesions. *Journal of Veterinary Diagnostic Investigation*, 9**:** 334-337.

THANAWONGNUWECH, R., THACKER, E.L. & HALBUR, P.G. 1997b. Effect of porcine reproductive and respiratory syndrome virus (PRRSV) (isolate ATCC VR-2385) infection on bactericidal activity of porcine pulmonary intravascular macrophages (PIMs): in vitro comparisons with pulmonary alveolar macrophages (PAMs). *Veterinary Immunology and Immunopathology*, 59**:** 323-335.

THEILER, A. 1910. The susceptibility of the dog to African horsesickness. *Journal of Comparative Pathology*, 23**:** 315-353.

THOMAS, A.D. & NEITZ, W.O. 1947. Further observations of the pathology of bluetongue in sheep. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 22**:** 27-40.

TSAI, K. & KARSTAD, L. 1970. Epizootic hemorrhagic disease virus of deer: an electron microscopic study. *Canadian Journal of Microbiology*, 16**:** 427-432.

TSAI, K. & KARSTAD, L. 1973. The pathogenesis of epizootic haemorrhagic disease of deer: an electron microscopic study. *American Journal of Pathology*, 70**:** 379-340.

TYLER, K.L. 1998. Pathogenesis of reovirus infections of the central nervous system, in *Reoviruses II, cytogenicity and pathogenesis*, edited by K.L. Taylor & M.B.A. Oldstone. New York: Springer-Verlag.

UITENWEERDE, J.M., THERON, J., STOLTZ, M.A. & HUISMANS, H. 1995. The multimeric nonstructural NS2 proteins of bluetongue virus, African horsesickness virus, and epizootic hemorrhagic disease virus differ in their single-stranded RNA-binding ability. *Virology*, 209**:** 624-632.

VAN ALSTINE, W.G., POPIELARCZYK, M. & ALBREGTS, S.R. 2002. Effects of formalin fixation on the immunohistochemical detection of PRRS virus antigen in experimentally and naturally infected pigs. *Journal of Veterinary Diagnostic Investigation*, 14**:** 504-507.

VAN DER LUGT, J.J., COETZER, J.A., SMIT, M.M. & CILLIERS, C. 1995. The diagnosis of Wesselsbron disease in a newborn lamb by immunohistochemical staining of viral antigen. *Onderstepoort Journal of Veterinary Research*, 62**:** 143-146.

VAN NIEKERK, M., SMIT, C.C., FICK, W.C., VAN STADEN, V. & HUISMANS, H. 2001. Membrane association of African horsesickness virus nonstructural protein NS3 determines its cytotoxicity. *Virology*, 279**:** 499-508.

VAN NIEKERK, M., FREEMAN, M., PAWESKA, J.T., HOWELL, P.G., GUTHRIE, A.J., POTGIETER, A.C., VAN STADEN, V. & HUISMANS, H. 2003. Variation in the NS3 gene and protein in South African isolates of bluetongue and equine encephalosis viruses. *Journal of General Virology*, 84**:** 581-590.

VAN RENSBURG, I.B., DE CLERK, J., GROENEWALD, H.B. & BOTHA, W.S. 1981. An outbreak of African horsesickness in dogs. *Journal of the South African Veterinary Association*, 52**:** 323-325.

VAN RENSBURG, R. 2004. Construction and structural evaluation of viral protein 7 of African horse sickness virus as a particulate, multiple peptide vaccine delivery system. M.Sc. thesis, University of Pretoria.

VAN STADEN, V., SMIT, C.C., STOLTZ, M.A., MAREE, F.F. & HUISMANS, H. 1998. Characterization of two African horse sickness virus nonstructural proteins, NS1 and NS3. *Archives of Virology*, 14, Supplement 1: 251-258.

VAN STADEN, V., THERON, J., GREYLING, B.J., HUISMANS, H. & NEL, L.H. 1991. A comparison of the nucleotide sequences of cognate NS2 genes of three different orbiviruses. *Virology*, 185**:** 500-504.

VAN WYNGAARDT, W., DU PLESSIS, D.H., VAN WYNGAARDT, S. & VERSCHOOR, J.A. 1992. Production and properties of monoclonal antibodies against African horsesickness virus, serotype 3. *Onderstepoort Journal of Veterinary Research*, 59**:** 129-133.

VENTER, G.J. & MEISWINKEL, R. 1994. The virtual absence of *Culicoides imicola* (Diptera: Ceratopogonidae) in a light-trap survey of the colder, high-lying area of the eastern Orange Free State, South Africa, and implications for the transmission of arboviruses. *Onderstepoort Journal of Veterinary Research*, 61**:** 327-340.

VERWOERD, D.W. & ERASMUS, B.J. 2004. Bluetongue, in *Infectious diseases of livestock*, 2nd ed., edited by J.A.W. Coetzer & R.C. Tustin. Cape Town: Oxford University Press Southern Africa.

VILJOEN, G.J. & HUISMANS, H. 1989. The characterization of equine encephalosis virus and the development of genomic probes. *Journal of General Virology*, 70**:** 2007-2015.

WADE-EVANS, A.M., PULLEN, L., HAMBLIN, C., O'HARA, R.S., BURROUGHS, J.N. & MERTENS, P.P.C. 1998. VP7 from African horse sickness virus serotype 9 protects mice against a lethal, heterologous serotype challenge. *Archives of Virology*, 14, Supplement 1: 211-219.

WADE-EVANS, A.M., WOOLHOUSE, T., O'HARA, R. & HAMBLIN, C. 1993. The use of African horse sickness virus VP7 antigen, synthesized in bacteria, and anti-VP7 monoclonal antibodies in a competitive ELISA. *Journal of Virological Methods*, 45**:** 179-188.

WADE-EVANS, A.M., PULLEN, L., HAMBLIN, C., O'HARA, R., BURROUGHS, J.N. & MERTENS, P.P.C. 1997. African horsesickness virus VP7 sub-unit vaccine protects mice against a lethal, heterologous serotype challenge. *Journal of General Virology*, 78**:** 1611-1616.

WANG, L., KEMP, M.C., ROY, P. & COLLISSON, E.W. 1988. Tissue tropism and target cells of bluetongue virus in the chicken embryo. *Journal of Virology*, 62**:** 887-893.

WARNER, A.E., BARRY, B.E. & BRAIN, J.D. 1986. Pulmonary intravascular macrophages in sheep: morphology and function of a novel constituent of the mononuclear phagocyte system. *Laboratory Investigation*, 55**:** 276-288.

WARNER, A.E. & BRAIN, J.D. 1990. The cell biology and pathogenic role of pulmonary intravascular macrophages. *American Journal of Physiology*, 258**:** L1-L12.

WARNER, A.E., DECAMP, M.M., MOLINA, R.M. & BRAIN, J.D. 1988. Pulmonary removal of circulating endotoxin results in acute lung injury in sheep. *Laboratory Investigation*, 59**:** 219-230.

WHEELDON, E.B. & HANSEN-FLASCHEN, J.H. 1986. Intravascular macrophages in the sheep lung. *Journal of Leukocyte Biology*, 40**:** 657-661.

WHETTER, L.E., MACLACHLAN, N.J., GEBHARD, D.H., HEIDNER, H.W. & MOORE, P.F. 1989. Bluetongue virus infection of bovine monocytes. *Journal of General Virology*, 70**:** 1663-1676.

WIEGERS, A.L. 2004. The quality assurance of proficiency testing programs for animal disease diagnostic laboratories. *Journal of Veterinary Diagnostic Investigation*, 16**:** 255-263.

WILLIAMS, C.F., INOUE, T., LUCUS, A.M., ZANOTTO, P. & ROY, P. 1998. The complete sequence of four major structural proteins of African horse sickness virus serotype 6: evolutionary relationships within and between the orbiviruses. *Virus Research*, 53**:** 53-73.

WILLIAMS, R., DU PLESSIS, D.H. & VAN WYNGAARDT, W. 1993. Group-reactive ELISAs for detecting antibodies to African horsesickness and equine encepahalosis viruses in horse, donkey and zebra sera. *Journal of Veterinary Diagnostic Investigation*, 5**:** 3-7.

WINKLER, G.C. 1988. Pulmonary intravascular macrophages in domestic animal species: review of structural and functional properties. *American Journal of Anatomy*, 181**:** 217-234.

WINKLER, G.C. 1989. Review of the significance of pulmonary intravascular macrophages with respect to animal species and age. *Experimental Cell Biology*, 57**:** 281-286.

WINKLER, G.C. & CHEVILLE, N.F. 1985. Monocytic origin and postnatal mitosis of intravascular macrophages in the porcine lung. *Journal of Leukocyte Biology*, 38**:** 471-480.

WITTMANN, E.J. & BAYLIS, M. 2000. Climate change: effects on *Culicoides*-transmitted viruses and implications for the UK. *Veterinary Journal*, 160**:** 107-117.

WOHLSEIN, P., POHLENZ, J.F., DAVIDSON, F.L., SALT, J.S. & HAMBLIN, C. 1997. Immunohistochemical demonstration of African horse sickness viral antigen in formalin-fixed equine tissues. *Veterinary Pathology*, 34**:** 568-574.

WOHLSEIN, P., POHLENZ, J.F., SALT, J.S. & HAMBLIN, C. 1998. Immunohistochemical demonstration of African horse sickness viral antigen in tissues of experimentally infected equines. *Archives of Virology*, 14, Supplement 1: 57-65.

WU, E. & NEMEROW, G.R. 2004. Virus yoga: the role of flexibility in virus host cell recognition. *Trends in Microbiology*, 12**:** 162-169.

ZIENTARA, S., SAILLEAU, C., MOULAY, S., WADE-EVANS, A. & CRUCIERE, C. 1995. Application of the polymerase chain reaction to the detection of African horse sickness viruses. *Journal of Virological Methods*, 53**:** 47-54.

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
1	1	1	1	-	Equine	-	N	-	-	1	-	3208-04	Healthy, abattoir case	New Zealand (NZ)	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
2	1	1	1	-	Equine	-	N	-	-	2	-	3209-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
3	1	1	1	-	Equine	-	N	-	-	3	-	3210-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
4	1	1	1	-	Equine	-	N	-	-	4	-	3211-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
5	1	1	1	-	Equine	-	N	-	-	5	-	3212-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
6	1	1	1	-	Equine	-	N	-	-	6	-	3213-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
7	1	1	1	-	Equine	-	N	-	-	7	-	3214-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
8	1	1	1	-	Equine	-	N	-	-	8	-	3215-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
9	1	1	1	-	Equine	-	N	-	-	9	-	3216-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
10	1	1	1	-	Equine	-	N	-	-	10	-	3217-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
11	1	1	1	-	Equine	-	N	-	-	11	-	3218-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
12	1	1	1	-	Equine	-	N	-	-	12	-	3219-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
13	1	1	1	-	Equine	-	N	-	-	13	-	3220-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
14	1	1	1	-	Equine	-	N	-	-	14	-	3221-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
15	1	1	1	-	Equine	-	N	-	-	15	-	3222-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
16	1	1	1	-	Equine	-	N	-	-	16	-	3223-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
17	1	1	1	-	Equine	-	N	-	-	17	-	3224-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
18	1	1	1	-	Equine	-	N	-	-	18	-	3225-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
19	1	1	1	-	Equine	-	N	-	-	19	-	3226-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
20	1	1	1	-	Equine	-	N	-	-	20	-	3227-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
21	1	1	1	-	Equine	-	N	-	-	21	-	3228-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
22	1	1	1	-	Equine	-	N	-	-	22	-	3229-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
23	1	1	1	-	Equine	-	N	-	-	23	-	3230-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
24	1	1	1	-	Equine	-	N	-	-	24	-	3231-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
25	1	1	1	-	Equine	-	N	-	-	25	-	3232-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
26	1	1	1	-	Equine	-	N	-	-	26	-	3233-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
27	1	1	1	-	Equine	-	N	-	-	27	-	3234-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
28	1	1	1	-	Equine	-	N	-	-	28	-	3235-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
29	1	1	1	-	Equine	-	N	-	-	29	-	3236-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
30	1	1	1	-	Equine	-	N	-	-	30	-	3237-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
31	1	1	1	-	Equine	-	N	-	-	31	-	3238-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
32	1/	1	1	-	Equine	-	N	-	-	32	-	3239-04	Healthy, abattoir case	NZ	-	M.G. Collett	Jul-04	1-2 days	2 yrs	Lung, spleen, heart	Mild
33	-	1	-	-	Equine	-	N	-	-	P97012	-	3240-04	Not reported	Scotland	-	E. McInnes	Jan-97	1-2 days	9 yrs	Lung	Mild
34	1	1	1	-	Equine	-	N	-	-	P97018	-	3241-04	Not reported	Scotland	-	E. McInnes	Jan-97	1-2 days	9 yrs	Lung, spleen, heart	Mild
35	1	1	-	-	Equine	-	N	-	-	9492-03-1	-	3242-04	Not reported	United States of America (USA)	-	J.A. Ramos-Vara	2003	1-2 days	3 yrs	Lung, spleen	Mild
36	1	1	-	-	Equine	-	N	-	-	9938-03-7	-	3243-04	Pneumonia	USA	-	J.A. Ramos-Vara	2003	1-2 days	3 yrs	Lung, spleen	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
37	1	1	1	-	Equine	-	N	-	-	N04-0380	-	3244-04	Trauma	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
38	1	1	1	-	Equine	-	N	-	-	N04-0382	-	3245-04	Grain overload	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
39	1	1	1	-	Equine	-	N	-	-	N04-0385	-	3246-04	Chronic lameness	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
40	1	1	1	-	Equine	-	N	-	-	N04-0390	-	3247-04	Trigeminal nerve injury	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Heart mild; rest moderate
41	1	1	1	-	Equine	-	N	-	-	N04-0396	-	3248-04	Rectal tear	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
42	1	1	1	-	Equine	-	N	-	-	N04-0398	-	3249-04	Premature, septicaemia	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
43	1	1	1	-	Equine	-	N	-	-	N04-0399	-	3250-04	Encephalitis	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
44	1	1	1	-	Equine	-	N	-	-	N04-0403	-	3251-04	Colic	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
45	1	1	1	-	Equine	-	N	-	-	N04-0405	-	3252-04	Pneumonia	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Lung mild; rest moderate

