PATHOLOGY OF FATAL NATURAL AFRICAN HORSESICKNESS VIRUS INFECTION IN DOGS

by

NICOLIZE O'DELL

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of

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THE WOMAN IN THE GLASS

When you get what you want as you struggle for self
And the world makes you queen for a day,
Just go to the mirror and look at yourself,
And see what that woman has to say.

For it isn't your father or mother or husband
Who's judgement upon you must pass;
The person whose verdict counts most in your life
Is the one staring back from the glass.

She's the person to please, never mind all the rest,
For she's with you clear up to the end.
And you've passed your most dangerous, difficult test
If the woman in the glass is your friend.

You may fool the whole world down the pathway of life,
And get pats on your back as you pass.
But your final reward will be heartache and tears
If you've cheated the woman in the glass.

~ Dale Wimbrow ~

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"O God, my only hope lies in Your invincible power to make me what I am not yet,

but what You know I can become.

Strengthen my will Lord. Make me firm, steadfast, consistent.

Control my impulses, my emotions. May I keep pursuing and never quit."

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DECLARATION

I, **Nicolize O'Dell**, hereby declare that the work on which this thesis is based is my own independent work and that neither the whole work nor part of it has been, is being, or shall be submitted for another degree at this or another university, institution for tertiary education or professional examination body.

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Nicolize O'Dell

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Date

October 2018

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LIST OF ABBREVIATIONS

ACP	Acute cor pulmonale
AHS	African horsesickness
AHSV	African horsesickness virus
ALI	Acute lung injury
ARC	Agricultural Research Council
ARDS	Acute respiratory distress syndrome
BALT	Bronchiolar-associated lymphoid tissue
BSA	Bovine Serum Albumin
BTV	Bluetongue virus
CT	Cycle threshold
DAFF	Department of Agriculture, Forestry and Fisheries
ERC	Equine Research Centre
DPS	Department of Paraclinical Sciences
FVS	Faculty of Veterinary Science
H&E	Haematoxylin & Eosin
HPF	High power field
IHC	Immunohistochemistry
IL-1	Interleukin-1

INF-1	Interferon-1
MRU	Malelane Research Unit
MSB	Martius Scarlet Blue
No	Number
NO	Nitrous oxide
OVAH	Onderstepoort Veterinary Academic Hospital
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate Buffered Saline
RT-qPCR	Duplex real-time reverse transcription quantitative polymerase chain reaction
SOPs	standard operating procedures
TEM	Transmission Electron Microscopy
TNFα	Tumour necrosis factor alpha
UP	University of Pretoria
VGL	Veterinary Genetics Laboratory
VHL	Veterinary Histopathology Laboratory

SUMMARY

PATHOLOGY OF FATAL NATURAL AFRICAN HORSESICKNESS VIRUS INFECTION IN DOGS

by

Nicolize O'Dell

Supervisor:	**Dr JCA Steyl**
Co-supervisor:	**Dr JH Williams**
Department:	**Paraclinical Sciences**
Degree:	**M.Med.Vet (Pathology)**

African horsesickness (AHS) is a highly fatal arthropod-transmitted viral disease affecting mainly equids. Dogs are known to contract fatal AHS by ingestion to AHS-infected horse meat. At the Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria a number of canine mortalities with no history of horse meat consumption have been diagnosed since 2006. Limited published information describing the pathological findings in AHSV-infected dogs however exists.

This study is a retrospective survey focussing on providing a full macroscopic, histologic (including immunohistochemistry) and ultrastructural description of the pathological changes observed in dogs naturally infected by AHS virus.

Clinically most dogs presented with severe dyspnoea that deteriorated rapidly and terminated in respiratory failure and death. Some of the dogs had a history of lethargy and anorexia of less than 24 hours.

Macroscopically the most significant lesions were observed in the thoracic cavity and characteristically included severe acute interstitial pneumonia associated with mild serofibrinous pleuritis and moderate mediastinal oedema. Histopathologically the pulmonary findings included severe protein rich alveolar and septal oedema with randomly scattered areas of haemorrhage. In addition, acute inflammatory changes were present, characterised by mononuclear interstitial pneumonia and alveolar exudation, with hyperactivation of the alveolar capillary endothelial cells, alveolar macrophage proliferation and moderate diffuse hyperaemia. Immunohistochemical labelling revealed AHSV-specific positive labelling of the microvascular endothelial cells as well as scattered mononuclear leukocytes (monocytes and macrophages). The ultrastructural changes observed were supportive of vascular injury similar to those observed in horses infected by AHSV.

The pathological findings observed in all cases were indicative of acute pulmonary inflammation of haematogenous origin, resulting in fatal immune- and pathogen induced vascular injury.

CHAPTER 1

INTRODUCTION

African horsesickness (AHS) is a highly fatal arthropod-transmitted viral disease affecting mainly equids. It is caused by African horsesickness virus (AHSV), an *Orbivirus* of the family *Reoviridae*.[48,65,71] Dogs are known to contract fatal AHS, proven by in-depth experiments performed by Sir Arnold Theiler in the early 20th century.[66] In these cases and other cases of AHS outbreaks in dogs, all the affected animals were either inoculated experimentally by the AHS virus or they were exposed by ingestion to AHS-infected horse meat.[8,28,57,66,69] Only one case without consumption of horse meat was reported by Van Sittert *et al.* in 2013.[70]

At the Section of Pathology, Department of Paraclinical Sciences (DPS), Faculty of Veterinary Science (FVS), University of Pretoria (UP) a number of canine mortalities ascribed to AHS virus have been diagnosed of which the first was already confirmed in 2006.[51] African horsesickness as the cause of death in 29 dogs was confirmed at this institution using a combination of diagnostic criteria obtained from macropathology-, histopathology-, immunohistochemistry- and PCR results.

Limited published information regarding the pathological findings in AHSV-infected dogs however exists. Only five reports exist discussing the macropathology, two mentioning the histopathology, one reporting on the immunohistochemistry and none that address the ultrastructural findings.[36,66,67,69,70]

The epidemiological role of dogs in the transmission of AHS is unclear and may possibly be underestimated because dogs are not recognized as a host that may become infected by means of arthropod-transmission as in the horse.

The focus of this study will be to provide a full macroscopic-, histologic- and ultrastructural description of the pathological changes observed in dogs naturally infected by AHS virus. Formalin fixed tissues submitted by private practitioners for diagnostic histopathology mainly consisted of thoracic viscera since clinical signs and macroscopic necropsy findings were suggestive of a primary pneumopathy. Heart, liver and spleen were also submitted in the majority of cases, since heart failure was another consideration by many resulting in the observed lesions and signs. Since the tropism of AHS virus is known to mainly include these tissues, it was decided to set the focus of the study on the lung, heart, spleen and liver.[20]

CHAPTER 2

LITERATURE REVIEW

2.1 African horsesickness overview

African horsesickness virus (AHSV), the causative agent of African horsesickness (AHS), is an arthropod-transmitted *Orbivirus* of the family *Reoviridae* and is morphologically similar to other Orbiviruses such as Bluetongue virus (BTV).[48,65,71] African horsesickness is a non-contagious infectious disease transmitted by various species of biting midges, most importantly *Culicoides imicola.*[22,44] Nine antigenically distinct serotypes have been identified.[22,27,38]

African horsesickness virus mainly affects *Equidae*, of which horses are the most susceptible, with African donkeys and zebras being more resistant.[68] Zebras may even serve as latent carriers.[13] Dogs have been shown to be susceptible to experimental intravenous infection using infected horse blood as well as by experimental and natural ingestion of infected horse meat.[8,11,54,66,67] Detection of antibody in other species such as camelids, bovids, African elephants (*Loxodonta africana*), and carnivores such as lions (*Panthera leo*), cheetah (*Acinonyx jubata*), jackal (*Canis* spp.), spotted hyena (*Crocuta crocuta*), African wild dogs (*Lycaon pictus*) and genets (*Genetta* spp.) suggest that these species may also play a role in the epidemiology of AHS.[3,5,23,49,70]

AHS is endemic to sub-Saharan Africa, but occasionally occurs in North Africa, the Middle East and even Southern Europe where the vector has become established apparently as a result of climate change.[22,42,48]

Four clinical forms of the disease are recognized. The peracute or pulmonary form, the subacute or cardiac form, the acute or mixed form and the mild AHS fever form.[20,22,42,48] Excluding the AHS fever form, the mortality rate in susceptible horses suffering from the first 3 clinical forms, may be as high as 95%.[15,20,22,48]

2.2 Occurrence of Orbivirus infection in dogs

Experiments performed by Sir Arnold Theiler in 1906 and subsequently in 1910 proved that dogs can contract AHS by experimental inoculation with viraemic horse blood.[66,67] Although the validity of these experiments was questioned by M'Fadyean in 1910 the susceptibility of dogs was confirmed in 1911 by Bevan when a number of dogs were infected through feeding of uncooked AHS infected horse meat.[8,36] Following these experiments a few outbreaks have been reported, all of which resulted from dogs consuming AHS infected horse meat.[28,54,57,69] Cases of AHS in dogs without a history of horse meat ingestion have been recorded since 2006 at the Section of Pathology, DPS, FVS, UP. It was not until 2013 when Van Sittert *et al*. (2013) from the Malelane Research Unit (MRU) Mpumalanga Province, South Africa reported the phenomenon for the first time in the literature. In this case no exposure to infected horse meat was possible as all research animals were housed in a semi-open enclosure with strictly controlled feeding practices.[70]

Bluetongue virus infection of two dogs following inoculation with contaminated Canine distemper virus vaccine that resulted in cardio-pulmonary failure and subsequent death has been reported by Akita *et al.* in 1994.[1] The detailed pathology for these cases has not been described. It is also known that carnivores, including domestic dogs may become subclinically infected with BTV following the consumption of infected meat from prey species.[4,14,53]

2.3 Comparative pathology of African horsesickness in horses and dogs

In the experiments performed by Sir Arnold Theiler in 1906 it was stated that the post mortem lesions observed in the dogs that succumbed after inoculation with viraemic horse blood were identical to lesions observed in affected horses. He also observed the course of AHS in dogs to be very rapid both in incubation (3-5 days) and temperature reaction.[25,67] This supports the finding that the presentation of AHS in dogs is similar to the peracute pulmonary form observed in horses.[22,42]

2.3.1 Clinical manifestation and Macropathology

In horses the disease is generally characterized by serofibrinous effusions and haemorrhages in various organs and tissues resulting from vascular injury.

The peracute pulmonary form is characterized clinically by fever and severe respiratory distress. Macroscopic lesions consist of severe pulmonary oedema and hydrothorax.

The subacute cardiac form is characterized clinically by intermittent fever, swelling of the supraorbital fossa and occasional signs of colic. Macroscopic lesions consist of subcutaneous and interfascial oedema of the neck, especially surrounding the ligamentum nuchae, serosal and mucosal haemorrhages and hydropericardium.

Lesions in the acute or mixed form are a combination of those of in the peracute pulmonary form and subacute cardiac form. This is the presentation most often observed in susceptible horses.

The mild AHS fever form is characterized by a transient fever with subsequent complete recovery. This is most likely the form that occurs in partially immune horses and other resilient species such as donkeys and zebras.[13,20,22,40,42,48]

In dogs the course of the disease resembles the peracute pulmonary form observed in horses characterized by fever and severe respiratory distress most often resulting in mortality.[42] The

first experiments performed by Sir Arnold Theiler were consistently characterized by pulmonary oedema and gastric congestion. Other less consistent post mortem findings included mild hydrothorax, mild splenomegaly, pleural petechiation, blood stained faeces and intestinal, renal, and hepatic congestion.[67] Subsequent studies by Sir Arnold Theiler in 1910 describe more complete post mortem findings that consisted of severe pulmonary oedema, multi-organ congestion, severe hydrothorax, mild hydropericardium, endocardial haemorrhages and mild bronchial lymphadenomegaly.[66]

With the outbreak reported by Van Rensburg *et al.* (1981) the macroscopical post mortem findings were consistent with those observed by Theiler in 1906. Van Rensburg *et al.* (1981) also reported that the fluid accumulated in the thoracic cavity coagulated on exposure to air, indicating that it was a highly proteinaceous exudate.[69]

The positive case that was naturally infected reported by Van Sittert *et al*. (2013) received supportive treatment which most likely modified the course of the disease. Therefore the pathological changes observed in this case may resemble the acute mixed form and may not be representative of the typical peracute pulmonary form of the disease usually observed in dogs.[70]

2.3.2 Histopathology

Histological changes are not considered to be helpful in the diagnosis or the understanding of the pathogenesis of AHS in horses.[42]

The histopathology associated with AHS in horses is characterized by extensive microvascular injury mostly affecting the lung and heart resulting in adventitial serocellular to fibrinous exudation and haemorrhage, associated with mononuclear leukocytosis and neutrophilia. Endothelial cell hypertrophy and occasional apoptosis may also be observed. These changes are usually accompanied by generalized fibrin-rich alveolar oedema in the lung and foci of myocardial necrosis in the heart.[19,50]

In the early experiments on AHSV infection in dogs performed by Theiler (1906, 1910), M'Fadyean (1910), Bevan (1911), Piercy (1951) and Haig (1956) no histological descriptions were provided.[8,28,36,54,66,67]

The outbreak reported by Van Rensburg *et al*. in 1981 was the first record of histological lesions observed in AHSV infected dogs.[69] Thereafter the only report of histological lesions was by Van Sittert *et al*. (2013) of one affected case.[70]

The histological lesions observed by Van Rensburg *et al.* (1981) included acute sero-fibrinous pneumonia with severe protein-rich oedema, exudation of macrophages and neutrophils into alveolar spaces, mild lymphocytic myocarditis and hepatic, intestinal and cerebral congestion.[69]

The histopathology results of the naturally infected dog reported by Van Sittert *et al*. (2013) revealed severe diffuse pulmonary congestion and protein-rich oedema, moderate to severe intra-alveolar haemorrhage, moderate leukocyte infiltration of the lungs, hepatic centrilobular sinusoidal congestion and moderate brain oedema. Multifocal sub-endocardial hyaline degeneration and necrosis were also noted.[70]

The clinical signs and necropsy lesions usually associated with AHSV infection, similar to BTV infection, can be attributed to changes in the capillary endothelial cell.[15]

2.3.3 Immunohistochemistry

Immunohistochemistry (IHC) is considered an accurate diagnostic method, especially for the diagnosis of infectious diseases in animals. In AHS it entails the demonstration of AHSV antigens within tissue sections by means of specific antibody binding. A coloured histochemical reaction visible by light microscopy demonstrates the specific antigen-antibody complex. Therefore the visualisation of specific cell types dependent upon specific cell antigens is possible and IHC facilitates a better understanding regarding antigen and tissue

interactions and localization of specific antigens. The latter contributes significantly to the understanding of disease pathogenesis.[19,56]

By utilizing a standardized and validated immunohistochemical assay for detection of AHS virus[21], Clift *et al.* (2010) confirmed heart and lung, and to a lesser degree the spleen, to be the main target tissues for AHS virus in horses.[20] The most important target cells for AHSV in the above mentioned tissues were confirmed to be mononuclear leukocytes (predominantly monocytes and macrophages) and microvascular endothelial cells.[21]

In the heart samples of affected horses, positive labelling was observed within the cytoplasm of capillary endothelial cells in the capillary network of loose connective tissue between cardiac myocytes. Less prominent granular to beaded cytoplasmic positivity was observed in circulating monocytes within interstitial blood vessels. In the lung, positive labelling was mostly present in pulmonary intravascular mononuclear leukocytes (monocytes), microvascular endothelial cells and rarely in the cytoplasm of macrophages in the pulmonary interstitial connective tissue.[20]

In the report of AHS in a dog by Van Sittert *et al.* (2013) scant positive labelling was observed within the myocardium only, as the presence of large amounts of acid-haematin pigment artefact interfered with the histological examination of other organs.[70]

2.3.4 Transmission electron microscopy

Ultrastructurally, the changes in AHS virus infected capillary endothelial cells of horses include: alteration of intercellular junctions resulting in increased permeability, viral particles (typical of *Orbivirus*), endothelial cell hypertrophy, -cytoplasmic projections, -degeneration, subendothelial cell debris and fibrin deposition with loss of endothelium and vascular repair.[26]. No ultrastructural studies regarding changes associated with AHSV infection in dogs have been conducted.

2.4 Pathogenesis of African horsesickness

The pathogenesis of the clinical signs and lesions associated with AHS is related to the sites of viral replication. Viral infection results in degeneration of endothelium in pulmonary and myocardial capillaries that consequently leads to increased vascular permeability, oedema, haemorrhage and microthrombosis.[42]

Vascular permeability of pulmonary capillaries in the horse specifically may be increased through the action of inflammatory mediators released by activated pulmonary intravascular macrophages (PIMs) and is not necessarily linked to the presence of virus in the injured endothelial cells.[17,19,26] Pulmonary intravascular macrophages however do not appear to perform a major function in the lungs of dogs, laboratory animals and humans. In these species the hepatic Kupffer cells fulfil the same role as PIMs in species such as cattle, horses, pigs, sheep, goats, cats and whales.[60,74]

2.5 Climate change and the occurrence of African horsesickness.

Temperature, amongst other climatic factors such as humidity, rainfall and wind, is an important factor that may directly affect vector biology and ecology. Increased temperatures accelerate the vector's metabolic rate and increase biting rates and feeding frequency, which subsequently result in enhanced egg production and population size increase.[47,61] With the current climatic trend towards global warming, the incidence of AHS is expected to increase, not only in horses but possibly also incidental hosts such as dogs. On the other hand, an increase in canine AHS cases may also be attributed to a change in viral virulence and associated host specificity.

Although *Culicoides* midges are thought not to prefer feeding on dogs, the absence of their preferred hosts, higher numbers of midges, and increased biting rates associated with global warming may compel them to revert to feeding on dogs. Studies performed by Blackwell *et al*. (1995) indicated that *Culicoides impunctatus* prefer feeding on dogs instead of sheep.[10] Although *C. impunctatus* has a different host range to *C. imicola*, these findings may suggest that other *Culicoides* species may also feed on dogs.[53] A number of other potential vectors such as mosquitos, biting flies and ticks have been implicated to transmit AHS, mostly under experimental conditions. Although these vectors do not currently appear to play an important role in AHS transmission, the effect of global warming may alter the disease ecology of AHS in the near future.[1, 25]

2.6 Epidemiological role of dogs in AHS transmission

Braverman *et al*. (1996) established in one study that the major AHSV vector, *Culicoides imicola* is probably not, or only very slightly attracted to dogs and is unlikely to transmit AHS to and from dogs.[12,73] McIntosh also found that the viraemia in dogs is very short (3-5 days), decreasing the probability of vector re-infection from dogs. Based on these studies and conclusions made by McIntosh in 1955 it is believed that the dog plays a minor role in the transmission of AHS. [12,43,73] Mellor however concluded in 1994, based on the AHS outbreaks in southern Europe that other arthropod-vectors such as mosquitos, biting flies and ticks, although not usually associated with AHS transmission, may become infected with AHSV and may play an important role in the epizootiology of AHS.[45] The role dogs and other wild canids in the epizootiology of AHS is yet to be established.[13]

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

This study was a retrospective descriptive analysis of the pathology resulting from AHSV infection in 29 dogs that died following natural exposure (i.e. no exposure to AHS infected horse meat). Ethical approval was granted by the University of Pretoria Ethics Committee (Ref. no: V069-16). Healthy control animals included two dogs that were electively euthanized using pentobarbitone at the Onderstepoort Veterinary Academic Hospital (OVAH). The relevant tissues from these animals were approved as healthy/normal controls in a concurrent research project for which ethical approval was granted by the UP Ethics committee (Ref no: V073-15).

3.2 Case definition

A diagnosis of fatal AHS infection in dogs was based on a history of acute respiratory failure, a positive AHSV-specific duplex real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) test result on submitted tissue and/or a positive AHS-specific immunohistochemistry (IHC) test result on formalin fixed lung tissue in association with significant pulmonary pathology.

3.2.1 Inclusion Criteria

Thirty-two dogs that were diagnosed with fatal natural AHSV infection at the Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, were included in the study. The pathology records for these animals were reviewed

and the epidemiological information collected. Dogs without a history of horse meat consumption were included in the study. A clinical presentation of acute respiratory distress was also used to select cases for further analysis.

The two control dogs were clinically healthy dogs admitted to the OVAH for elective euthanasia. Informed owner consent was granted for research purposes.

3.2.2 Exclusion Criteria

Cases were excluded from the study if AHSV infection could not be confirmed using either RT-qPCR or IHC. Two cases that displayed moderate to advanced post mortem changes were excluded, in spite of them testing positive on both RT-qPCR and IHC.

3.3 Laboratory procedures

All the materials of animal origin that were used for this study were obtained from cases that died following a clinical history of severe pulmonary distress and were subsequently submitted to the Section of Pathology, DPS, FVS, UP for diagnostic purposes. In cases where the whole carcass was submitted for post mortem, a complete post mortem examination was performed and a comprehensive set of tissues submitted for histopathological examination. The post mortem examination entailed a thorough systematic dissection of the carcass with macroscopic evaluation of all organs. In a number of cases submitted by private practitioners, however, only limited information about gross findings is reported. In addition, only selected, but key tissues (lung, heart, liver and spleen) were available for histological examination. Immunohistochemical analysis was performed on the available key tissues.[20,37] Ultrastructural description of AHSV-induced lesions on pulmonary tissue of selected positive qPCR and IHC cases were also conducted. The results are compared with those described in naturally infected horses and dogs infected by the consumption of infected horse meat.

3.3.1 Post mortem examination and sample collection

Of the 29 dogs used in this study 14 presented as whole carcasses at the Section of Pathology, DPS, FVS, UP for diagnostic purposes. A complete diagnostic post mortem was performed in these cases which included descriptive and digital recording of the macroscopic lesions and collection of samples for microscopic and molecular analysis. For histological examination, 1cm^3 tissue sections fixed in 10% buffered formalin were collected. In 12 of the 14 cases, sterile collected fresh lung and/or liver and/or spleen kept cool (4 to 10 ˚C) or frozen (-20˚C) was submitted for RT-qPCR testing.

The remainder of the 29 cases consisted of specimens (fresh and formalin fixed) and gross necropsy information submitted by private practitioners. The inclusion of these cases depended on the inclusion and exclusion criteria as set out under the study design.

3.3.2 Sample processing

The formalin-fixed samples were sectioned into standard tissue cassettes used for histological tissue processing. Tissue processing and staining for light microscopic examination was performed by the Section of Pathology, DPS, FVS, UP, according to The Department of Agriculture, Forestry and Fisheries (DAFF) accredited standard operating procedures (SOPs).

3.3.3 Tissue sections and stains

From each case, including the two control cases, five sections were produced for histological examination using a light microscope. Two of the sections were submitted for haematoxylin & eosin (H&E) and Martius scarlet blue (MSB)-staining[7]. The remainder of the sections were used for immunohistochemical (IHC)-labelling. Immunohistochemistry was performed utilizing antibodies/sera against AHSV-specific NS4[77] and -VP7[21] proteins to confirm the presence of AHS viral antigens. Immunohistochemical testing for bluetongue virus (BTV) was also conducted to rule out co-infection and cross reactivity, as this closely related Orbivirus

cause a similar disease syndrome to AHSV in domestic ruminants.[4,14,46] Immunohistochemical sera against the BTV-4-specific VP7 protein[58] was used for this purpose.