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
46	1	1	1	-	Equine	-	N	-	-	N04-0419	-	3253-04	Aggressive - normal tissues	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Heart moderate; rest mild
47	1	1	-	-	Equine	-	N	-	-	N04-0424	-	3254-04	Colitis	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
48	1	1	1	-	Equine	-	N	-	-	693017	-	3255-04	Torsion	USA	-	J.F. Edwards	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
49	-	1	-	-	Equine	-	N	-	-	H04-26	04-042	3259-04 C	Phlegmon	Australia	-	A. O'Hara	2004	1-2 days	2 yrs	Lung	Mild
50	1	1	1	-	Equine	-	N	-	-	H04-157	04-253	3260-04 A,B,C	Annual rye grass toxicity	Australia	-	A. O'Hara	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
52	-	1	1	-	Equine	-	N	-	-	H04-264	04-412	3262-04 A	Cranial contusions	Australia	-	A. O'Hara	2004	1-2 days	2 yrs	Lung, Heart	Mild
53	1	-	-	-	Equine	-	N	-	-	H04-324	04-484	3263-04 A	Splenic lymphoma	Australia	-	A. O'Hara	2004	1-2 days	2 yrs	Spleen	Mild
54	1	1	1	-	Equine	-	N	-	-	H03-85	03-82	3264-04 A,C,E	*Rhodococcus* bronchopneumonia	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung, heart, spleen	Mild
55	-	1	-	-	Equine	-	N	-	-	H03-136	03-132	3265-04 D	Melanomas & arytenoid chondropathy	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
56	1	1	1	-	Equine	-	N	-	-	H03-137	03-135	3266-04 A, B	Snake evenomation	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung, heart, spleen	Moderate
57	-	1	1	-	Equine	-	N	-	-	H03-280	03-273	3267-04 B, C	Septic peritonitis	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung, Heart	Mild
58	-	1	-	-	Equine	-	N	-	-	H03-298	03-289	3268-04 H	Eosinophilic gastroenteritis	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung	Mild
59	-	1	1	-	Equine	-	N	-	-	H03-384	03-365	3269-04 A, Q	Anaethetic death	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Heart, Lung	Mild
60	1	1	1	-	Equine	-	N	-	-	H03-545	03-553	3271-04A,C	Melanomas	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung, heart, spleen	Mild
61	-	1	-	-	Equine	-	N	-	-	H03-683	03-692	3272-04 E	Ischaemic enteritis	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung	Mild
62	-	1	1	-	Equine	-	N	-	-	H03-803	03-831	3273-04 A, B	Asphyxiation	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung, Heart	Mild
63	1	-	-	-	Equine	-	N	-	-	H03-903	03-963	3274-04 E	Leukemia	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Spleen	Mild
64	-	1	1	-	Equine	-	N	-	-	H03-923	03-984	3275-04 B, D	Acute pneumonia - acute respiratory distress syndrome (ARDS)	Australia	-	A. O'Hara	2003	1-2 days	3 yrs	Lung, Heart	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
65	1	1	-	-	Equine	-	N	-	-	H02-200	02-152	3276-04 A, B	Cyathostomiasis	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Lung, Spleen	Mild
66	-	1	-	-	Equine	-	N	-	-	H02-228	02-177	3277-04 B	Cyathostomiasis	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Lung, Spleen	Mild
67	1	1	-	-	Equine	-	N	-	-	H02-229	02-180	3278-04 A, H, O	Pleuropneumonia	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Lung, Spleen	Mild
68	-	1	-	-	Equine	-	N	-	-	H02-278	02-236	3279-04 A	Aborted foetus	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Lung	Mild
69	-	-	1	-	Equine	-	N	-	-	H02-325	02-294	3280-04 A	Dilated cardiomyopathy	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Heart	Mild
70	1	1	1	-	Equine	-	N	-	-	H02-829	02-805	3281-04 A, B	Mycotic rhinitis & encephalitis	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Spleen, Lung, Heart	Mild
71	-	1	-	-	Equine	-	N	-	-	H02-973	02-944	3282-04 H	Myocarditis	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Lung	Mild
72	-	1	-	-	Equine	-	N	-	-	H02-1005	02-978	3283-04 A	*Rhodococcus* bronchopneumonia	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Lung	Mild
73	-	-	1	-	Equine	-	N	-	-	H02-1070	02-1053	3284-04 K	Acute cranial trauma	Australia	-	A. O'Hara	2002	1-2 days	4 yrs	Heart	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
74	1	1	-	-	Equine	-	N	-	-	H01-85	01-89	3285-04 A, C	Systemic cryptococcosis	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Lung, Spleen	Mild
76	1	1	-	-	Equine	-	N	-	-	H01-196	01-195	3287-04 C, D	Enteritis	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Lung, Spleen	Mild
77	-	1	1	-	Equine	-	N	-	-	H01-322	01-316	3288-04 B,C	Endotoxaemia	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Lung, heart	Mild
78	-	1	-	-	Equine	-	N	-	-	H01-327	01-317	3289-04 B	Colonic volvulus	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Lung	Mild
79	1	1	1	-	Equine	-	N	-	-	H01-405	01-412	3290-04 A, E,	Endotoxaemia	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Spleen, lung, heart	Mild
80	-	1	-	-	Equine	-	N	-	-	H01-531	01-525	3291-04 A	Cleft palate & aspiration pneumonia	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Lung	Mild
81	-	1	-	-	Equine	-	N	-	-	H01-574	01-567	3292-04 A	Mesothelioma	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Lung	Mild
82	1	1	1	-	Equine	-	N	-	-	H01-797	01-782	3292-04 L, P, J	Heart failure	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Heart, Lung, Spleen	Mild
83	1	1	1	-	Equine	-	N	-	-	H01-862	01-845	3294-04 B, D	Foetal abortion	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Spleen, heart	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
84	1	1	1	-	Equine	-	N	-	-	H01-929	01-895	3295-04 A, B	Immune-mediated haemolytic anaemia and thrombocytopaenia	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Spleen, lung, heart	Mild
85	-	1	1	-	Equine	-	N	-	-	H01-1019	01-1034	3296-04 A, B	Acute death post-racing-dysrrhythmia?	Australia	-	A. O'Hara	2001	1-2 days	5 yrs	Lung, Heart	Mild
86	-	1	-	-	Equine	-	N	-	-	H00-562	00-575	3297-04 A	Drenching pneumonia	Australia	-	A. O'Hara	2000	1-2 days	6 yrs	Lung	Mild
87	-	1	-	-	Equine	-	N	-	-	H00-630	00-654	3298-04 A	Pleuritis	Australia	-	A. O'Hara	2000	1-2 days	6 yrs	Lung	Mild
88	-	1	-	-	Equine	-	N	-	-	H00-662	00-687	3299-04 A, B, C	Interstitial pneumonia	Australia	-	A. O'Hara	2000	1-2 days	6 yrs	Lung	Mild
89	1	1	1	-	Equine	-	N	-	-	H00-764	00-799	3300-04 A, C	Intestinal strangulation & peritonitis	Australia	-	A. O'Hara	2000	1-2 days	6 yrs	Spleen, heart, lung	Mild
90	1	1	1	-	Equine	-	N	-	-	H00-790	00-821	3301-04 B, C	Gastric ulceration	Australia	-	A. O'Hara	2000	1-2 days	6 yrs	Spleen, heart, lung	Mild
91	1	-	-	-	Equine	-	N	-	-	H00-874	00-903	3302-04 E	*Rhodococcus* pneumonia and encephalitis	Australia	-	A. O'Hara	2000	1-2 days	6 yrs	Spleen	Mild
92	1	1	-	-	Equine	-	N	-	-	H99-2774	99-923	3303-04 A, F	Haemangiosarcoma of skeletal muscle	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Lung, Spleen	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
93	1	1	1	-	Equine	-	N	-	-	H99-2834	99-980	3304-04 G, H, K	Multicentric lymphoma	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Spleen, heart, lung	Mild
94	1	1	1	-	Equine	-	N	-	-	H99-3000	99-1154	3305-04 B, C, F	Foetal death	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Spleen, heart, lung	Mild
95	1	-	-	-	Equine	-	N	-	-	H99-77	-	3306-04 B, C	Not reported	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Spleen	Mild & acid hematin
96	1	-	-	-	Equine	-	N	-	-	H99-1932	99-525	3307-04 B	Acute typhlocolitis	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Spleen	Mild
98	1	-	1	-	Equine	-	N	-	-	H99-2276	99-624	3308-04 A H	Septic arthritis & granulosa cell tumour	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Spleen, heart	Mild
99	1	1	-	-	Equine	-	N	-	-	H99-2662	99-813	3309-04 A	Hepatopathy	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Lung, spleen	Mild
100	1	1	1	-	Equine	-	N	-	-	H99-2692	99-844	3310-04 A, B	*Actinobacillus* pneumonia	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Lung, spleen, heart	Mild
101	1	1	1	-	Equine	-	N	-	-	H99-2695	99-846	3311-04 B, C	Septicaemia	Australia	-	A. O'Hara	1999	1-2 days	7 yrs	Lung, spleen, heart	Mild
102	-	1	1	-	Equine	-	N	-	-	H00-846	00-877	277-05 A, C	Alimentary sarcoma	Australia	-	A. O'Hara	2000	1-2 days	6 yrs	Lung, heart	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
104	1	1	1	-	Equine	-	N	-	-	603-04 1, 2, 3	-	1061-05	Euthanasia due to traumatic fracture (#)	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
105	1	1	1	-	Equine	-	N	-	-	614-04 1, 2, 3	-	1062-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
106	1	1	1	-	Equine	-	N	-	-	615-04 1, 2, 3	-	1063-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
107	1	1	1	-	Equine	-	N	-	-	618-04 1, 2, 3	-	1064-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
108	1	1	1	-	Equine	-	N	-	-	646-04 1, 2, 3	-	1065-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
109	1	1	1	-	Equine	-	N	-	-	659-04 1, 2, 3	-	1066-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
110	1	1	1	-	Equine	-	N	-	-	661-04 1, 2, 3	-	1067-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
111	1	1	1	-	Equine	-	N	-	-	662-04 1, 2, 3	-	1068-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
112	1	1	1	-	Equine	-	N	-	-	664-04 1, 2, 3	-	1069-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
113	1	1	1	-	Equine	-	N	-	-	674-04 1, 2, 3	-	1070-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
114	1	1	1	-	Equine	-	N	-	-	682-04 1, 2, 3	-	1071-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
115	1	1	1	-	Equine	-	N	-	-	686-04 1, 2, 3	-	1072-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
116	1	1	1	-	Equine	-	N	-	-	700-04 1, 2, 3	-	1073-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
117	1	1	1	-	Equine	-	N	-	-	735-04 1, 2, 3	-	1074-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
118	1	1	1	-	Equine	-	N	-	-	807-04 1, 2, 3	-	1075-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
119	1	1	1	-	Equine	-	N	-	-	808-04 1, 2, 3	-	1076-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
120	1	1	1	-	Equine	-	N	-	-	898-04 1, 2, 3	-	1077-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
121	1	1	1	-	Equine	-	N	-	-	942-04 1, 2, 3	-	1078-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild

APPENDIX 1: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-NEGATIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
122	1/-	1	1	-	Equine	-	N	-	-	961-04 1, 2, 3	-	1079-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild
123	1	1	1	-	Equine	-	N	-	-	962-04 1,2,3	-	1080-05	Euthanasia due to traumatic #	Australia	-	J.A. Charles	2004	1-2 days	2 yrs	Lung, heart, spleen	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
124	3	3	3	14 yrs	Equine, Thoroughbred (TB)	F	Y	2	-	-	317-02	798-02	African horsesickness (AHS)	South Africa (SA)	Vet Faculty, Onderstepoort, Gauteng	Equine Research Centre, Vet Faculty, Onderstepoort	Mar-02	1-2 days	4 yrs	Lung, heart, spleen	Mild
125	-	3	3	10-11 yr	Equine, Boerperd X	M	Y	3	-	Histopath no: Stell: 97.672	PM no: Stell: 9712262	1899-02	AHS	SA	Worcester, via the Western Cape Provincial Veterinary Laboratory (WCPVL)	J. Bredenham	Dec-97	1-2 days	9 yrs	Heart, lung	Mild
126	3	3	3	4-5 months	Equine	-	Y	1	-	Vet Faculty virology no: E141/2000	-	1256.00	AHS	SA	Via P. Rogers, Hoedspruit, Northern Province	Smith	May-00	1 day	6 yrs	Lung, heart, spleen	Mild
127	3	3	-	5 yrs	Equine, paint horse	M	Y	4	-	-	181-02	510-02	AHS	SA	Waterval, Gauteng	J.P. Smit	Feb-02	1-2 days	4 yrs	Spleen, lung	Mild
128	3	3	-	3 months	Equine, Nooitgedacht pony	M	Y	4	-	-	392.00	1282.00	AHS	SA	Onderstepoort, Gauteng	Vet Faculty, Onderstepoort	Apr-00	1-2 days	6 yrs	Spleen, lung	Mild
129	2	3	3	12 yrs	Equine, Boerperd	M	Y	2	-	-	384.00	1270.00	AHS	SA	Bon Accord, Pretoria, Gauteng	L. de Villiers	Apr-00	1-2 days	6 yrs	Lung, heart, spleen	Mild
130	-	2	-	-	Equine, Boerperd X Friesian	F	Y	3	-	-	96-02	159-02 A	AHS	SA	Onderstepoort, Gauteng	J. de Villiers	Feb-02	1-2 days	4 yrs	Lung	Mild
131	-	-	3	8 yrs	Equine, TB	F	Y	2	-	-	348.00	1194.00	AHS	SA	Vet Faculty, Onderstepoort, Gauteng	Equine Research Centre, Vet Faculty, Onderstepoort	Mar-00	1-2 days	6 yrs	Heart	Mild to moderate
132	3	3	-	5 yrs	Equine	F	Y	2	-	-	81-02	137-02	AHS	SA	Onderstepoort, Gauteng	J. de Villiers	Jan-02	1-2 days	4 yrs	Lung, spleen	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
133	3	3	-	2,5 yrs	Equine, Welsh pony	F	Y	8	-	-	17-02	29-02	AHS	SA	Via P. Page, Equine Clinic, Vet Faculty, Onderstepoort, Gauteng	F. Jones	Jan-02	1-2 days	4 yrs	Lung, spleen	Mild
134	-	3	3	14 yrs	Equine, Nooitgedacht pony	F	Y	7	-	-	124-02	208-02	AHS	SA	Onderstepoort, Gauteng	Vet Faculty, Onderstepoort, Gauteng	Jan-02	1-2 days	4 yrs	Lung, heart	Mild
135	3	2/3	3	8 yrs	Equine, Miniature horse	F	Y	9	-	-	162-02	280-02	AHS	SA	Totiusdal, Gauteng	P.S. Snyman	Feb-02	1-2 days	4 yrs	Heart, lung, spleen	Mild
136	3	3	3	1 yr	Equine, Friesian	F	Y	6	-	-	6-02	11-02	AHS	SA	Onderstepoort, Gauteng	South African National Defence Force (SANDF)	Jan-02	1-2 days	4 yrs	Lung, heart, spleen	Mild
137	3	3	3	-	Equine, TB	F	Y	8	-	Vet Faculty virology no: E140/02	344-02	858-02	AHS	SA	Via C. Janisch, Pyramid, Gauteng	M. vd Merwe	Apr-02	1-2 days	4 yrs	Heart, lung, spleen	Mild
138	-	3	-	6 months	Equine, X-breed (skewbald)	M	Y	6	-	-	574-01	1349-01	AHS	SA	Pretoria, Gauteng	H.V. Gurtel	Jul-01	1-2 days	5 yrs	Lung	Mild
139	3	3	3	16 months	Equine, Arab	M	Y	7	-	-	307-04	832-04	AHS	SA	Pretoria, Gauteng	R. de Meillon	Mar-04	1-2 days	2 yrs	Lung, heart, spleen	Mild
140	2	3	-	7 yrs	Equine, X-breed	F	Y	7	-	-	426-04	1196-04	AHS	SA	Via P. Page, Equine Clinic, Vet Faculty, Onderstepoort, Gauteng	D.J.T. van Niekerk	May-04	1-2 days	2 yrs	Spleen, lung	Mild to moderate
141	3	3	-	-	Equine, Nooitgedacht pony	M	Y	9	-	Vet Faculty virology no: E245/04	447-04	1270-04	AHS	SA	Onderstepoort, Gauteng	Z.Z.Z. Majokweni, Onderstepoort Biological Products (OBP)	May-04	1-2 days	2 yrs	Spleen, lung	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
142	3	3	3	4 yrs	Equine, Nooitgedacht pony	F	Y	6	-	OVI registration no: 2001-D-2938	397-01	967-01	AHS	SA	Onderstepoort, Gauteng	Vet Faculty, Onderstepoort, Gauteng	Apr-01	1-2 days	5 yrs	Lung, heart, spleen	Mild
143	3	3	-	6 months	Equine	-	Y	9	-	OVI virology no: HS22/03	-	949-03	AHS	SA	Not recorded	Via T. Gerdes, virology OVI, Onderstepoort, Gauteng	Apr-03	1 week	3 yrs	Spleen, lung	Lung mild; spln moderate
144	3	3	-	-	Equine	-	Y	2	-	OVI virology no: HS25/03	-	1145-03	AHS	SA	Not recorded	Via T. Gerdes, virology OVI, Onderstepoort, Gauteng	May-03	1 week	3 yrs	Spleen, lung	Lung mild; spln moderate
145	3	3	-	-	Equine	-	Y	-	-	OVI virology no: HS24/03	-	1070-03	AHS	SA	Not recorded	Via T. Gerdes, virology OVI, Onderstepoort, Gauteng	May-03	1 week	3 yrs	Spleen, lung	Mild
146	3	3	-	-	Equine, Arab	-	Y	2	-	OVI virology no: HS21/03	-	948-03	AHS	SA	Not recorded	Via T. Gerdes, virology OVI, Onderstepoort, Gauteng	Apr-03	1 week	3 yrs	Spleen, lung	Mild
147	3	-	-	-	Equine	-	Y	9	-	OVI virology no: HS2/03	-	431-03	AHS	SA	Not recorded	Via T. Gerdes, virology OVI, Onderstepoort, Gauteng	Feb-03	1 week	3 yrs	Spleen	Mild to moderate
157	3	3	3	5 yrs	Equine, Boerperd	M	Y	2	-	OVI no: OP77089	64-97	123-97	AHS	SA	Gauteng	L. Bekker	Jan-97	1-2 days	9 yrs	Heart, lung, spleen	Lung mild; spln moderate
158	3	3	3	15 months	Equine, Welsh pony X	M	Y	1	-	OVI no: OP77755	107-97	227-97	AHS	SA	Johannesburg, Gauteng	S. Dittmann	Feb-97	1-2 days	9 yrs	Heart, lung, spleen	Mild
159	3	3	3	7,5 months	Equine, Arab	F	Y	2	-	OVI no: OP78990	213-97	565-97	AHS	SA	Grootvlei, Gauteng	B. Burger	Mar-97	1-2 days	9 yrs	Spleen, heart, lung	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
160	3	3	3	18 months	Equine, Arab	F	Y	2	-	OVI no: OP79306	243-97	618-97	AHS	SA	Pretoria, Gauteng	I.P.L. Dannheimer	Mar-97	1-2 days	9 yrs	Lung, heart, spleen	Mild
161	3	3	-	-	Equine	-	Y	2	-	Vet Faculty virology no: E66/03	-	1089-03 (C)	AHS	SA	Not recorded	Via P.G. Howell, Vet Faculty, Onderstepoort	Apr-03	1-2 days	3 yrs	Spleen, lung	Mild
162	3	3	-	-	Equine	-	Y	2	-	Vet Faculty virology no: E32/03 (3)	-	1089-03 (D)	AHS	SA	Not recorded	Via P.G. Howell, Vet Faculty, Onderstepoort	Early 2003	1-2 days	3 yrs	Spleen, lung	Mild
163	-	3	3	-	Equine	-	Y	4	-	Vet Faculty virology no: E50/03	-	1089-03 (J)	AHS	SA	Not recorded	Via P.G. Howell, Vet Faculty, Onderstepoort	Mar-03	1-2 days	3 yrs	Heart, lung	Mild
164	-	-	3	4,5 yrs	Equine, Nooitgedacht pony	F	Y	7	-	-	436-04	1230-04 AF	AHS	SA	Pretoria, Gauteng	Vet Faculty, Onderstepoort	May-04	2 days	2 yrs	Heart, liver	Mild
165	-	-	3	< 6 months	Equine, Boerperd	M	Y	7	-	-	458-04	1304-04 AG	AHS	SA	Bon Accord, Pretoria, Gauteng	R. Labuschagne	May-04	2 days	2 yrs	Heart, liver	Mild
166	2	3	3	15 yrs	Equine, TB	F	Y	7	-	-	545-04	1560-04	AHS	SA	Gauteng	N. Storm	Jun-04	1-2 days	2 yrs	Lung, heart, spleen	Mild
167	2	2	-	7 months	Equine, Arab	M	Y	-	-	Vet Faculty virology no: E73/01	406-01	976-01	AHS	SA	Pretoria, Gauteng	L. Hannekom	Apr-01	1-2 days	5 yrs	Spleen, lung	Mild to moderate
168	2	3	3	3 yrs	Equine, Nooitgedacht pony	F	Y	9	-	-	102-03	243-03	AHS	SA	Onderstepoort, Gauteng	Vet Faculty, Onderstepoort, Gauteng	Feb-03	2 days	3 yrs	Lung, heart, spleen	Mild to moderate