For IHC-labelling the wax-embedded tissues were sectioned (4µm-thick sections) and submitted for manual immunohistochemical staining following a standard IHC labelling method used at the Veterinary Histopathology Laboratory (VHL) of the Section of Pathology, DPS, FVS, UP. This method includes the following steps: Wax-embedded tissue sections are mounted on positively charged SuperFrost® Plus (Menzel-Glasser®) microscope slides and placed in a 40°C incubator overnight. Following this, the sections are de-waxed in xylene for ten minutes and subsequently rehydrated in 100%, 96% and 70% ethanol, respectively, for three minutes at a time. Thereafter slides are rinsed three times using distilled water and incubated in 3% hydrogen peroxide in methanol at room temperature for 15 minutes to inhibit endogenous peroxidase activity. For antigen retrieval, the sections labelled with the anti-NS4 antibody are microwaved for 14 minutes at 96°C in citrate buffer (pH 6), while those labelled with anti-VP7 serum and anti-BTV-4 serum are incubated in a solution of protease XIV in PBS-BSA (0.1 molar phosphate buffered saline with added 0.1% bovine serum albumin) buffer for 15 minutes at 37°C. Sections are then allowed to cool down at room temperature. A triple distilled water- and PBS-BSA buffer solution rinse for ten minutes follows, prior to being incubated with the primary antibody (Table 1). The triple distilled water- and 10 minute PBS-BSA buffer solution rinsing process is repeated after which the sections are subjected to the respective polymer kit processing (Table 1). For the anti-NS4 antibody stained sections, the EnVision™ detection system was used, while for those stained with anti-VP7 serum and anti-BTV serum, the EXPOSE® detection system was used. The triple distilled water- and 10 minute PBS-BSA buffer solution rinsing process is repeated. Development of the colour reaction by incubation of the slides using the NovaRED® chromogen is monitored under a light microscope until positive labelling of the control tissue can be visualised. All sections are

then rinsed with distilled water to inhibit the NovaRED® reaction and counterstained with Mayer's haematoxylin for 20 seconds. Lastly, all sections are rinsed for 10 minutes under running tap water, dehydrated in 70%, 96% and 100% ethanol respectively for three minutes at a time, cleared in xylene and mounted with a coverslip using Entallen® (Merck, KGaA, 64271, Darmstadt, Germany, Catalogue nr. HX57063261).

Table 1: Antibodies and immunohistochemical methods used.

Antibody	**AHSV (NS4)**	**AHSV (VP7)**	**BTV-4**
Manufacturer	Dr. C.A. Potgieter, Deltamune (Pty) Ltd, South Africa	Dr. C. Hamlin, Institute for Animal Health, Pirbright Laboratory, UK	Agricultural Research Council, Onderstepoort Veterinary Institute, South Africa
Animal source	Rabbit polyclonal	Rabbit polyclonal	Rabbit polyclonal
Dilution	1:12000	1:2000	1:200
Incubation time	40 minutes	120 minutes	Overnight
Antigen retrieval	Citrate buffer pH 6	Pronase[¤]	Pronase[¤]
Detection system	EnVision™[¥]	EXPOSE®[§]	EXPOSE®[§]
Chromogen	NovaRED®[¶]	NovaRED®	NovaRED®

[¤]Protease from Streptomyces griseus, Type XIV (code no. P5147-5G, Sigma Chemical Co, St. Louis, Missouri, USA)

[¥]Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Catalogue no. K5007, Dako, Glostrup, Denmark)

[§]Abcam® EXPOSE® Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (code no. ab80436, Abcam, Cambridge, UK)

[¶]VECTOR®, NovaRED®, (Catalogue no. SK-4800, Vector Laboratories, Burlingame, California, USA)

3.3.4 Histological examination

The H&E-stained sections were evaluated using a light microscope (Olympus® BH-2 Binocular microscope) at the Section of Pathology, DPS, FVS, UP. All submitted tissues were examined for light microscopic changes. From the clinical history and pathology records of the included cases (Section of Pathology case database, DPS, FVS, UP,) the lung was reported to be the principal organ that was most significantly affected in all cases. In order to facilitate a comprehensive histological evaluation of the lung, a scoring sheet was prepared listing all the anatomic areas susceptible to possible pathological changes and a basic grading system for lesion severity (APPENDIX 1). A description and digital image illustrating interpretation of lesion severity is provided in APPENDIX 2.

3.3.5 Immunohistochemical examination

The AHS-specific IHC labelling (NS4 and VP7) of the test cases were compared to a positive horse lung control that was labelled with the same antiserum respectively. For the anti-NS4 serum the positive control was characterised by fine dust-like to coarse granular cytoplasmic labelling as well as diffuse nuclear labelling by burnt-sienna particles (Figure 57).[77] A positive control for the anti-VP7 serum was indicated by the presence of small bead- or dot-like to fine granular bright red to burnt-sienna particles in the cytoplasm of capillary endothelial cells and mononuclear leukocytes (monocytes and macrophages) as described by Clift *et al.* in 2008 (Figure 59).[19] The BTV-4-specific IHC labelling of the test cases were compared to a positive sheep tongue tissue control. A anti-BTV-4 serum positive control was identified by dark-red to burnt-sienna fine- to coarse cytoplasmic granules in the microvascular endothelial cells (Figure 61).[58] Utilising the same scoring sheet that was used for the histological evaluation (APPENDIX 1) a category was created to assess the IHC labelling in the lung tissue. For both the NS4 and VP7 antibodies, the cell type, location, degree and nature of labelling was recorded. By inspecting 10 high power fields (HPF – 400x magnification) the degree of

labelling was graded as 0, 1+, 2+ or 3+ according to criteria specified in APPENDIX 2. For the BTV antibody, only presence or absence was recorded.

3.3.6 Transmission electron microscopy

Wax-embedded lung tissue samples of four cases were used for routine transmission electron microscopic (TEM) examination at the Electron Microscopy Laboratory of the Department of Anatomy and Physiology, FVS, UP. The samples were selected based on the histopathological changes, ensuring a representative range of the lesions observed. The samples were processed from the submitted wax blocks used for histopathological examination. Small tissue blocks were carefully removed from the wax-embedded material and de-waxed in 1% osmium tetroxide in xylene. Following this the tissue were infiltrated with resin and incubated at 65°C overnight. Ultrathin sections (70-90nm) were cut with a Leica EM UC7 ultramicrotome using a diamond knife and stained with lead citrate and uranyl acetate. The sections were then viewed in a Philips CM10 transmission electron microscope operated at 80kV.

Electron microscopic detectable cellular lesions associated with AHSV infection are described.

3.3.7 Molecular testing

Nested reverse transcription polymerase chain reaction (RT-PCR) was performed at the Onderstepoort Veterinary Institute (OVI), Agricultural Research Council (ARC) on 4 of the 29 cases, while duplex real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed at the Equine Research Centre (ERC) at the Veterinary Genetics Laboratory (VGL), UP, in 8 of the 29 cases.[27] Serotyping or typing of the AHSV strain and the determination of cycle threshold (CT)-values of respective organs were done where possible.

3.4 Data capture and analysis

All the canine cases diagnosed with AHS at the Section of Pathology, DPS, FVS, UP since 2006 were evaluated using the score sheet (APPENDIX 1) in conjunction with the grading criteria (APPENDIX 2). The findings recorded for each case can be found in the results section. Although the focus is mostly on the histopathology of the lung, the signalment, lesions observed (macroscopic, histologic and ultrastructural), and RT-PCR results for each case is captured, analysed and summarised in the results section.

3.5 Record keeping

The clearly identified wax embedded tissues and H&E stained tissue sections used in this study are permanently archived in a designated storage facility at the VHL, section of Pathology, DPS, FVS, UP. All the formalin fixed tissues have been disposed off according to the standard operating disposal procedures for formalin fixed tissues followed by the Section of Pathology. The documentation of all the cases is stored electronically by the primary investigator, supervisors and on the VHL electronic database at the Section of Pathology, DPS, FVS, UP.

CHAPTER 4

RESULTS

4.1 Gross pathology

The macroscopic appearance of the lungs, hearts, livers and spleens from the healthy control dogs conformed to the norm. The lungs were pink, well-aerated, filling the thoracic cavity with no fluid present in the pleural space. The hearts were of normal size and no abnormalities could be observed to affect the epicardium, myocardium, endocardium or valves. The livers was red-brown, meaty with a smooth surface. The spleens were dark red, flat, and did not bulge on cut-surface.

From the 29 cases included in this study, 14 was submitted as whole carcasses for full post mortem examination. The gross pathological findings observed in the AHSV-infected dogs was consistently similar throughout all of the 14 cases submitted for post mortem examination. The universal findings in these cases can be described as follows and differences are highlighted where they existed: The most significant lesions were observed in, or related to, the lungs and thoracic cavity. The mucous membranes were generally cyanotic (Figure 1) and in some cases serous and/or frothy fluid was oozing from the nostrils (Figure 2). Upon opening of the thoracic cavity protein-rich hydrothorax/serous pleuropneumonia varying from mild to severe was a consistent finding (Figure 3). The lungs were diffusely affected by severe interstitial pneumonia that was characterised by severe dorso-caudal variable hyperaemia (Figure 4) with serous fluid exudation on cut section and foam in the trachea (Figure 5). On palpation the lungs were firm and rubbery in consistency and significantly heavier than normal. In many cases scant fibrin strands were loosely attached to the pleural surface of the

lung suggestive of a fibrinous pleuritis and when in association with serous thoracic exudation and interstitial pneumonia (see earlier) could be classified as serofibrinous pleuropneumonia (Figure 6). Marked protein-rich mediastinal oedema/serous mediastinitis was uniquely present in most cases (Figure 7). Multifocal areas of pulmonary haemorrhage were observed in some cases.

Mild to moderate visceral congestion especially affecting the liver and spleen was observed in most cases. This resulted in mild enlargement of the respective organs with oozing of blood on cut surface. Subcutaneous petechiation and ecchymosis was rarely observed (Figure 8). No significant gross findings could be found in any of the other tissues or organs examined during full necropsy.

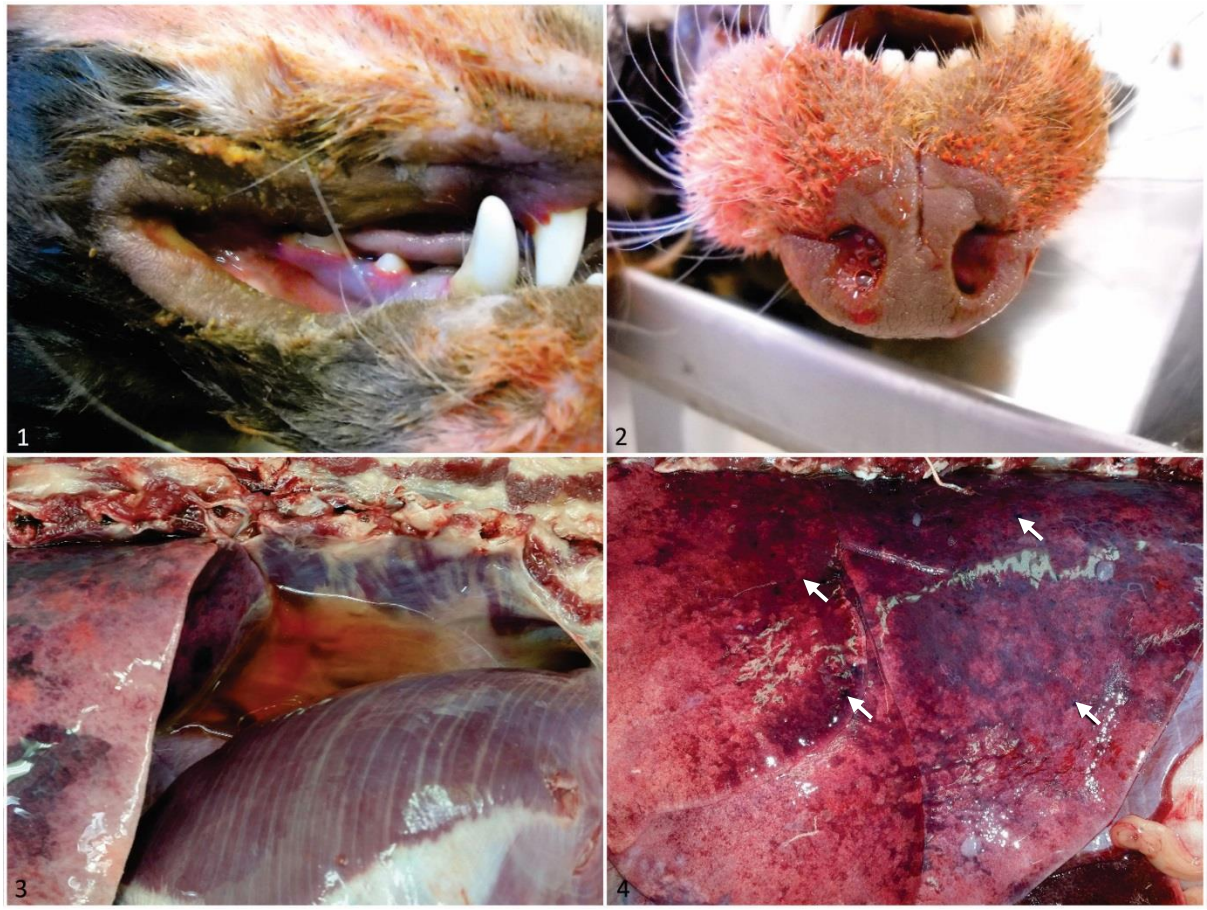

Figure 1: Cyanosis of oral mucous membranes.

Figure 2: Serous/frothy fluid oozing from nostril.

Figure 3: Severe protein-rich hydrothorax.

Figure 4: Severe interstitial pneumonia characterised by severe dorso-caudal hyperaemia and multifocal areas of pulmonary haemorrhage (*arrows*).

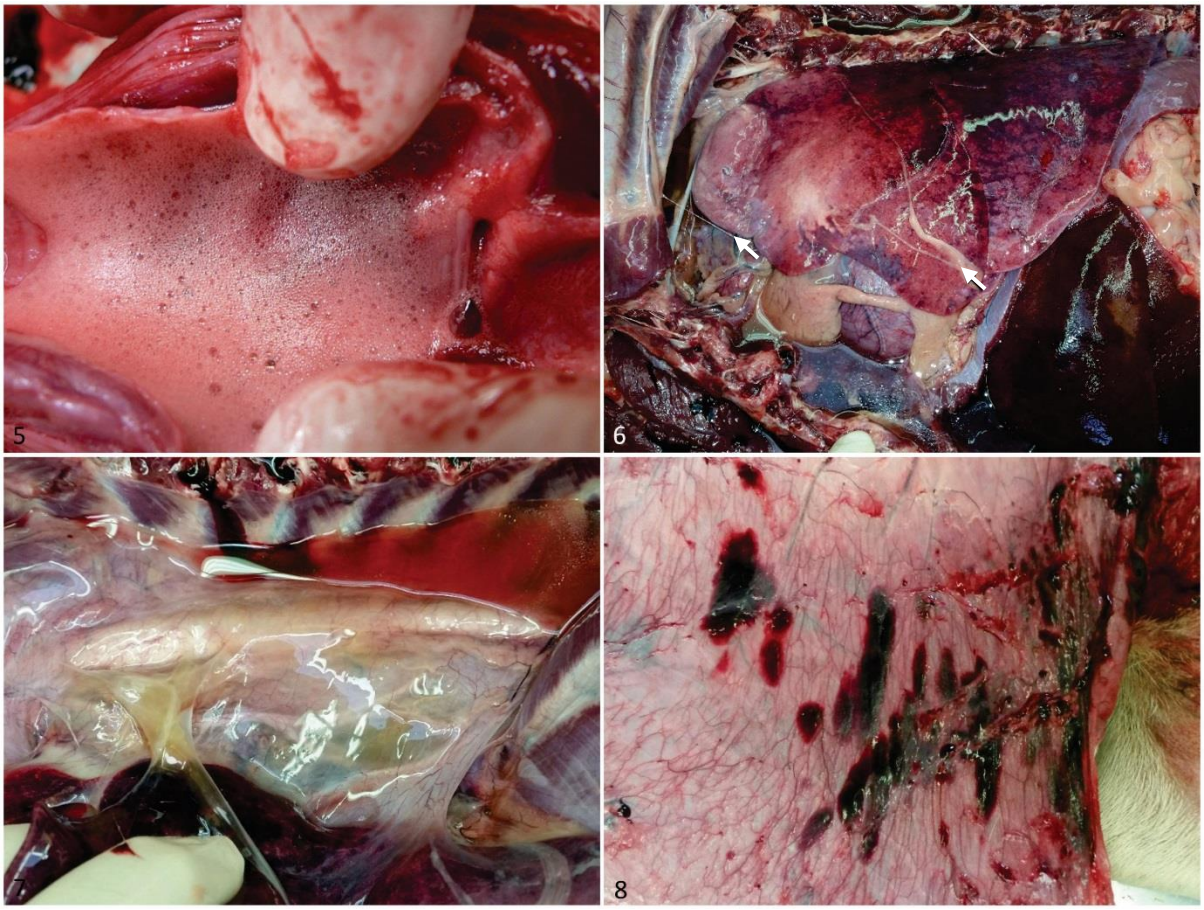

Figure 5: Severe pulmonary oedema indicated by foam in the trachea.

Figure 6: Loosely-attached fibrin strands (*arrows*) on pleural surface suggestive of fibrinous pleuritis.

Figure 7: Severe protein-rich mediastinal oedema.

Figure 8: Subcutaneous petechiation and ecchymosis.

4.2 Histopathology

Lung, heart, liver and spleen were the key organs most consistently received for examination from referring veterinarians that submitted the bulk of the cases and therefore the focus of the histological investigation of the study. The lung was submitted for histological examination in all cases. For the other tissues under investigation, 24 of the 29 (83%) cases had heart, 24 (83%) cases had liver and 22 (76%) cases had spleen available for histological examination.

4.2.1 Lung

By utilising the scoring sheet (APPENDIX 1) in conjunction with the grading criteria (APPENDIX 2) the following results were obtained.

All changes recorded were judged in comparison with the normal control lung (Figure 9 and 10). Both the control animals displayed mild congestion and mild alveolar oedema. In addition, mild perivascular and sub-pleural oedema was observed in the case of Control 2. The mild oedema and congestion in these cases were deemed to be secondary to circulatory failure as a result of pentobarbitone induced euthanasia. The anatomical location with its relevant changes are firstly described and thereafter summarised in a table format. All the relevant pathological changes were globally distributed throughout the associated anatomical regions examined in the lung in all 29 cases (Figure 11 and 12). Panvascular congestion was present in most cases ranging from mild to severe (Figure 13 and 14)..

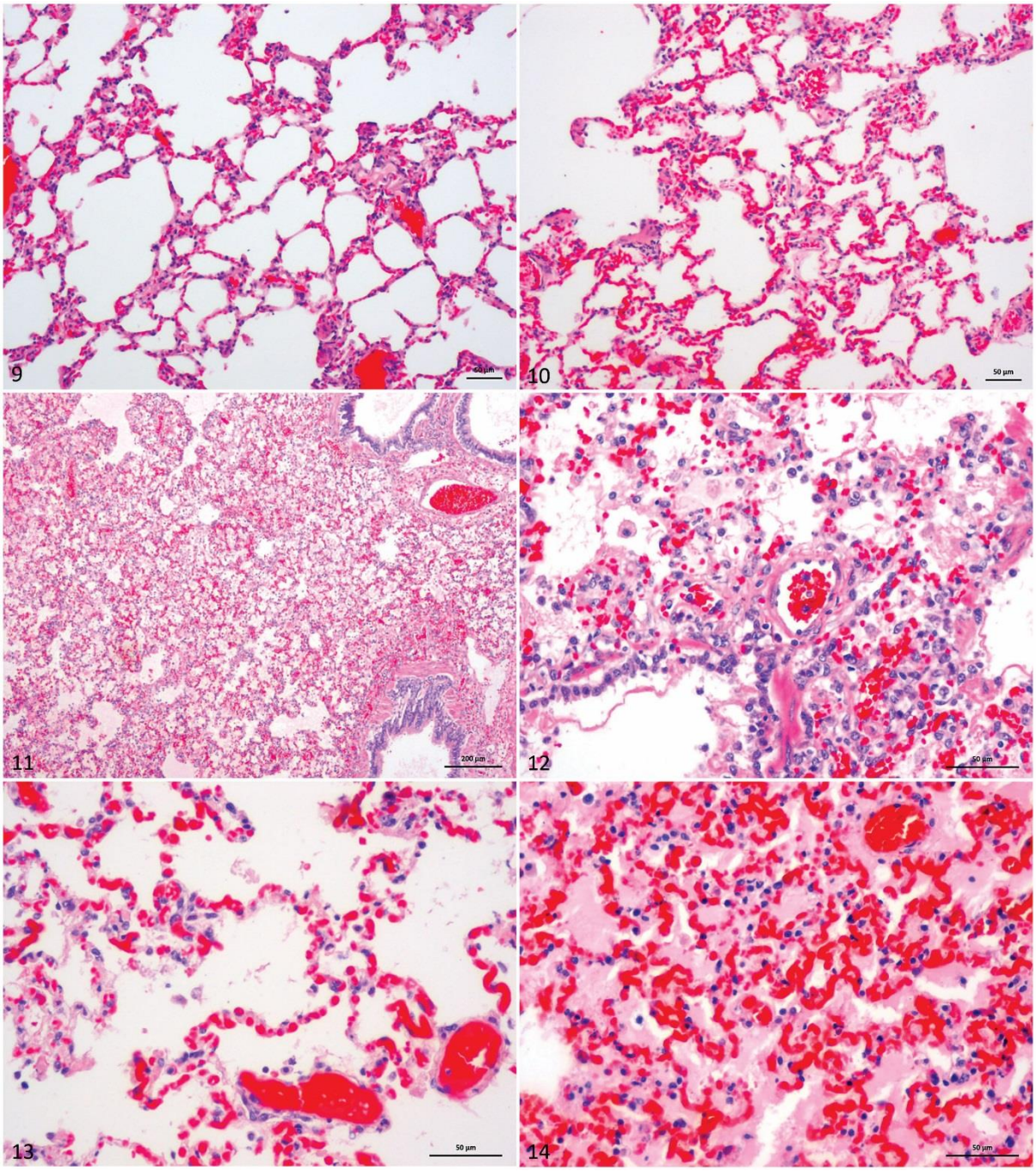

Figure 9: Normal lung histology of control dog 1 (HE stain, 100x magnification).

Figure 10: Normal lung histology of control dog 2 (HE stain, 100x magnification).

Figure 11: Diffuse distribution of lesion in the lung affecting all anatomical structures (HE stain, 40x magnification).

Figure 12: Diffuse distribution of lesion in the lung affecting all anatomical structures (HE stain, 200x magnification).

Figure 13: Mild congestion/hyperaemia of pulmonary vasculature (HE stain, 200x magnification).

Figure 14: Moderate-severe congestion/hyperaemia of pulmonary vasculature (HE stain, 200x magnification).

4.2.1.1 Alveolar wall

The changes observed in the alveolar walls are shown in Table 2.

Inflammation of the alveolar wall was present in all cases. The inflammatory cell infiltration was characterised by mild monocyte-macrophage infiltration in 59% of cases and moderate monocyte-macrophage infiltration in 41% of cases. Mild infiltration by lymphocytes was present in 97% of cases while one case (3%) displayed moderate lymphocytosis. Eighty-six percent of cases revealed mild infiltration by plasma cells and neutrophils was evident in the alveolar walls of 69% of cases (62% mild and 7% moderate) (Figure 15, 16 and 17).

Apoptosis (clusters of closely aggregated dark purple nuclear fragments) was clearly present in virtually all cases except for one. Forty-five percent of cases revealed mild apoptosis, 45% moderate apoptosis and 7% revealed severe apoptosis (Figure 18 and 19).

In all the cases hypertrophy of the microvascular (capillary) endothelial cells could be observed (Figure 20). In 45% the hypertrophy was mild, while in 48% it was moderate and in 7% the hypertrophy was severe.

Table 2: Summary of changes affecting the alveolar walls.