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
169	3/2	3	3	2 yrs	Equine, TB	M	Y	2	-	-	-	1269-03	AHS	SA	Gillitts, Kwazulu Natal (KZN)	via J.M.P. Fleming	May-03	2 days	3 yrs	Lung, heart, spleen	Spln mod; rest mild
170	3	2/3	3/-	3 yrs	Equine, Arab	M	Y	7	-	-	74-99	177-99	AHS	SA	Vet Faculty, Onderstepoort, Gauteng	E. van Dyk	Jan-99	1-2 days	7 yrs	Lung, heart, spleen	Mild
171	3	3	3	6 yrs	Equine, Boerperd	M	Y	7	-	-	75-99	178-99	AHS	SA	Onderstepoort, Gauteng	J.P. de Villiers	Jan-99	1-2 days	7 yrs	Lung, heart, spleen	Mild
172	2	3	3	7 yrs	Equine, Arab	F	Y	8	-	Vet Faculty virology no: E29/98	165-98	459-98	AHS	SA	Rosslyn near Pretoria, Gauteng	J.A. de Gouvera	Feb-98	1-2 days	8 yrs	Lung, heart, spleen	Mild
173	2	3	-	3 yrs	Equine, Arab	F	Y	7	-	Vet Faculty virology no: E19/02	99-02	167-02	AHS	SA	Gauteng	B. Phillips	Jan-02	1-2 days	4 yrs	Spleen, lung	Mild to moderate
174	3	3	3	-	Equine, Boerperd	F	Y	7	-	-	136-99	340-99	AHS	SA	Pretoria, Gauteng	N. Meyer	Feb-99	1-2 days	7 yrs	Lung, heart, spleen	Spln mod; rest mild
175	3	3	-	-	Equine	M	Y	6	-	-	575-01	1367-01	AHS	SA	Boekenhoutkloof, Gauteng	R. Wagner	Jul-01	1-2 days	5 yrs	Spleen, lung	Lung mild; spln moderate
176	3	3	3	5 months	Equine, Friesian	M	Y	2	-	-	471-02	1140-02	AHS	SA	Pretoria, Gauteng	J. Birrell, SANDF	May-02	1-2 days	4 yrs	Lung, heart, spleen	Mild
177	3	3	-	2 yrs	Equine	M	Y	7	-	-	360-98	1027-98	AHS	SA	Pretoria, Gauteng	W. van Rensburg	Apr-98	1-2 days	8 yrs	Spleen, lung	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
181	3	3	3	2 yrs	Equine, TB	F	Y	5	-	Vet Faculty virology no: E7/05	-	170-05	AHS	SA	Mooi River, KZN	P. du Toit	Jan-05	2 days	1 yr	Heart, lung, spleen	Mild
189	3	3	-	4,5 yrs	Equine	M	Y	7	-	OVI virology no: HS7/05	-	547-05	AHS	SA	Dundee, KZN	Pretorius	Feb-05	2 days	1 yr	Lung, spleen	Mild
193B 1	3	-	-	14 yrs	Equine, TB	F	Y	7	-	OVI virology no: HS17/05	-	652-05	AHS	SA	KZN	Via Vetdiagnostix Pathology Laboratory	Feb-05	2 days	1 yr	Spleen	Mild
194	3	3	3	18 months	Equine, Miniature horse	F	Y	5	-	OVI virology no: HS18/05 05-D1929	-	653-05	AHS	SA	Cascades, KZN	N.K. Armour, via Allerton Provincial Veterinary Laboratory	Mar-05	1 week	1 yr	Spleen, lung, heart	Mild
195B 2	3	-	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E31/05	-	668-05	AHS	SA	Not recorded	Not recorded	Feb-05	4 days	1 yr	Spleen	Mild
196B 3	3	3	-	4 yrs	Equine	M	Y	7	-	OVI virology no: HS19/05	-	714-05	AHS	SA	Newcastle, KZN	Zaal	Feb-05	8 days	1 yr	Spleen, lung	Mild
198B 1	3	3	3	1 yr	Equine, Friesian	M	Y	1	-	Vet Faculty virology no: E35/05	226-05	731-05	AHS	SA	Roodeplaat near Pretoria, Gauteng	J. Birrell, SANDF	Feb-05	2 days	1 yr	Spleen, lung, heart	Mild
200B 2	-	3	3	-	Equine	-	Y	2	-	OVI virology no: HS25/05	-	855-05	AHS	SA	Graaff-Reinett, Eastern Cape	Owner not recorded; sample via T. Gerdes, OVI	Mar-05	4 days	1 yr	Lung, heart	Mild
202B 3	3	3	-	-	Equine	-	Y	2	-	OVI virology no: HS26/05	-	919-05	AHS	SA	Clanwilliam, Western Cape	Nel, via T. Gerdes, OVI	Mar-05	8 days	1 yr	Spleen, lung	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
203B 1	3	3	-	-	Equine	-	Y	5	-	OVI virology no: HS29/05	-	920-05	AHS	SA	Western Cape	Muller, via T. Gerdes, OVI	Mar-05	2 days	1 yr	Spleen, lung	Mild
204B 1	3	3	3	10 yrs	Equine, TB	M	Y	8	-	-	323-05	999-05	AHS	SA	Pretoria, Gauteng	Viljoen, SANDF	Apr-05	2 days	1 yr	Lung, heart, spleen	Mild
205B 1	3	3	3	4 months	Equine, TB	M	Y	6	-	-	325-05	1008-05	AHS	SA	Pretoria, Gauteng	M. van den Berg	Apr-05	2 days	1 yr	Lung, heart, spleen	Mild
207B 1	3	3	3	Adult	Equine	M	Y	6	-	-	343-05	1096-05	AHS	SA	Not recorded	Owner not recorded, horse was optional post mortem	Apr-05	2 days	1 yr	Lung, heart, spleen	Mild
209	3	3	3	Foal	Equine	-	Y	7	-	OVI virology no: HS42/05 Histopathology no: VD05/1160	-	1127-05	AHS	SA	Via Allerton Provincial Veterinary Laboratory and Vetdiagnostix, KZN	March	Apr-05	2 days	1 yr	Lung, heart, spleen	Mild
211	3	3	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E72/05	-	1139-05	AHS	SA	Mooi River, KZN	Clarken	Apr-05	1-2 days	1 yr	Lung, spleen	Mild
212	3	-	3	7 yrs	Equine	M	Y	7	-	Vet Faculty virology no: E93/05	-	1231-05	AHS	SA	Lidgeton, KZN	Via Howick Veterinary Clinic	Apr-05	1-2 days	1 yr	Spleen (spln), heart (hrt)	Mild
213	2	3	3	12 yrs	Equine	F	Y	7	-	Vet Faculty virology no: E94/05	-	1232-05	AHS	SA	Mooi River, KZN	Via T. Hughes, Equi-Touch Veterinary Services	Apr-05	1-2 days	1 yr	Spleen, heart, lung	Mild
214	2/3	3	3	-	Equine	-	Y	7	-	OVI virology no: HS39/05 histopath no: VD05/3824	-	1296-05	AHS	SA	Gillitts, KZN	Via Summerveld Equine Hospital and Vetdiagnostix	May-05	1-2 days	1 yr	Lung, spln, hrt, liver (liv), kidney (kid)	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
218	2	3	-	-	Equine	-	Y	5	-	OVI virology no: HS33/05	-	1529-05	AHS	SA	Ellisras, Northern Province	Matthews	May-05	1-2 days	1 yr	Lung, spleen	Mild
219	-	3	-	-	Equine	-	Y	3	-	OVI virology no: HS35/05	-	1530-05	AHS	SA	East London, Eastern Cape	Owner not recorded; sample via T. Gerdes, OVI	May-05	1-2 days	1 yr	Lung	Mild
220	3	-	-	-	Equine	-	Y	7	-	OVI virology no: HS42/05	-	1531-05	AHS	SA	KZN	March	May-05	1-2 days	1 yr	Spleen, lymph node (lnn)	Spln moderate
222	3	3	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E77/05	-	1533-05	AHS	SA	Pinetown, KZN	Baksa	May-05	1-2 days	1 yr	Spleen, lung	Mild
224	3	-	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E87/05	-	1535-05	AHS	SA	Mooi River, KZN	Norman	May-05	2 days	1 yr	Spleen	Mild
225	2	3	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E88/05	-	1536-05	AHS	SA	Mooi River, KZN	Winter	May-05	2 days	1 yr	Spleen, lung	Mild
227	3	-	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E90/05	-	1538-05	AHS	SA	Mooi River, KZN	Winter	May-05	2 days	1 yr	Spleen	Mild
229	3	3	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E101/05	-	1540-05	AHS	SA	Mooi River, KZN	Winter	May-05	2 days	1 yr	Spleen, lung, liver	Mild to moderate & acid hematin
230	3	3	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E102/05	-	1541-05	AHS	SA	Mooi River, KZN	Winter	May-05	2 days	1 yr	Lung, spleen	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
233	2	-	-	Foal	Equine	-	Y	7	-	Vet Faculty virology no: E122/05	-	1544-05	AHS	SA	Mooi River, KZN	Invermooi Stud	May-05	2 days	1 yr	Spleen	Mild with acid hematin
234	3	-	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E125/05	-	1545-05	AHS	SA	Mooi River, KZN	Scott	May-05	2 days	1 yr	Spleen	Mild
236	3	3	3	15 yrs	Equine, X-breed	M	Y	7	-	Histopathology no: Stell: 99.106	PM no: Stell: 9904195	2784-05	AHS	SA	Stellenbosch, via Western Cape Provincial Veterinary Laboratory (WCPVL)	H. du Toit	Apr-99	2 days	7 yrs	Lung, spleen, heart	Mild
237	3	3	3	-	Equine	F	Y	4	-	Histopath no: Stell: 99.081	PM no: Stell: 9903299	2785-05	AHS	SA	Hout Bay, via WCPVL	C. del Castilloz	Mar-99	2 days	7 yrs	Heart, lung, spleen	Moderate
238	1	2/3	3	4 yrs	Equine, American Saddler	F	Y	7	-	Histopath no: Stell: 99.094	PM no: Stell: 9903318	2786-05	AHS	SA	Stellenbosch, via WCPVL	J.C. Botha	Mar-99	2 days	7 yrs	Heart, lung, spleen	Mild to moderate
239	3	3	3	-	Equine, Arab	-	Y	7	-	Histopath no: Stell: 99.077	PM no: Stell: 9903290	2787-05	AHS	SA	Stellenbosch, via WCPVL	Rozendal Farm	Mar-99	2 days	7 yrs	Heart, lung, spleen	Mild
241	3	3	3	8 months	Equine, TB	M	Y	7	-	Histopath no: Stell: 99.111	PM no: Stell: 9904246	2789-05	AHS	SA	Somerset West, via WCPVL	Avontuur Stud	Apr-99	1-2 days	7 yrs	Heart, lung, spleen	Mild to moderate
242	3	3	3	Adult	Equine	F	Y	1	-	Histopath no: Stell: 04-057A	PM no: Stell: 2004/02-0282	2790-05	AHS	SA	Elsenburg, via WCPVL	Elsenburg Stables	Feb-04	1-2 days	2 yrs	Heart, lung, spleen	Mild to moderate
243	3	3	3	Adult	Equine	F	Y	1	-	Histopath no: Stell: P04-057B	PM no: Stell: 2004/02-0282	2791-05	AHS	SA	Elsenburg, via WCPVL	Elsenburg Stables	Feb-04	1-2 days	2 yrs	Heart, lung, spleen	Mild to moderate