Alveolar wall	**(n-29)**	**Absent**	**Mild**	**Moderate**	**Severe**
Inflammatory cells					
Monocyte-macrophages		-	17 (59%)	12 (41%)	-
Lymphocytes		-	28 (97%)	1 (3%)	-
Plasma cells		4 (14%)	25 (86%)	-	-
Neutrophils		9 (31%)	18 (62%)	2 (7%)	-
Eosinophils		29 (100%)	-	-	-
Apoptosis/necrosis		1 (3%)	13 (45%)	13 (45%)	2 (7%)
Microvascular endothelial cell activation/hypertrophy		-	13 (45%)	14 (48%)	2 (7%)

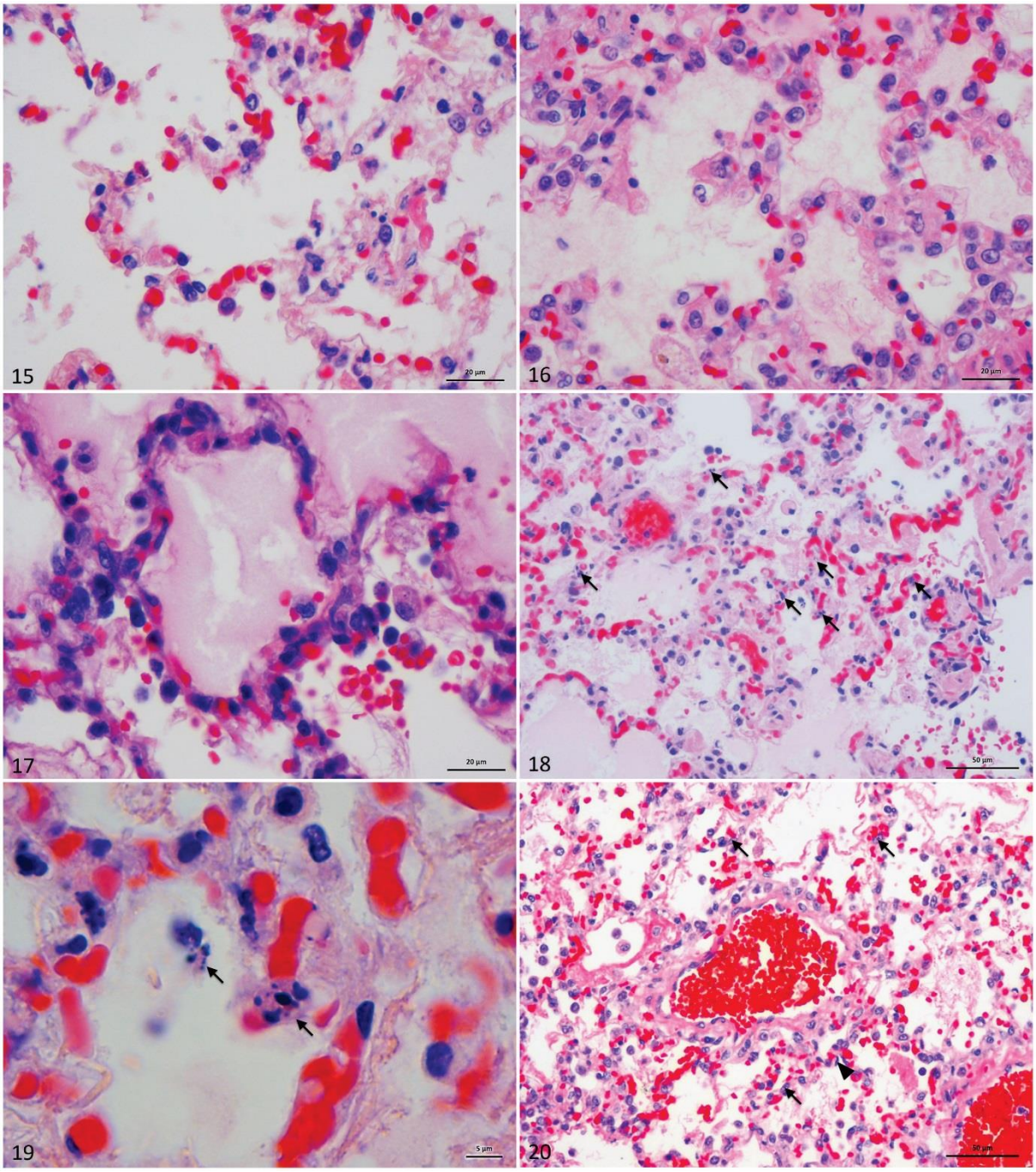

Figure 15: Mild inflammatory cell infiltration of alveolar walls (HE stain, 400x magnification).

Figure 16: Moderate inflammatory cell infiltration of alveolar walls (HE stain, 400x magnification).

Figure 17: Severe inflammatory cell infiltration of alveolar walls (HE stain, 400x magnification).

Figure 18: Marked apoptosis of alveolar vascular endothelial cells (*arrows*) (HE stain, 200x magnification).

Figure 19: Close-up of figure 18 illustrating apoptosis (*arrows*) (HE stain, 400x magnification).

Figure 20: Alveolar vascular endothelial cell hypertrophy (*arrows*) compared to normal endothelial cell (*arrow head*) (HE stain, 200x magnification).

4.2.1.2 Alveolar lumen

The changes observed in the alveolar lumens are shown in Table 3.

The alveolar lumens of all cases displayed some degree of inflammatory cell exudation which was mostly mild (Figure 21) to moderate except for one case (3%) where the presence of inflammatory cell exudation was severe (Figure 22). The inflammatory cells consisted mostly of mild to moderate numbers of macrophages while the lymphocyte, plasma cell and neutrophil exudation was generally mild.

Fibrin within the alveolar lumens, which could be clearly visualised with the help of MSB staining, could be observed in all cases. The accumulation was generally mild to moderate (86%), but in 14% the fibrin accumulation affected more than two thirds of the lung section and was considered severe (Figure 23 and 24). Most of the cases (62%) displayed only small numbers of extravasated erythrocytes (haemorrhage) and usually affected less than half of the lung surface examined (Figure 25). In 17% of cases small multifocal areas could be observed where the alveolar lumens were completely filled with extravasated erythrocytes (Figure 26) and in one case these areas coalesced to result in effacement of more than 50% of all the alveolar lumens in the lung section (Figure 27). Oedema (amorphous extravascular fluid accumulation) was present in all cases and was generally moderate to severe (76%) with a mostly moderate (59%) eosinophilia (Figure 28).

Table 3: Summary of changes affecting the alveolar lumens.

Alveolar lumen	(n-29)	Absent	Mild	Moderate	Severe
Inflammatory cells					
Macrophages		-	13 (45%)	15 (52%)	1 (3%)
Lymphocytes		2 (7%)	26 (90%)	1 (3%)	-
Plasma cells		5 (17%)	23 (79%)	1 (3%)	-
Neutrophils		8 (28%)	19 (66%)	2 (7%)	-
Eosinophils		29 (100%)	-	-	-
Fibrin		-	14 (48%)	11 (38%)	4 (14%)
Haemorrhage		5 (17%)	18 (62%)	5 (17%)	1 (3%)
Oedema		-	7 (24%)	11 (38%)	11 (38%)

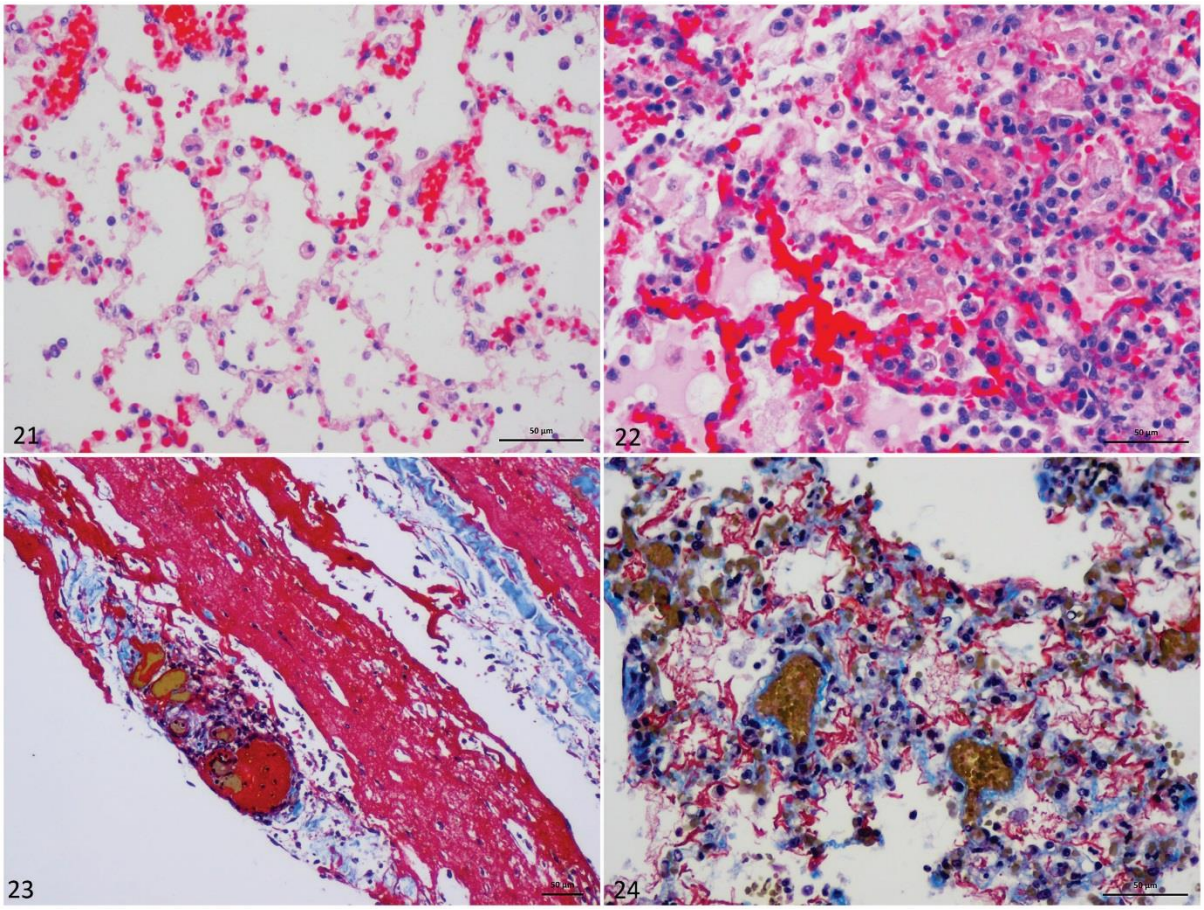

Figure 21: Mild inflammatory cell exudation into alveolar lumens (HE stain, 200x magnification).

Figure 22: Severe inflammatory cell exudation into alveolar lumens (HE stain, 200x magnification).

Figure 23: Martius scarlet blue (MSB) control, fibrin indicated by bright red staining (MSB stain, 100x magnification).

Figure 24: Moderate fibrin accumulation within the alveolar lumens indicated by red staining (MSB stain, 200x magnification).

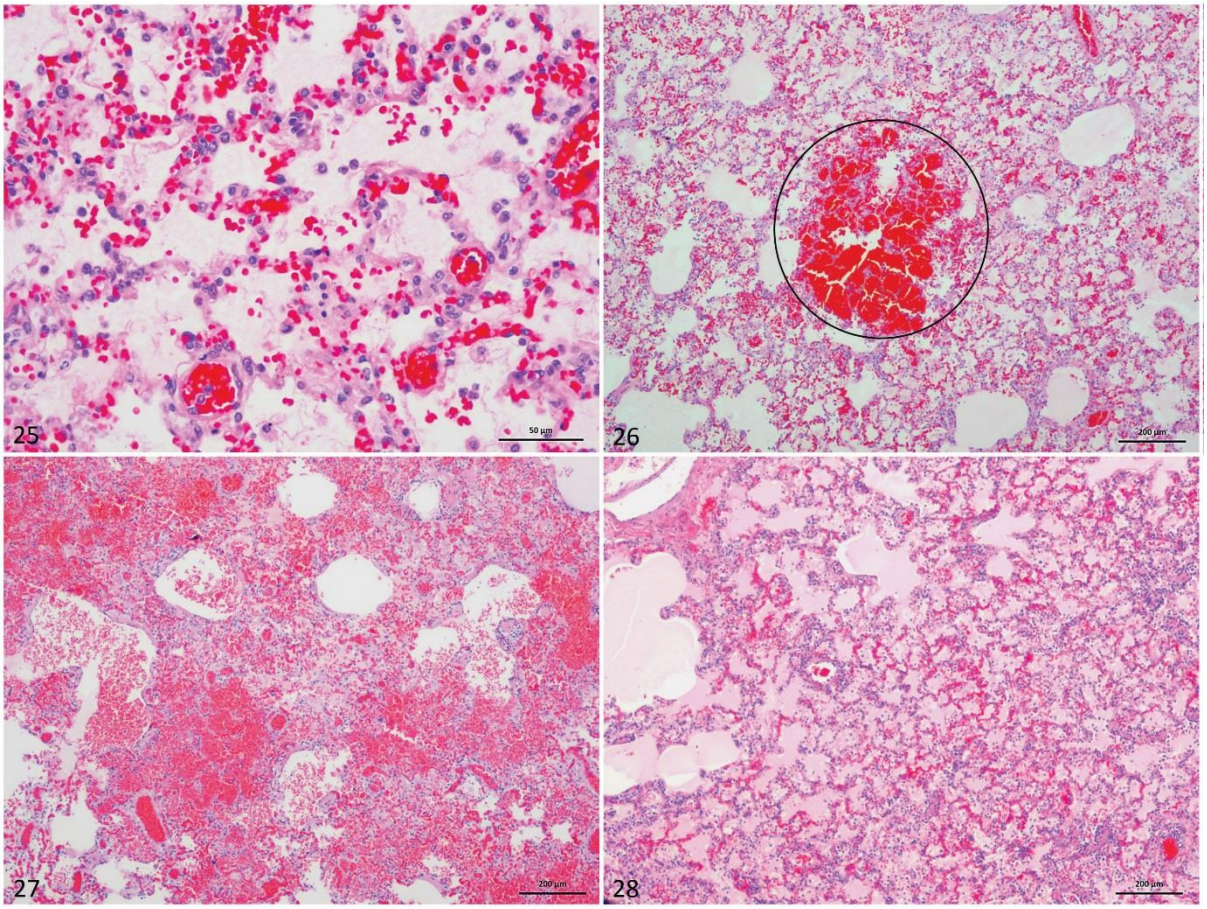

Figure 25: Mild intra-alveolar haemorrhage characterised by few extravasated erythrocytes within alveolar lumens (HE stain, 200x magnification).

Figure 26: Moderate intra-alveolar haemorrhage characterised by focal area (*circle*) where the alveolar lumens are completely filled with extravasated erythrocytes (HE stain, 40x magnification).

Figure 27: Severe intra-alveolar haemorrhage characterised by effacement of >50% of alveolar lumens by extravasated erythrocytes (HE stain, 40x magnification).

Figure 28: Severe oedema displaying moderate eosinophilia filling most of the alveolar lumens (HE stain, 40x magnification).

4.2.1.3 Perivascular interstitium

The changes observed in the perivascular interstitium are shown in Table 4.

In all cases the perivascular interstitium displayed some level of inflammatory cell infiltrate which was generally mild (Figure 29) except for 17% of cases that displayed moderate inflammation (Figure 30). The cell infiltrate was predominated by macrophages, with fewer lymphocytes, plasma cells and neutrophils.

Varying degrees of fibrin exudation could be observed within the perivascular interstitium with the majority of cases (69%) showing mild accumulation of fibrin between the perivascular connective tissue that was clearly expanded by oedema fluid (Figure 31). Twenty-four percent of the cases displayed moderate to severe fibrin accumulation (Figure 32). Haemorrhage was a consistent finding in 48% of cases with one case showing severe haemorrhage with marked expansion of the perivascular interstitium by extravasated erythrocytes (Figure 35). Most of the cases where haemorrhage was observed however showed only mild (Figure 33) to moderate (Figure 34) expansion of the perivascular interstitium by extravasated erythrocytes. All cases showed the presence of some degree of oedema characterised by variable degree of eosinophilia that resulted in expansion of the perivascular interstitium. The expansion of the interstitium ranged from mild in 34% of cases (Figure 36), moderate in 48% of cases (Figure 37) to severe in 17% of cases (Figure 38).

Mild to moderate lymphatic distention was observed in all cases. The lymphatic vessels were mostly distended by oedema fluid, but in a small number of cases drained erythrocytes, fibrin and leukocytes could be observed within the lymphatic lumens (Figure 39 and 40).

All the larger blood vessels displayed some degree of endothelial cell nuclear hypertrophy, of which the vast majority of the cases (97%) displayed mild to moderate nuclear hypertrophy and in one case (3%) the hypertrophy was classified to be severe (Figure 41 and 42).

Table 4: Summary of changes affecting the perivascular interstitium.

Perivascular interstitium	**(n-29)**	**Absent**	**Mild**	**Moderate**	**Severe**
Inflammatory cells					
Macrophages		-	24 (83%)	5 (17%)	-
Lymphocytes		3 (10%)	24 (83%)	2 (7%)	-
Plasma cells		8 (28%)	21 (72%)	-	-
Neutrophils		13 (45%)	16 (55%)	-	-
Eosinophils		29 (100%)	-	-	-
Fibrin		2 (7%)	20 (69%)	4 (14%)	3 (10%)
Haemorrhage		15 (52%)	7 (24%)	6 (21%)	1 (3%)
Oedema		-	10 (34%)	14 (48%)	5 (17%)
Lymphatic vessel distention		-	23 (79%)	6 (21%)	-
Venous/arteriolar endothelial cell activation/hypertrophy		-	23 (79%)	5 (17%)	1 (3%)

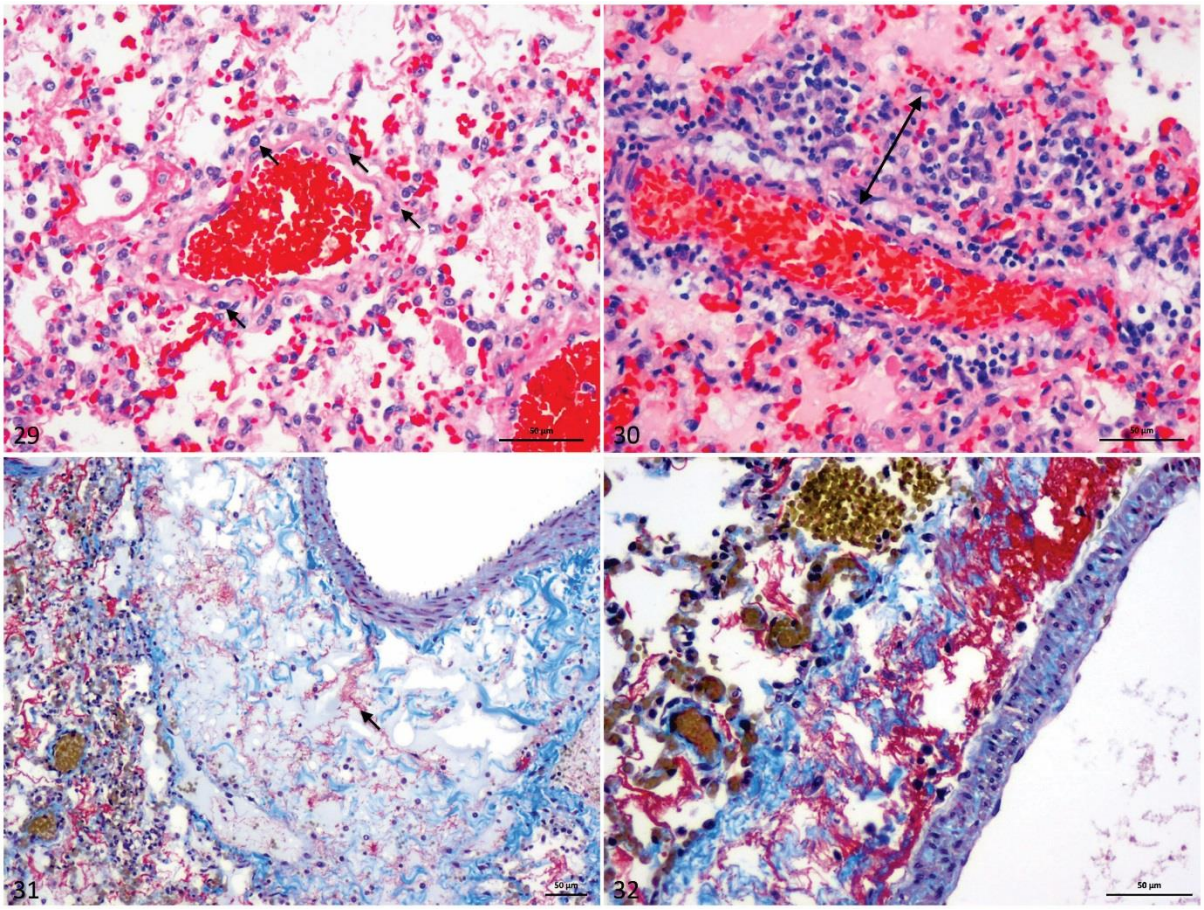

Figure 29: Mild perivascular inflammatory cell infiltrate characterised by only a few mononuclear leukocytes (*arrows*) (HE stain, 200x magnification).

Figure 30: Moderate-severe perivascular inflammatory cell infiltrate characterised by marked expansion (*double arrow*) (HE stain, 200x magnification).

Figure 31: Mild fibrin accumulation in perivascular space staining red with MSB (*arrow*) (MSB stain, 100x magnification).

Figure 32: Moderate-severe fibrin accumulation in perivascular space staining red with MSB (MSB stain, 100x magnification).

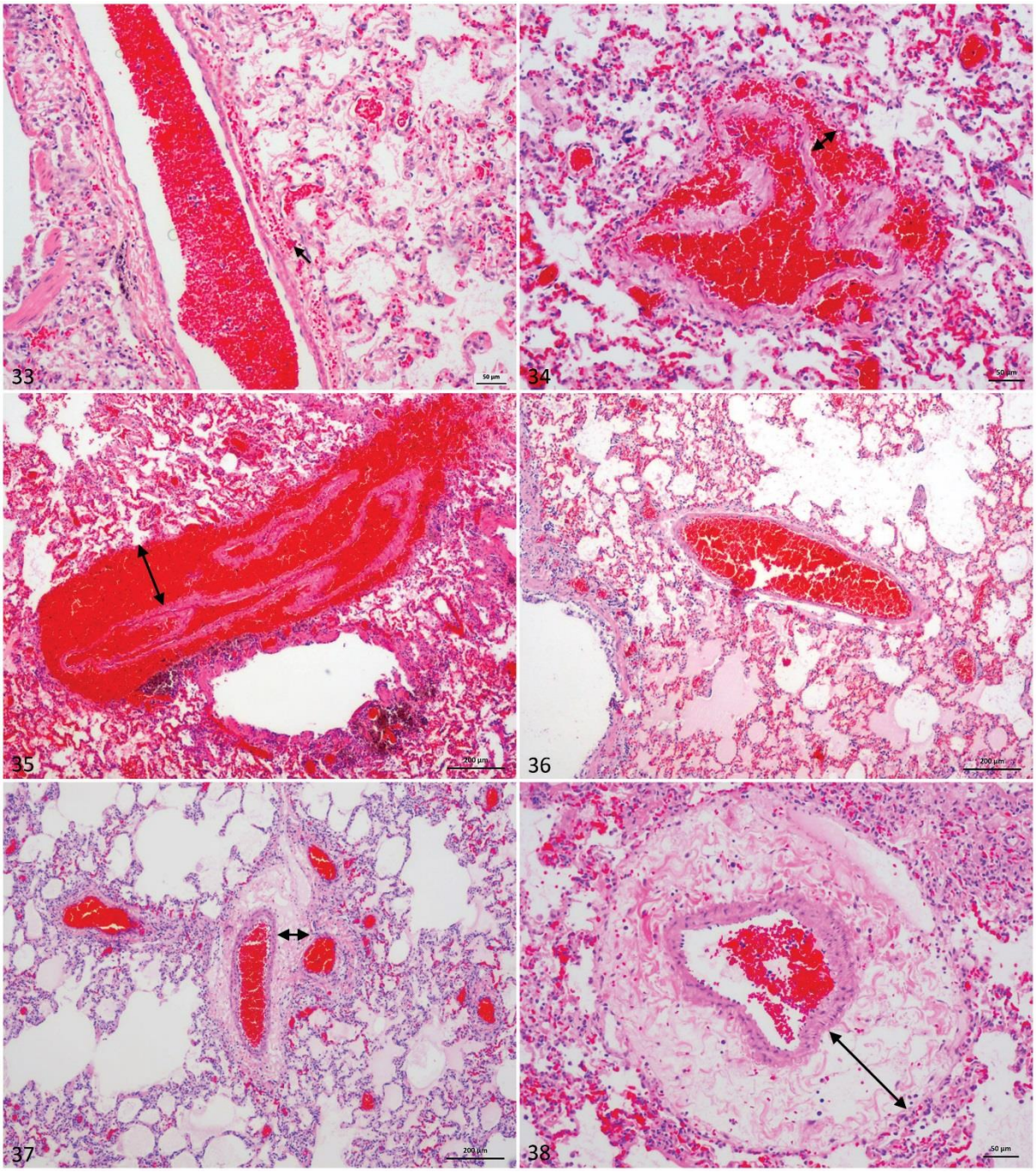

Figure 33: Mild perivascular haemorrhage (*arrow*) without expansion of the perivascular interstitium (HE stain, 100x magnification).