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
244	3	3	3	12 yrs	Equine, Arab	F	Y	7	-	Histopath no: Stell: 99.072A	PM no: Stell: 9903261	910-02A	AHS	SA	Stellenbosch, via WCPVL	Rozendal Farm	Mar-99	1-2 days	7 yrs	Heart, lung, spleen	Mild
245	3	-	-	4 yrs	Equine, Arab	M	Y	7	-	Histopath no: Stell: 99.072B	PM no: Stell: 9903261	910-02B	AHS	SA	Stellenbosch, via WCPVL	Rozendal Farm	Mar-99	1-2 days	7 yrs	Spleen	Mild to moderate
246	3	3	3	-	Equine, Arab X	F	Y	7	-	Histopath no: Stell: 99.072C	PM no: Stell: 9903261	910-02C	AHS	SA	Stellenbosch, via WCPVL	Rozendal Farm	Mar-99	1-2 days	7 yrs	Heart, lung, spleen	Mild
247	3	3	-	-	Equine, Warmblood	M	Y	4	-	Histopath no: Stell: 99.074	PM no: Stell: 9903276	911-02	AHS	SA	Hout Bay, via WCPVL	N. Ruthenberg	Mar-99	1-2 days	7 yrs	Lung, spleen	Mild
248	3	3	3	20 yrs	Equine, X-breed	M	Y	7	-	Histopath no: Stell: 99.075	PM no: Stell: 9903283	1730-02	AHS	SA	Stellenbosch, via WCPVL	Gabler	Mar-99	1-2 days	7 yrs	Heart, lung, spleen	Mild
250	3	3	3	3 yrs	Equine, American Saddler	F	Y	7	-	Histopath no: Stell: 99.098	PM no: Stell: 9904167	3256-05	AHS	SA	Stellenbosch, via WCPVL	J.C. Botha	Apr-99	1-2 days	7 yrs	Heart, lung, spleen	Mild
255	3	3	3	2 yrs	Equine, American Saddler	M	Y	7	-	Histopath no: Stell: 99.093	PM no: Stell: 9903305	3296-05	AHS	SA	Stellenbosch, via WCPVL	J.C. Botha	Mar-99	1-2 days	7 yrs	Heart, lung, spleen	Mild
256	3	3	3	-	Equine	-	Y	7	-	Histopath no: Stell: 99.128	PM no: Stell: 9905131	3297-05	AHS	SA	Stellenbosch, via WCPVL	K. Bezuidenhout	May-99	1-2 days	7 yrs	Heart, lung, spleen	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
257	2	2/3	3	-	Equine	-	Y	7	-	Histopath no: Stell: 99.132	PM no: Stell: 9905144	3298-05	AHS	SA	Stellenbosch, via WCPVL	J.C. Botha	May-99	1-2 days	7 yrs	Heart, lung, spleen	Moderate
258	3	3	3	-	Equine	-	Y	1	-	Histopath no: Stell: 04.067	PM no: Stell: 04020370	3299-05	AHS	SA	Stellenbosch, via WCPVL	P. Botha	Feb-04	1-2 days	2 yrs	Heart, lung, spleen	Mild
260	-	2/3	-	15 months	Equine	M	Y	2	-	-	369-01	896-01	AHS	SA	Rietfontein, Pretoria, Gauteng	L.C. Strydom	Apr-01	2 days	5 yrs	Lung	Mild
262	3	3	3	2,5 yrs	Equine, Nooitgedacht pony	F	Y	7	-	-	262-04	744-04	AHS	SA	Onderstepoort, Gauteng	Z.Z.Z. Majokweni (OBP)	Mar-04	2 days	2 yrs	Lung, spleen, heart	Mild
329	3	3	3	Adult	Equine	-	Y	-	-	AFIP Acc. 2584231	-	2678-06	AHS	Middle East	Not recorded	AFIP	Oct-97	2 days	9 yrs	Lung, heart, spleen	Mild
330	3	3	3	Adult	Equine	-	Y	-	-	AFIP Acc. 2582826	-	2679-06	AHS	Middle East	Not recorded	AFIP	Apr-97	2 days	9 yrs	Lung, heart, spleen	Mild
331	3	3	3	-	Equine	-	Y	5	-	OVI virology no: HS3/06 Histopath no: Idexx 579/06	-	105-06	AHS	SA	Via Allerton Provincial Veterinary Laboratory, KZN	K. Baynes	Jan-06	2 days	< 1yr	Lung, heart, spleen	Mild
332	3	3	3	2 yrs	Equine, Nooitgedacht pony	F	Y	5	-	OVI virology no: HS64/05	1020-05	4000-05	AHS	SA	Onderstepoort, Gauteng	Z.Z.Z. Majokweni (OBP)	Dec-05	2 days	< 1yr	Lung, heart, spleen	Mild
333	3	3	3	2 yrs	Equine, Nooitgedacht pony	F	Y	5	-	OVI virology no: HS65/05	1029-05	4039-05	AHS	SA	Onderstepoort, Gauteng	Z.Z.Z. Majokweni (OBP)	Dec-05	2 days	< 1yr	Lung, heart, spleen	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
335	-	3	3	-	Equine	-	Y	2	-	OVI virology no: HS19/06 Histopath no: Idexx 1799/06	-	641-06	AHS	SA	Not recorded	Van der Merwe	Feb-06	2 days	< 1yr	Heart, lung	Mild with acid hematin
343	3	3	-	-	Equine	-	Y	7	-	OVI virology no: HS89/06 Histopath no: VD1049/06	-	1272-06	AHS	SA	Not recorded	Naude	Mar-06	2 days	< 1yr	Lung, spleen, liver	Mild
344	-	3/-	-/3	5 yrs	Equine	F	Y	9	-	Histopath no: Idexx 3551/06	-	1301-06	AHS	SA	Mpumalanga	Via Steyn	Mar-06	1-2 days	< 1yr	Heart only	Mild
345	1	3	3	3 yrs	Equine, Nooitgedacht pony	F	Y	2	-	-	340-06	1354-06	AHS	SA	Onderstepoort, Gauteng	Vet Faculty, Onderstepoort	Apr-06	1-2 days	< 1yr	Heart, lung, spleen	Mild
346	3	3	3	-	Equine, Boerperd	-	Y	2	-	Histopath no: Idexx 3829/06	-	1443-06	AHS	SA	Not recorded	Southey	Apr-06	1-2 days	< 1yr	Lung, heart, spleen, liver	Mild with acid hematin
348	-	3	2	3 yrs	Equine	M	Y	2	-	Histopath no: Idexx 3830/06	-	1442-06	AHS	SA	Colesberg, Western Cape	Badenhorst	Apr-06	2 days	< 1yr	Lung, heart	Mild
351	3	3	3	-	Equine, Palomino	-	Y	1	-	Histopath no: Idexx 3837/06	-	1455-06	AHS	SA	Robertson, Western Cape	F.J. Bruwer	Apr-06	2 days	< 1yr	Lung, spleen, heart, liver, lnn, kidney	Mild
353	3	3	-	6 yrs	Equine, TB	F	Y	2	-	Histopath no: Idexx 4264/06	-	1584-06	AHS	SA	Addo, Eastern Cape	Willowtree stud	Apr-06	2 days	< 1yr	Spleen, lung	Mild
355	3	3	3	5-6 months	Equine, Arab X Saddler	F	Y	-	-	Vet Faculty virology no: E102/06	399-06	1626-06	AHS	SA	Pretoria, Gauteng	P. Miller	Apr-06	2 days	< 1yr	Spleen, lung, heart	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
356	3	3	3	4 months	Equine, Friesian	F	Y	1	-	OVI virology no: 20/06	139-06	566-06	AHS	SA	Pretoria, Gauteng	C.J.M. Botha	Feb-06	2 days	< 1yr	Spleen, lung, heart	Mild
357	3	3	3	2 yrs	Equine	F	Y	5	-	OVI virology no: 64/05	982-05	3915-05	AHS	SA	Onderstepoort, Gauteng	Z.Z.Z. Majokweni (OBP)	Dec-05	2 days	< 1yr	Spleen, lung, heart, liver, kidney	Mild to moderate
359	3	3	-	-	Equine	-	Y	9	-	Histopath no: Idexx 4782/06	-	1722-06	AHS	SA	Plettenberg Bay, Western Cape	Via George Rex Veterinary Clinic	Apr-06	2 days	< 1yr	Lung, spleen, liver, kidney	Mild
360	3	3	3	6 yrs	Equine, Miniature Horse	M	Y	1	-	OVI virology no: 100/06	323-06	1246-06	AHS	SA	Pretoria, Gauteng	Pretorius	Mar-06	2 days	< 1yr	Heart, lung, spleen	Moderate to advanced
362	3	3	-	18 months	Equine	F	Y	5	-	Histopath no: Idexx 4789/06A	-	1737-06A	AHS	SA	Robertson, Western Cape	Dageraad Stoet	May-06	2 days	< 1yr	Spleen, lung	Moderate
363	3	3	3	4 months	Equine	F	Y	5	-	Histopath no: Idexx 4789/06B	-	1737-06B	AHS	SA	Robertson, Western Cape	Dageraad Stoet	May-06	2 days	< 1yr	Lung, spleen, heart, liver, kidney	Moderate
364	-	3	3	-	Equine, TB	F	Y	5	-	Histopath no: Idexx 4866/06	-	1763-06	AHS	SA	Robertson, Western Cape	Highland Stud	May-06	2 days	< 1yr	Heart, lung, kidney	Mild
365	2	2	-	6 yrs	Equine	F	Y	5	-	Histopath no: Idexx 4867/06	-	1764-06	AHS	SA	Robertson, Western Cape	Dageraad Stoet	May-06	2 days	< 1yr	Spleen, lung	Spln moderate; lung mild
366	-	3	-	18 months	Equine, Dutch warmblood	M	Y	5	-	Histopath no: Idexx 4868/06	-	1765-06	AHS	SA	Robertson, Western Cape	Dageraad Stoet	May-06	2 days	< 1yr	Lung, liver, stomach (stom), small intestine (sml int)	Mild to moderate

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
368	-	2	3	3 yrs	Equine	F	Y	2	-	Histopath no: Idexx 5008/06	-	1778-06	AHS	SA	Fouriesburg, Free State	Moolmanshoek	May-06	1-2 days	< 1yr	Lung, heart, Inn, small intestine	Mild with acid hematin
369	3	3	3	20 yrs	Equine	F	Y	2	-	Histopath no: Idexx 5006/06	-	1780-06	AHS	SA	Eastern Cape	Muller	May-06	1-2 days	< 1yr	Spleen, heart, lung, liver, adrenal, stom, thyroid, kid	Mild
370	3	3	3	3 yrs	Equine	F	Y	2	-	Histopath no: Idexx 4863/06	-	1782-06	AHS	SA	Eastern Cape	Steynberg	May-06	2 days	< 1yr	Lung, spleen, heart, liver, stomach, kidney	Mild
371	3	3	-	1 yr	Equine	-	Y	2	-	Histopath no: Idexx 5126/06	-	1804-06	AHS	SA	Christiana, Free State	J. Fourie	May-06	2 days	< 1yr	Lung, spleen, liver	Mild
372	3	3	3	-	Equine	-	Y	2	-	Histopath no: Idexx 5125/06	-	1805-06	AHS	SA	Eastern Cape	Buys	May-06	2 days	< 1yr	Lung, spleen, heart, stomach	Mild
373	3	3	-	Foal	Equine	-	Y	2	-	Histopath no: Idexx 5127/06	-	1806-06	AHS	SA	Christiana, Free State	J. Fourie	May-06	2 days	< 1yr	Spleen, lung	Moderate with acid hematin
376	3	3	-	7 yrs	Equine	M	Y	5	-	Histopath no: Idexx 5531/06	-	1929-06	AHS	SA	Winterton, KZN	The Nest Hotel	May-06	2 days	< 1yr	Spleen, lung	Mild
377	3	3	-	9 months	Equine, TB	F	Y	3	-	Histopath no: Idexx 5760/06	-	2024-06	AHS	SA	Walmer, Eastern Cape	Milkwood Stud	May-06	2 days	< 1yr	Spleen, lung	Mild
378	3	3	-	6 months	Equine	-	Y	5	-	Histopath no: Idexx 5866/06	-	2042-06	AHS	SA	Not recorded	Highland Stoet	Jun-06	2 days	< 1yr	Lung, spleen, stomach, small intestine	Mild