Figure 34: Moderate perivascular haemorrhage resulting in slight expansion of the perivascular interstitium (*double arrow*) (HE stain, 100x magnification).

Figure 35: Severe perivascular haemorrhage resulting in marked expansion of the perivascular interstitium (*double arrow*) (HE stain, 40x magnification).

Figure 36: Mild perivascular oedema resulting in slight expansion of the perivascular interstitium (HE stain, 40x magnification).

Figure 37: Moderate perivascular oedema resulting in moderate expansion of the perivascular interstitium (*double arrow*) (HE stain, 40x magnification).

Figure 38: Severe perivascular oedema resulting in marked expansion of the perivascular interstitium (*double arrow*) (HE stain, 100x magnification).

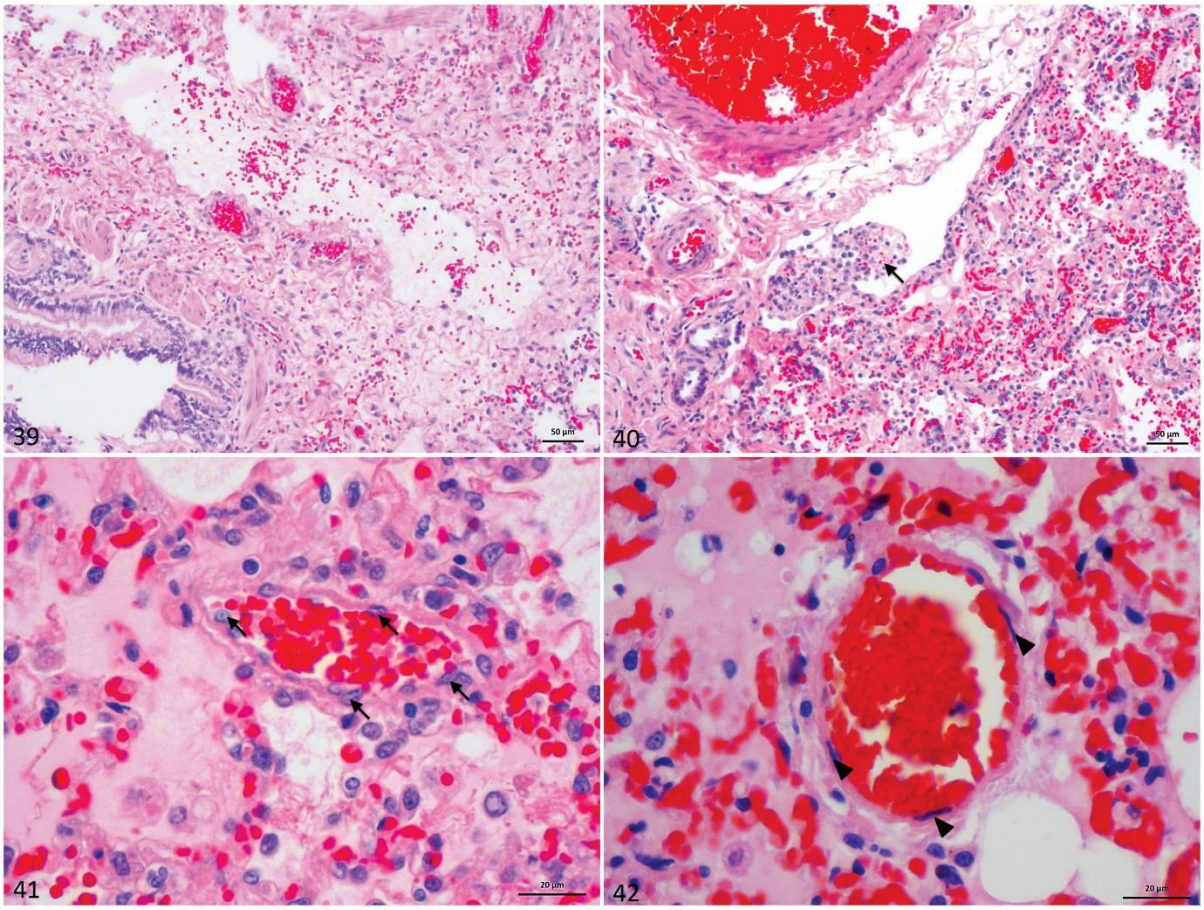

Figure 39: Lymphatic vessel moderately distended by oedema, fibrin and erythrocytes (HE stain, 100x magnification).

Figure 40: Lymphatic vessel moderately distended by leukocytes (*arrow*) and oedema (HE stain, 100x magnification).

Figure 41: Endothelial cell hypertrophy (*arrows*), compare with figure 42 for normal endothelial cells (HE stain, 400x magnification).

Figure 42: Normal endothelial cells appear flattened (*arrow heads*), compare with figure 41 (HE stain, 400x magnification).

4.2.1.4 Sub-pleural interstitium

The changes observed in the sub-pleural interstitium are shown in Table 5.

In 76% of cases the sub-pleural interstitium displayed inflammatory cell infiltration. This reaction was mild in all cases and consisted mainly of macrophages with fewer lymphocytes, plasma cells and neutrophils (Figure 43).

The presence of mild fibrin accumulation in the sub-pleural interstitium was observed in 45% of cases. Haemorrhage was not a consistent finding, affecting only 31% of cases. When present it manifested only multifocally, and in some cases coalescing (Figure 44). Oedema was present in all cases and were mild in 72% of cases (Figure 45) with a mild to moderate degree of eosinophilia. Twenty-one percent of cases displayed moderated oedema (Figure 46) in this location and two cases (7%) displayed severe oedema (Figure 47) with marked expansion of the interstitial connective tissue.

Pleural mesothelial cells displayed some degree of hypertrophy in 76% of cases (Figure 48).

Table 5: Summary of changes affecting the sub-pleural interstitium.

Sub-pleural interstitium	(n-29)	Absent	Mild	Moderate	Severe
Inflammatory cells					
Macrophages		7 (24%)	22 (76%)	-	-
Lymphocytes		20 (69%)	9 (31%)	-	-
Plasma cells		23 (79%)	6 (21%)	-	-
Neutrophils		25 (86%)	4 (14%)	-	-
Eosinophils		29 (100%)	-	-	-
Fibrin		16 (55%)	13 (45%)	-	-
Haemorrhage		20 (69%)	6 (21%)	3 (10%)	-
Oedema		-	21 (72%)	6 (21%)	2 (7%)

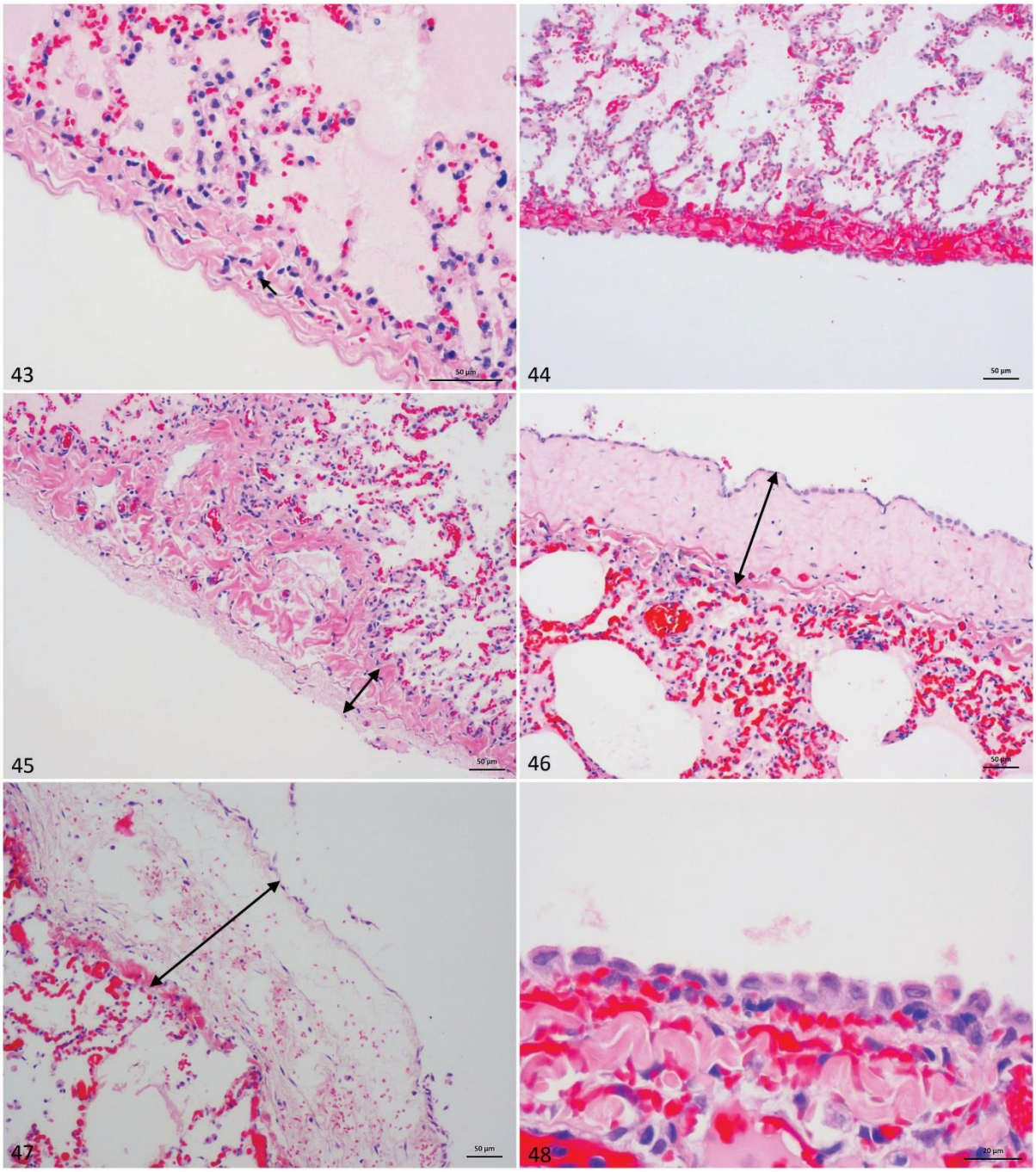

Figure 43: Mild inflammatory cell infiltration in the sub-pleural interstitium characterised by a few mononuclear leukocytes (*arrow*) (HE stain, 200x magnification).

Figure 44: Haemorrhage of the sub-pleural interstitium (HE stain, 100x magnification).

Figure 45: Mild oedema of the sub-pleural interstitium resulting in slight expansion (*double arrow*) (HE stain, 100x magnification).

Figure 46: Moderate oedema of the sub-pleural interstitium resulting in moderate expansion (*double arrow*) (HE stain, 100x magnification).

Figure 47: Severe oedema of the sub-pleural interstitium resulting in marked expansion (*double arrow*) (HE stain, 100x magnification).

Figure 48: Mesothelial hypertrophy/activation characterised by a cuboidal appearance (HE stain, 400x magnification).