APPENDIX 2: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE TEST CASES (WITH TISSUES LESS THAN 10 YEARS OLD) USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
380	3	3	3	-	Equine	-	Y	-	-	Vet Faculty virology no: E103/06	-	1819-06	AHS	SA	KZN	M. van Vuuren	Apr-06	1 week	< 1yr	Spleen, heart, lung	Mild
381	3	3	3	6 yrs	Equine	M	Y	2	-	Histopath no: Idexx 5034/06	-	1781-06	AHS	SA	Eastern Cape	Deysel	May-06	2 days	< 1yr	Spleen, lung, heart, kidney, liver	Mild
382	-	3	3	20 months	Equine, Arab	F	Y	5	-	Histopath no: Idexx 6088/06	-	2204-06	AHS	SA	Piet Retief, Mpumalanga	Yollaris & Labuschagne	Jun-06	2 days	< 1yr	Lung, heart, liver, kidney	Mild

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
148	3	3	3	4 months	Equine, Arab	F	Y	2	-	-	258-90	357-90	AHS	SA	Pretoria, Gauteng	C. McCrindle	Feb-90	1-2 days	16 yrs	Lung, heart, spleen	Mild
149	-	3	-	-	Equine	-	Y	2	-	-	500-90	625-90	AHS	SA	Gauteng	H.G. Robertson	Apr-90	1-2 days	16 yrs	Lung	Mild
150	-	-	3	18 months	Equine, Arab	F	Y	6	-	-	509-90	631-90	AHS	SA	Pretoria, Gauteng	K. Nieman	Apr-90	1-2 days	16 yrs	Heart	Mild
151	3	-	-	5-7 yrs	Equine, Friesian	F	Y	4	-	OVI no: OP62086	65-96	148-96	AHS	SA	Pretoria, Gauteng	South African Police Equine Unit	Jan-96	1-2 days	10 yrs	Spleen	Mild
152	3	-	-	5 yrs	Equine, Friesian	F	Y	4	-	OVI no: OP062967	133-96	327-96	AHS	SA	Pretoria, Gauteng	South African Police Equine Unit	Feb-96	1-2 days	10 yrs	Spleen	Mild
153	3	3	-	3 months	Equine, Lippizaner	M	Y	4	-	Vet Faculty virology no: E34/96; OVI no: OPO63309	169-96	446-96	AHS	SA	Lippizaner Centre, Johannesburg, Gauteng	A. Chester	Feb-96	1-2 days	10 yrs	Spleen, lung	Mild
154	3	3	3	18 months	Equine, TB X Hanoverian	M	Y	4	-	Vet Faculty virology no: E42/96; OVI no: OPO63422	187-96	492-96	AHS	SA	Bryanston, Johannesburg, Gauteng	R. Boyce	Feb-96	1-2 days	10 yrs	Lung, heart, spleen	Mild
155	3	-	-	5 months	Equine, Nooitgedacht pony	F	Y	1	-	Vet Faculty virology no: E36/96 & 39/96	186-96	474-96	AHS	SA	Onderstepoort, Gauteng	Vet Faculty, Onderstepoort, Gauteng	Feb-96	1-2 days	10 yrs	Spleen	Mild
156	3	3	3	5 yrs	Equine, TB	M	Y	2	-	OVI no: OP067613	541-96	1518-96	AHS	SA	Pretoria, Gauteng	J.H. Breytenbach	May-96	1-2 days	10 yrs	Heart, lung, spleen	Mild

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
178	3	3	3	18 months	Equine, TB	F	Y	4	-	Vet Faculty virology no: E5/96	9-96	26-96	AHS	SA	Pretoria, Gauteng	J.M. Breytenbach	Jan-96	1-2 days	10 yrs	Lung, heart, spleen	Mild
179	3	3	3	4 yrs	Equine, Miniature horse	F	Y	4	-	Vet Faculty virology no: E22/96	150-96	357-96	AHS	SA	Pretoria, Gauteng	V. Joubert	Feb-96	1-2 days	10 yrs	Lung, heart, spleen	Mild
180	3	-	-	9 months	Equine	M	Y	4	-	Vet Faculty virology no: E95/96; OVI no: OP64463	265-96	830-96	AHS	SA	Grootvlei near Pretoria, Gauteng	A.C. Loock	Mar-96	1-2 days	10 yrs	Spleen	Mild to moderate
249	3	3	3	1 yr	Equine	-	Y	5	-	Histopath no: Stell: 93.780	PM no: Stell: 9310002	177-03	AHS	SA	Paarl, via WCPVL	J. van Streepan	Oct-93	1-2 days	13 yrs	Heart, lung, spleen	Mild
254	2	3	3	-	Equine	-	Y	5	-	Histopath no: Stell: 93.764	PM no: Stell: 9309484	3295-05	AHS	SA	Ashton, via WCPVL	Zandvliet Stoet	Sep-93	1-2 days	13 yrs	Heart, lung, spleen	Moderate
259	-	3	3	8 months	Equine, Palomino	M	Y	5	-	-	1024-93	2308-93	AHS	SA	Gauteng	J. Coetzee	Oct-93	2 days	13 yrs	Heart, lung	Mild
264	-	-	3	3,5 months	Equine, Arab	F	Y	4	-	-	7558-81	470-81	AHS	SA	Pretoria, Gauteng	S.G. Oosthuizen	Mar-81	2 days	25 yrs	Heart	Mild
265	3	3	3	Adult	Equine, Shetland pony	M	Y	1	-	-	421-85	628-85	AHS	SA	Rosslyn near Pretoria, Gauteng	D. Sumpton	Apr-85	2 days	21 yrs	Heart, lung, spleen	Mild
266	2	3	3	15 yrs	Equine	F	Y	1	-	-	486-85	578-85	AHS	SA	Onderstepoort, Gauteng	E.L. Cox	Apr-85	2 days	21 yrs	Heart, lung, spleen	Mild

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
267	3	3	-	Adult	Equine, TB	F	Y	4	-	-	266-88	353-88	AHS	SA	Pretoria, Gauteng	D.H. Viljoen	Feb-88	2 days	18 yrs	Lung, spleen	Mild
268	3	3	3	Foal	Equine	-	Y	5	-	Histopath no: OVI: 93-3747	PM no: OVI: 33052	3459-05	AHS	SA	Bronkhorstspruit, Gauteng	Grobler Boerdery, via the OVI	Oct-93	2 days	13 yrs	Heart, lung, spleen	Spln moderate; rest mild
269	3	3	3	-	Equine, Arab	-	Y	5	-	Histopath no: OVI: 93-3763	PM no: OVI: 33137	3460-05	AHS	SA	Middelburg, Mpumalanga	P.A. Welken, via the OVI	Oct-93	2 days	13 yrs	Heart, lung, spleen	Mild
270	3	3	3	8 months	Equine, Apalloosa	M	Y	5	-	Histopath no: OVI: 93-3761	PM no: OVI: 33109	3461-05	AHS	SA	Bronkhorstspruit, Gauteng	Grobler Boerdery, via the OVI	Nov-93	2 days	13 yrs	Heart, lung, spleen	Mild
271	2	3	3	4 months	Equine, Nooitgedacht pony	F	Y	1	-	Histopath no: OVI: 96-0435	-	3462-05	AHS	SA	Kaalplaas, near Onderstepoort, Gauteng	G.P. de Koker, via the OVI	Feb-96	1-2 days	10 yrs	Heart, lung, spleen	Spln moderate; rest mild
272	3	3	3	3-4 months	Equine	F	Y	6	-	Histopath no: OVI: 96-518	-	3463-05	AHS	SA	Onderstepoort, Gauteng	Onderstepoort, Gauteng Biological Products, via the OVI	Mar-96	1-2 days	10 yrs	Heart, lung, spleen	Mild
273	3	3	3	-	Equine	-	Y	2	-	Histopath no: OVI: 96-595	-	3464-05	AHS	SA	Kaalplaas, near Onderstepoort, Gauteng	G.P. de Koker, via the OVI	Feb-96	2 days	10 yrs	Heart, lung, spleen	Mild
274	3	3	3	-	Equine	-	Y	2	-	Histopath no: OVI: 96-575	PM no: OVI: 63460	3465-05	AHS	SA	Pretoria, Gauteng	E. Viviers, via the OVI	Feb-96	2 days	10 yrs	Heart, lung, spleen	Spln moderate; rest mild
275	2	3	3	-	Equine	-	Y	1	-	Histopath no: OVI: 96-597	PM no: OVI: 63534	3466-05	AHS	SA	Pretoria, Gauteng	E. Viviers, via the OVI	Feb-96	1-2 days	10 yrs	Heart, lung, spleen	Moderate

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
276	3	3	3	Adult	Equine	-	Y	2	-	Histopath no: OVI: 96-730	PM no: OVI: 64086	3467-05	AHS	SA	Pretoria, Gauteng	S. Rufus, via the OVI	Mar-96	1-2 days	10 yrs	Heart, lung, spleen	Mild to moderate
277	2	3	3	Adult	Equine, Nooitgedacht pony	F	Y	2	-	Histopath no: OVI: 96-754	PM no: OVI: 64159	3468-05	AHS	SA	Kaalplaas, near Onderstepoort, Gauteng	G.P. de Koker, via the OVI	Mar-96	2 days	10 yrs	Heart, lung, spleen	Mild to moderate
278	3	3	3	4 months	Equine	F	Y	2	-	Histopath no: OVI: 96-981	PM no: OVI: 64941	3469-05	AHS	SA	Onderstepoort, Gauteng	Onderstepoort, Gauteng Biological Products, via the OVI	Apr-96	2 days	10 yrs	Heart, lung, spleen	Moderate
279	-	2	2	6 months	Equine, Hanoverian X	M	Y	4	-	Histopath no: OVI: 96-1020	PM no: OVI: 65086	3470-05	AHS	SA	Pretoria, Gauteng	S.H. Rufus, via the OVI	Mar-96	2 days	10 yrs	Heart, lung	Mild
280	-	3	3	5 yrs	Equine, Boerperd	M	Y	2	-	Histopath no: OVI: 96-1084	PM no: OVI: 65379	3471-05	AHS	SA	Pretoria, Gauteng	J.C. Kruger, via the OVI	Apr-96	2 days	10 yrs	Heart, lung	Moderate
281	3	3	2	7 yrs	Equine, Namib horse	F	Y	1	-	Histopath no: OVI: 96-1277	-	3472-05	AHS	SA	Pretoria, Gauteng	P.C. Colyn, via the OVI	Apr-96	2 days	10 yrs	Heart, lung, spleen	Mild
285	3	-	-	-	Equine	-	?	-	-	Histopath no: OVI: 23-3211	PM no: OVI: 23-3197	1508-06	AHS, dikkop form	SA	Not recorded	AHS reference collection, Arnold Theiler museum, OVI	Nov-23	Not known	83 yrs	Spleen	Mild to moderate
286	1	1	1	-	Equine	-	Y	1	**E**	Histopath no: OVI: 62-1424	PM no: OVI: 62-39897	1509-05	Experimental AHS - intranasal inoculation of A501 (serotype 1) neurotropic strain	SA	Experimental, OVI, Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Jul-62	Not known	44 yrs	Spln, lung, hrt, sk musc, brain	Mild
287	1	1	-	-	Equine	-	Y	9	**E**	Histopath no: OVI: 62-2015	PM no: OVI: 62-40553	1510-06	Experimental AHS - intranasal inoculation of neurotropic strain 7/60 (serotype 9)	SA	Experimental, OVI, Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Aug-62	Not known	44 yrs	Spln, lung, liv, kid, brain	Mild to moderate

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
288	1	1	1	-	Equine	-	Y	4	-	Histopath no: OVI: 63-318	PM no: OVI: 63-41223	1511-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Jan-63	Not known	43 yrs	Spln, lung, hrt, liver	Mild
289	1	1	1	18 months	Equine	-	Y	7	**E**	Histopath no: OVI: 63-374	PM no: OVI: 63-41100	1512-06	Experimental AHS - intranasal inoculation of the neurotropic Karen strain (serotype 7)	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Feb-63	Not known	43 yrs	Hrt, lung, spln, liv, brain	Mild
290	1	1	1	-	Equine	-	Y	7	**E**	Histopath no: OVI: 63-375	PM no: OVI: 63-41104	1513-06	Experimental AHS - intranasal inoculation of the neurotropic Karen strain (serotype 7)	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Feb-63	Not known	43 yrs	Hrt, lung, spln, liv, kid, brain	Mild
292	3	3	3	3 yrs	Equine	M	Y	-	**E**	Histopath no: OVI: 64-4252	PM no: OVI: 64-44276	1515-06	Experimental AHS - intravenous inoculation with splenic suspension	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Nov-64	Not known	42 yrs	Spln, lung, hrt, adrenal, thyroid	Mild to moderate
293	3	3	3	-	Equine	-	Y	1	**E**	Histopath no: OVI: 64-4254	PM no: OVI: 64-44049	1516-06	Experimental AHS - intravenous inoculation with splenic suspension, serotype 1	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Nov-64	Not known	42 yrs	Heart, lung, spleen	Mild
294	3	3	-	-	Equine	-	Y	-	**E**	Histopath no: OVI: 64-4395	PM no: OVI: 64-44142	1517-06	Experimental AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Nov-64	Not known	42 yrs	Spln, lung, lnn, small intestine	Mild to moderate
295	-	3	3	-	Equine	-	?	-	-	Histopath no: OVI: 65-700	PM no: OVI: 65-44631	1520-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Mar-65	Not known	41 yrs	Lung, heart, liver, kid	Moderate with acid hematin
296	1	2	1	-	Equine	-	?	-	-	Histopath no: OVI: 65-905	PM no: OVI: 65-45241	1521-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Mar-65	Not known	41 yrs	Spleen, lung, heart	Moderate
297	3	3	3	-	Equine	-	?	-	-	Histopath no: OVI: 65-1031	PM no: OVI: 65-45251	1522-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Mar-65	Not known	41 yrs	Heart, lung, spleen	Mild

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
298	3	3	3	-	Equine	-	?	-	-	Histopath no: OVI: 65-1120	PM no: OVI: 65-44391	1523-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-65	Not known	41 yrs	Spln, lung, hrt, liv, stomach, sml intestine, sk musc, bladder, prostate, testes	Mild
299	2	3	3	-	Equine	-	?	-	-	Histopath no: OVI: 65-1230	PM no: OVI: 65-44962	1524-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-65	Not known	41 yrs	Spln, lung, hrt, liver, lnn, thymus	Mild
300	3	2	2	2 yrs	Equine	F	Y	2	**E**	Histopath no: OVI: 65-1231	PM no: OVI: 65-44955	1525-06	Experimental AHS - intravenous inoculation with splenic suspension, serotype 2	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-65	Not known	41 yrs	Spleen, lung, heart, liver	Mild
301	3	3	3	3 yrs	Equine	F	Y	-	**E**	Histopath no: OVI: 65-1233	PM no: OVI: 65-45267	1526-06	Experimental AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-65	Not known	41 yrs	Spln, lung, heart, lnn, sk musc, kidney	Mild to moderate
302	3	3	3	-	Equine	-	Y	-	**E**	Histopath no: OVI: 65-1602	PM no: OVI: 65-45294	1545-06	Experimental AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	May-65	Not known	41 yrs	Spleen, lung, heart, lymph node	Mild
303	2	3	3	-	Equine	-	Y	-	**E**	Histopath no: OVI: 65-2135	PM no: OVI: 65-45827	1605-06	Experimental AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Jul-65	Not known	41 yrs	Spleen , lung, heart, liver	Mild to moderate
304	3	3	3	-	Equine	-	Y	-	**E**	Histopath no: OVI: 65-3996	PM no: OVI: 65-45483	1606-06	Experimental AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Nov-65	Not known	41 yrs	Heart, lung, spleen	Mild
305	3	3	3	-	Equine	-	?	-	-	Histopath no: OVI: 66-3726	PM no: OVI: 66-47483	1607-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Oct-66	Not known	40 yrs	Heart, lung, spleen, lymph node	Mild
306	3	3	2	-	Equine	-	?	-	-	Histopath no: OVI: 69-1416	PM no: OVI: 69-51423	1608-06	AHS	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Jun-69	Not known	37 yrs	Heart, lung, spleen, kid, sk musc	Mild

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
307	1	1	1	-	Equine	-	?	-	-	Histopath no: OVI: 76-451	PM no: OVI: 76-221	1610-06	AHS	SA	Kaalplaas, near Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Feb-76	Not known	30 yrs	Spln, lung, hrt, liv, lnn, sk musc, adrenal	Mild
308	-	3	2	-	Equine	-	?	-	-	Histopath no: OVI: 80-2027	-	1611-06	AHS	SA	Springs, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Jun-80	Not known	26 yrs	Lung, heart, thymus	Mild
309	3	3	3	18 months	Equine	F	Y	3	**E**	Histopath no: OVI: 81-776	PM no: OVI: 81-5684	1612-06	Experimental AHS - intravenous inoculation with suspension of BHK21 cells, serotype 3	SA	Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI (S.J. Newsholme)	Jan-81	Not known	25 yrs	Spleen , lung, heart, adrenal	Mild
310	3	3	3	-	Equine	-	?	-	-	Histopath no: OVI: 81-840	PM no: OVI: 81-5690	1613-06	AHS	SA	Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Mar-81	Not known	25 yrs	Spln, lung, hrt, liver, lnn, brain	Mild
312	3	3	3	6 yrs	Equine	F	?	8	-	Histopath no: OVI: 81-1272	PM no: OVI: 81-5733	1615-06	AHS, mixed form	SA	Kaalplaas, near Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI (S.J. Newsholme)	May-81	Not known	25 yrs	Spln, lung, hrt, liv, brain	Mild
313	-	3	-	-	Equine	-	?	4	-	Histopath no: OVI: 88-795	PM no: OVI: 88-2427	1616-06	AHS	SA	Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-88	Not known	18 yrs	Lung	Mild
314	-	3	2	-	Equine	-	?	6	-	Histopath no: OVI: 88-850	PM no: OVI: 88-2435	1617-06	AHS	SA	Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-88	Not known	18 yrs	Lung, heart, stomach	Mild
315	-	-	1	-	Equine	-	Y	2	-	Histopath no: OVI: 88-852	-	1618-06	AHS	SA	Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-88	Not known	18 yrs	Heart	Mild
316	1	3	2	-	Equine	-	?	-	-	Histopath no: OVI: 94-122	PM no: OVI: 94-1061	1619-06	AHS	SA	Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Jan-94	1-2 days	12 yrs	Spleen, lung, heart, kidney	Mild to moderate

APPENDIX 3: DATABASE OF ALL ARCHIVE AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES (WITH TISSUES 10 YEARS AND OLDER) USED IN THE STUDY, INCLUDING THEIR SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
317	2	3	3	-	Equine	-	?	-	-	Histopath no: OVI: 94-1482	PM no: OVI: 94-6430	1620-06	AHS	SA	Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Mar-94	1-2 days	12 yrs	Spleen, lung, heart, lnn, kidney	Mild to moderate
318	1	-	3	-	Equine	-	?	-	-	Histopath no: OVI: 94-1604	PM no: OVI: 94-6466	1621-06	AHS	SA	Kaalplaas, near Onderstepoort, Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Apr-94	1-2 days	12 yrs	Spleen, heart, adrenal	Moderate
319	3	3	3	-	Equine	-	?	5	**E**	Histopath no: OVI: 94-2972	PM no: OVI: 94-2863	1622-06	Experimental AHS	SA	Gauteng	AHS reference collection, Arnold Theiler museum, OVI	Aug-94	1-2 days	12 yrs	Spleen, lung, heart, liver	Mild
323	3	-	3	12 yrs	Equine	M	Y	-	**E**	AFIP Acc. 1003791	-	1730-06	Experimental AHS (Elazig strain) - mixed form, predominantly pulmonary type	Middle East	Not recorded	Via the Armed Forces Institute of Veterinary Pathology / AFIP (Maurer & McCully)	Apr-61	Not known	45 yrs	Spleen, heart, stomach, small intestine	Mild
324	3	3	-	Old	Equine	F	Y	-	**E**	AFIP Acc. 1003792	-	1731-06	Experimental AHS (Elazig strain) - mixed form, predominantly cardiac type	Middle East	Not recorded	AFIP (Maurer & McCully)	Apr-61	Not known	45 yrs	Spleen, lung, stomach	Mild
325	3	3	1	12 yrs	Equine	M	Y	-	-	AFIP Acc. 1003793	-	1732-06	Natural AHS, cardiac form	Middle East	Not recorded	AFIP (Maurer & McCully)	May-61	Not known	45 yrs	Spleen, lung, heart	Mild
326	2	3	3	13 yrs	Equine	F	Y	-	-	AFIP Acc. 1003794	-	1733-06	Natural AHS, cardiac form	Middle East	Not recorded	AFIP (Maurer & McCully)	May-61	Not known	45 yrs	Spleen, lung, heart, stomach	Mild
327	1	3	3	4 yrs	Equine	M	Y	-	-	AFIP Acc. 1008356	-	1734-06	AHS	Middle East	Not recorded	AFIP (Maurer & McCully)	May-61	Not known	45 yrs	Spleen, lung, heart, stomach	Mild
328	-	1	1	-	Equine	-	Y	-	-	AFIP Acc. 2318960	-	2677-06	AHS	Middle East	Not recorded	AFIP	Mar-91	2 days	15 yrs	Lung, heart, stomach, small intestine	Mild

APPENDIX 4: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES USED FOR THE FORMALIN-FIXATION KINETICS TRIAL, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
193B 1	3	-	-	14 yrs	Equine, TB	F	Y	7	-	OVI virology no: HS17/05	-	652-05	AHS	SA	KZN	Via Vetdiagnostix Pathology Laboratory	Feb-05	2 days	1 yr	Spleen	Mild
195B 2	3	-	-	-	Equine	-	Y	7	-	Vet Faculty virology no: E31/05	-	668-05	AHS	SA	Not recorded	Not recorded	Feb-05	4 days	1 yr	Spleen	Mild
196B 3	3	3	-	4 yrs	Equine	M	Y	7	-	OVI virology no: HS19/05	-	714-05	AHS	SA	Newcastle, KZN	Zaal	Feb-05	8 days	1 yr	Spleen, lung	Mild
198B 1	3	3	3	1 yr	Equine, Friesian	M	Y	1	-	Vet Faculty virology no: E35/05	226-05	731-05	AHS	SA	Roodeplaat near Pretoria, Gauteng	J. Birrell, SANDF	Feb-05	2 days	1 yr	Spleen, lung, heart	Mild
200B 2	-	3	3	-	Equine	-	Y	2	-	OVI virology no: HS25/05	-	855-05	AHS	SA	Graaff-Reinett, Eastern Cape	Owner not recorded; sample via T. Gerdes, OVI	Mar-05	4 days	1 yr	Lung, heart	Mild
202B 3	3	3	-	-	Equine	-	Y	2	-	OVI virology no: HS26/05	-	919-05	AHS	SA	Clanwilliam, Western Cape	Nel, via T. Gerdes, OVI	Mar-05	8 days	1 yr	Spleen, lung	Mild
203B 1	3	3	-	-	Equine	-	Y	5	-	OVI virology no: HS29/05	-	920-05	AHS	SA	Western Cape	Muller, via T. Gerdes, OVI	Mar-05	2 days	1 yr	Spleen, lung	Mild
204B 1	3	3	3	10 yrs	Equine, TB	M	Y	8	-	-	323-05	999-05	AHS	SA	Pretoria, Gauteng	Viljoen, SANDF	Apr-05	2 days	1 yr	Lung, heart, spleen	Mild
205B 1	3	3	3	4 months	Equine, TB	M	Y	6	-	-	325-05	1008-05	AHS	SA	Pretoria, Gauteng	M. van den Berg	Apr-05	2 days	1 yr	Lung, heart, spleen	Mild

APPENDIX 4: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE HORSE CASES USED FOR THE FORMALIN-FIXATION KINETICS TRIAL, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
207B 1	3	3	3	Adult	Equine	M	Y	6	-	-	343-05	1096-05	AHS	SA	Not recorded	Owner not recorded, horse was optional post mortem	Apr-05	2 days	1 yr	Lung, heart, spleen	Mild

APPENDIX 5: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE AND -NEGATIVE DOG CASES USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
383	3	3	3	4 yrs	Canine, Rottweiler	M	N	-	-	-	-	2341-08	AHS	SA	Pretoria, Gauteng	P. Maree	May-08	1-2 days	3 days	Spleen, lung, heart, liver, kidney	Mild
384	3	3	3	5 yrs	Canine, GSD	M	Y	6	-	-	328-06	1270-06	AHS	SA	Onderstepoort, Gauteng	J. Prinsloo	May-06	2 days	< 1yr	Spleen, lung, heart, liver, small intestine	Mild
393	1	1	1	1 yr	Canine, boerboel	M	N	-	-	-	1092-04	1092-04	Babesiosis and distemper	SA	Gauteng	C.G.J. Bezuidenhout, via the academic hospital, Onderstepoort	Dec-04	2 days	3 yrs	Spleen, lung, heart, liver	Mild
394	1	1	1	5 yrs	Canine, GSD	F	N	-	-	-	1090-04	3501-04	Aldicarb intoxication	SA	Gauteng	A.J. Portwig, via the academic hospital, Onderstepoort	Dec-04	2 days	3 yrs	Lung, heart, spleen	Mild
395	1	1	1	-	Canine, bulldog	M	N	-	-	-	576-02	1372-02	Congestive heart failure	SA	Gauteng	P.W. Prinsloo, via the academic hospital, Onderstepoort	Jun-02	2 days	5 yrs	Lung, heart, spleen, liver	Mild
396	1	1	1	10 yrs	Canine, Staffordshire bull terrier X	F	N	-	-	-	626-02	1482-02	Haemorrhagic diathesis	SA	Gauteng	S. de Beer, via the academic hospital, Onderstepoort	Jun-02	2 days	5 yrs	Lung, heart, spleen, liver, stomach	Mild
397	1	1	-	9 weeks	Canine, fox terrier	M	N	-	-	-	682-02	1609-02	Canine distemper virus	SA	Gauteng	M. Steenkamp, via the academic hospital, Onderstepoort	Jul-02	2 days	5 yrs	Lung, spleen, kidney, brain	Mild
398	1	1	1	-	Canine, Jack Russell terrier	M	N	-	-	-	709-02	1720-02	Spirocercosis	SA	Gauteng	A. Buys, via the academic hospital, Onderstepoort	Jul-02	2 days	5 yrs	Lung, spleen, heart, liver	Mild
399	1	1	1	15 months	Canine, boerboel	F	N	-	-	-	970-02	2420-02	Bladder carcinoma and bilateral hip dysplasia	SA	Gauteng	J. Coetzee, via the academic hospital, Onderstepoort	Sep-02	2 days	5 yrs	Lung, spleen, heart	Mild

APPENDIX 5: DATABASE OF ALL AFRICAN HORSESICKNESS VIRUS-POSITIVE AND -NEGATIVE DOG CASES USED IN THE STUDY, INCLUDING THEIR TARGET TISSUE SCORES (SPLEEN, LUNG AND HEART)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
400	1	1	1	3,5 yrs	Canine, bull terrier	F	N	-	-	-	973-02	2423-02	Spirocercosis	SA	Gauteng	I.M. Rautenbach, via the academic hospital, Onderstepoort	Sep-02	2 days	5 yrs	Lung, heart, spleen	Mild
401	1	1	1	2 yrs	Canine, X-breed	M	N	-	-	-	1109-02	2761-02	Canine distemper virus and parvovirus	SA	Gauteng	J.H. van Wyk, via the academic hospital, Onderstepoort	Nov-02	2 days	5 yrs	Lung, heart, spleen, brain, small intestine	Mild
402	1	1	1	9 months	Canine, dachshund	M	N	-	-	-	691-03	1889-03	Canine parvovirus, complicated by septicaemia and DIC	SA	Gauteng	A.P. du Plessis, via the academic hospital, Onderstepoort	Aug-03	2 days	4 yrs	Lung, heart, spleen, liver	Mild
403	-	1	1	3 months	Canine, beagle	F	N	-	-	-	342-08	1685-08	Viraemia or septicaemia	SA	Onderstepoort, Gauteng	P. Irons, via the academic hospital, Onderstepoort	Apr-08	1-2 days	2 weeks	Heart, lung, liver, stomach, brain	Mild

APPENDIX 6: DATABASE OF ALL EQUINE ENCEPHALOSIS VIRUS-POSITIVE HORSE CASES USED IN THE STUDY, INCLUDING SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
251	-	3	-	7 yrs	Equine, TB	M	Y	1	-	Histopath no: Stell: 99.105	PM no: Stell: 9904189	1760-02	Suspected equine encephalosis (EE)	SA	Somerset West, via WCPVL	D. Nel	Apr-99	1-2 days	7 yrs	Lung	Mild
350	-	2	-	-	Equine	-	Y	-	-	Histopath no: Idexx 3918/06	-	1456-06	Equine encephalosis (EE)	SA	Drummond, KZN	B. Lattimer	Apr-06	2 days	< 1yr	Liver, lung	Mild
352	3	2	2	-	Equine	-	Y	4	-	OVI virology no: 113/06 Histopath no: Idexx 4337/06	-	1604-06	EE	SA	Springfontein, Free State	Irwin	Apr-06	2 days	< 1yr	Spleen, lung, heart, liver	Mild
385	3	3	-	-	Equine, Basotho pony	-	Y	1	E	-	-	2605-00	Experimental EE	SA	Vet Faculty, Onderstepoort, Gauteng	A. Pardini (researcher)	Jan-00	1-2 days	6 yrs	Lung, spleen, liver	Mild
386	-	3	-	-	Equine, Basotho pony	-	Y	1	E	-	-	2637-00	Experimental EE	SA	Vet Faculty, Onderstepoort, Gauteng	A. Pardini (researcher)	Jan-00	1-2 days	6 yrs	Lung	Mild
387	3	-	-	-	Equine, Basotho pony	-	Y	1	E	-	-	2638-00	Experimental EE	SA	Vet Faculty, Onderstepoort, Gauteng	A. Pardini (researcher)	Jan-00	1-2 days	6 yrs	Spleen	Mild
388	3	3	-	-	Equine	-	Y	1	-	Histopath no: Stell 7-101	-	2025-07	EE	SA	Simondium, Western Cape	River World Stud, via WCPVL	Mar-07	2 days	6 mo	Spleen, lung, lymph node	Mild
389	3	3	-	-	Equine, TB	-	Y	1	-	Histopath no: Stell 7-120	-	2026-07	EE	SA	Windmeul, Western Cape	G. Kotzen, via WCPVL	Apr-07	2 days	6 mo	Spleen, lung, liver	Mild
390	3	3	-	-	Equine, TB	-	Y	1	-	Histopath no: Stell 7-126	-	2027-07	EE	SA	Windmeul, Western Cape	G. Kotzen, via WCPVL	Apr-07	2 days	6 mo	Spleen, lung, liver, lymph node	Mild

APPENDIX 6: DATABASE OF ALL EQUINE ENCEPHALOSIS VIRUS-POSITIVE HORSE CASES USED IN THE STUDY, INCLUDING SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
391	3	3	-	-	Equine, TB	F	Y	1	-	Histopath no: Stell 7-149	-	2029-07	EE	SA	Windmeul, Western Cape	G. Kotzen, via WCPVL	Apr-07	2 days	6 mo	Spleen, lung, liver	Mild
392	3	3	-	-	Equine, Arab	F	Y	1	-	Histopath no: Stell 7-164	-	2031-07	EE	SA	Sumondium, Western Cape	F. Brown, Elkana Trust, via WCPVL	May-07	2 days	6 mo	Spleen, lung, liver	Moderate

APPENDIX 7: DATABASE OF ALL BLUETONGUE VIRUS-POSITIVE SHEEP CASES USED IN THE STUDY, INCLUDING SPECIFIED TARGET TISSUE SCORES (IF SPLEEN, LUNG AND/OR HEART SAMPLES WERE PRESENT)

Case No.	Spleen score A/B	Lung score A/B	Heart score A/B	Age	Species and Breed	Sex	Virus isolated (Yes/No)	Serotype	Experimental case? (E)	Original reference number	Post mortem (PM) number	Onderstepoort histology acc. number	Cause of death	Country	Place/Prov	Sender / Place of origin	Tissue collection date	Formalin fixation (FF) time	Wax block storage time	Tissue(s) present	Level of autolysis and if acid hematin present
261	-	3	-	2 tooth	Ovine, Merino	F	Y	-	-	-	435-05	1564-05	Experimental bluetongue (BT) virus infection	SA	Vet Faculty, Onderstepoort, Gauteng	J. Terblanche	Jun-05	1-2 days	1 yr	Coronary skin, lung, skeletal muscle (sk musc)	Mild
282	-	-	2	Adult	Ovine, Merino	F	Y	-	-	-	441-05	1570-05	Experimental bluetongue (BT) virus infection	SA	Vet Faculty, Onderstepoort, Gauteng	J. Terblanche	May-05	3 days	1 yr	Heart	Mild
283	-	-	-	Adult	Ovine, Merino	F	Y	-	-	-	447-05	1591-05	Experimental BT infection	SA	Vet Faculty, Onderstepoort, Gauteng	J. Terblanche	Jun-05	2 days	1 yr	Skeletal muscle (sk musc)	Mild
284	-	-	-	2 Tooth	Ovine, Merino	F	Y	-	-	-	448-05	1592-05	Experimental BT infection	SA	Vet Faculty, Onderstepoort, Gauteng	J. Terblanche	Jun-05	2 days	1 yr	Skeletal muscle	Mild
291	-	-	-	-	Ovine, Merino	-	Y	-	-	Histopath no: OVI: 64-3246	PM no: OVI: 64-43085	1514-06	Bluetongue	SA	Onderstepoort, Gauteng	Bluetongue reference collection, Arnold Theiler museum, OVI	Jan-64	Not known	42 yrs	Skeletal muscle	Mild
320	-	-	3	-	Ovine, Merino	-	Y	-	-	Histopath no: OVI: 94-3281	PM no: OVI: 94-2043	1637-06	Experimental BT	SA	Onderstepoort, Gauteng	Bluetongue reference collection, Arnold Theiler museum, OVI	Jan-94	1-2 days	12 yrs	Heart, skeletal muscle	Mild
321	-	3	3	-	Ovine, Merino	-	Y	-	-	Histopath no: OVI: 94-3326	PM no: OVI: 94-2041	1638-06	Experimental BT	SA	Onderstepoort, Gauteng	Bluetongue reference collection, Arnold Theiler museum, OVI	Jan-94	1-2 days	12 yrs	Heart, lung, skeletal muscle	Mild
322	-	-	-	-	Ovine, Merino	-	Y	-	-	Histopath no: OVI: 94-3389	PM no: OVI: 94-2048	1639-06	Experimental BT	SA	Onderstepoort, Gauteng	Bluetongue reference collection, Arnold Theiler museum, OVI	Jan-94	1-2 days	12 yrs	Skeletal muscle	Mild

APPENDIX 8: Phosphate buffered saline (PBS)/bovine serum albumin (BSA) buffer composition (Prophet, Mills, Arrington & Sobin 1992; Bancroft & Gamble 2002).

To 2 litres of distilled water add 17.42g chemically pure sodium chloride (NaCl) (product no: 6878, Merck & Co., Inc., Whitehouse Station, New Jersey, United States of America/USA), 5.8g disodium-hydrogenphosphate-12-hydrate (Na_2HPO_4.12H2O) (product no: 6879, Merck & Co., Inc., Whitehouse Station, New Jersey, USA), 0.52g potassium-dihydrogenphosphate (KH_2PO_4) (product no: 4871, Merck & Co., Inc., Whitehouse Station, New Jersey, USA), and 2g albumin (fraction V) from bovine serum lyophilizate (product no: 10735094001, Roche Diagnostics, Indianapolis, USA). The final pH of the buffer should be 7.6.