4.2.1.5 Peribronchiolar interstitium

The changes observed in the peribronchiolar interstitium are shown in Table 6.

A low degree of inflammatory cell infiltrate of the peribronchiolar interstitium was a consistent finding in 90% of the cases. The majority of inflammatory cells consisted of macrophages and lymphocytes with fewer plasma cells and neutrophils (Figure 49).

The accumulation of fibrin in the peribronchiolar interstitium was only observed in 34% of cases and was mild in all instances. Haemorrhage was only observed in 21% of cases and except for one case (3%) that displayed moderate numbers of extravasated erythrocytes. The degree of haemorrhage was usually only mild (17%) and did not contribute significantly to expansion of the peribronchiolar interstitium (Figure 50). Seventy-nine percent of cases showed a mild degree of oedema, of low eosinophilic staining intensity (Figure 51).

In 86% of cases anthracosis (black pigmented carbon particles within macrophages in the peri-bronchiolar areas) was observed and in 12% of these cases hyperplastic bronchiolar-associated lymphoid tissue (BALT) could be associated with the accumulation of carbon particles in the peribronchiolar interstitium (Figure 52).

Table 6: Summary of changes affecting the peribronchiolar interstitium.

Peribronchiolar interstitium	(n-29)	Absent	Mild	Moderate	Severe
Inflammatory cells					
Macrophages		3 (10%)	22 (76%)	4 (14%)	-
Lymphocytes		7 (24%)	21 (72%)	1 (3%)	-
Plasma cells		15 (52%)	14 (48%)	-	-
Neutrophils		19 (66%)	10 (34%)	-	-
Eosinophils		29 (100%)	-	-	-
Fibrin		19 (66%)	10 (34%)	-	-
Haemorrhage		23 (79%)	5 (17%)	1 (3%)	-
Oedema		6 (21%)	22 (76%)	1 (3%)	-

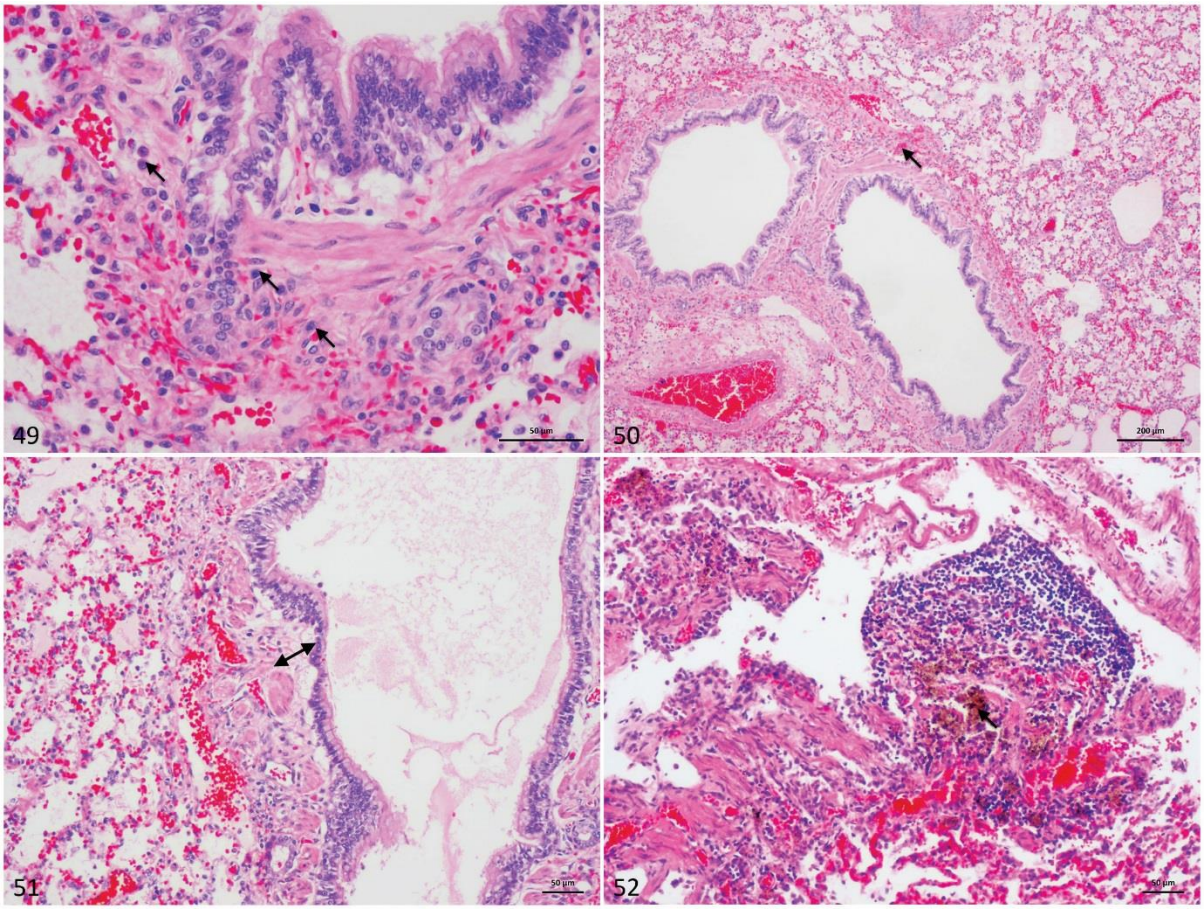

Figure 49: Mild inflammatory cell infiltration of the peri-bronchiolar interstitium characterised by scattered mononuclear leukocytes (*arrows*) (HE stain, 200x magnification).

Figure 50: Mild peri-bronchiolar haemorrhage (*arrow*) (HE stain, 40x magnification).

Figure 51: Mild peri-bronchiolar oedema (*double arrow*) (HE stain, 100x magnification).

Figure 52: Mild bronciolar-associated lymphoid tissue (BALT) hyperplasia associated with anthracosis (*arrow*) (HE stain, 100x magnification).

4.2.1.6 Bronchiolar lumen

The changes observed in the bronchiolar lumens are shown in Table 7.

The bronchiolar lumen of only 21% of cases displayed some degree of inflammatory cell exudate. In all cases the inflammation was mild and consisted of macrophages in 21%, lymphocytes in 14%, plasma cells in 10% and neutrophils in 3% of cases (Figure 53).

The bronchiolar lumens of 48% of cases contained fibrin. In 41% of cases less than a third of the lumens were affected, while in 7% a third to two-thirds were affected (Figure 53 and 54). In 31% of cases small numbers of extravasated erythrocytes were present, affecting <50% of the bronchiolar lumens in the examined sections (Figure 55). Oedematous fluid exudation into the bronchiolar lumens was a more consistent finding affecting the majority (90%) of bronchiolar lumens with mild oedema in 55%, moderate oedema in 25% and severe oedema in 7% of cases. Significant variation in eosinophilic staining intensity was noted with the degree of eosinophilia classified as mild in 28%, moderate in 55% and severe in 7% of cases (Figure 56).

Table 7: Summary of changes affecting the bronchiolar lumen.

Bronchiolar lumen	**(n-29)**	**Absent**	**Mild**	**Moderate**	**Severe**
Inflammatory cells					
Macrophages		23 (79%)	6 (21%)	-	-
Lymphocytes		25 (86%)	4 (14%)	-	-
Plasma cells		26 (90%)	3 (10%)	-	-
Neutrophils		28 (97%)	1 (3%)	-	-
Eosinophils		29 (100%)	-	-	-
Fibrin		15 (52%)	12 (41%)	2 (7%)	-
Haemorrhage		20 (69%)	9 (31%)	-	-
Oedema		3 (10%)	16 (55%)	8 (28%)	2 (7%)

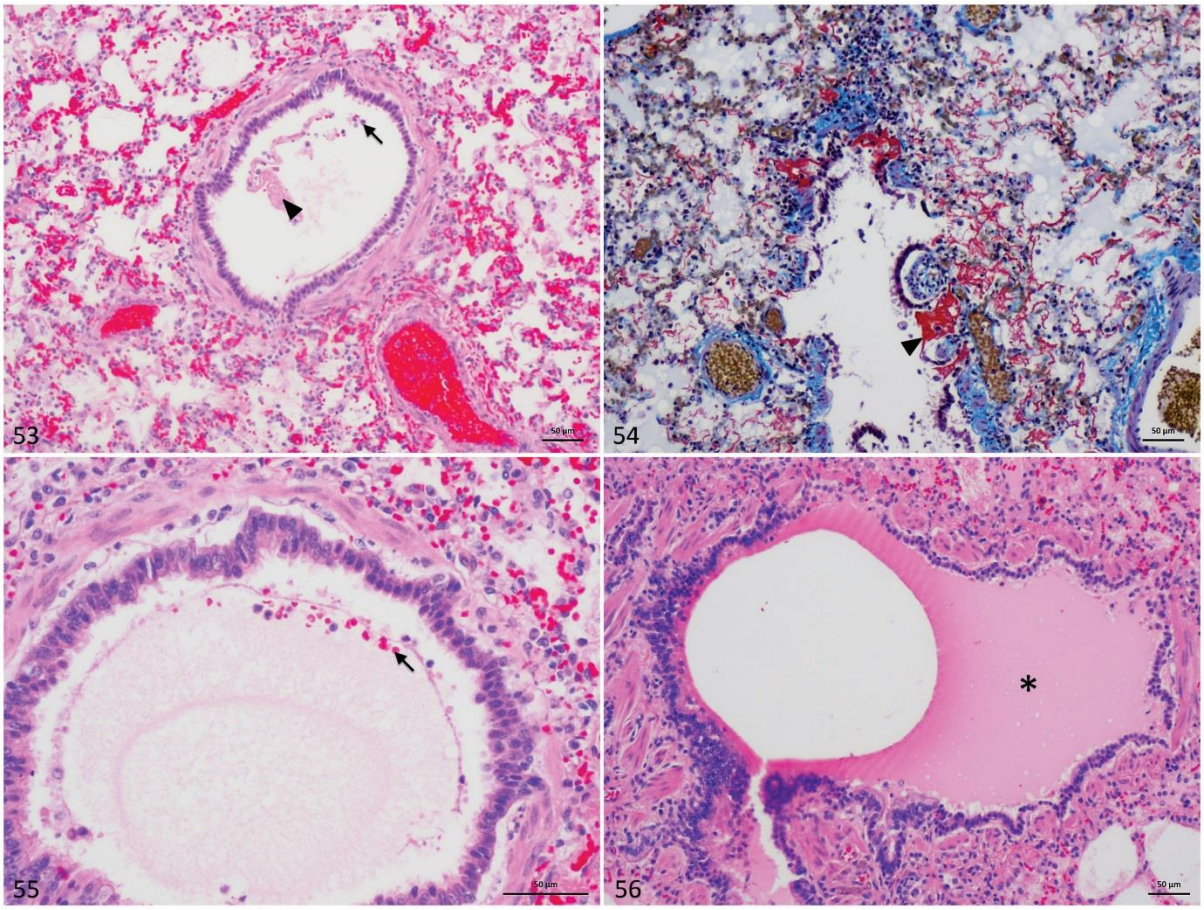

Figure 53: Mild exudation of fibrin (*arrow head*) and mononuclear leukocytes (*arrow*) into bronchiolar lumen (HE stain, 100x magnification).

Figure 54: Mild exudation of fibrin (*arrow head*) into bronchiolar lumen (MSB stain, 100x magnification).

Figure 55: Small numbers of extravasated erythrocytes in bronchiolar lumen (HE stain, 200x magnification).

Figure 56: Oedema in bronchiolar lumen displaying marked eosinophilia (*asterisk*) (HE stain, 100x magnification).

4.2.2 Heart

The changes observed in the heart is shown in Table 8.

The most consistent histological finding in the heart was mild to moderate interstitial oedema that affected 75% of cases. In 21% of cases mild haemorrhage, mostly in the sub-endocardial location, was observed; while 17% displayed mild generalised congestion. In three cases (13%) focal areas of mild mononuclear (macrophages and lymphocytes) inflammatory cell infiltration could be observed. In two of these cases these foci were located sub-endocardially, while in one case it was situated in the myocardial interstitium associated with a focal area of fibrosis.

Table 8: Summary of changes affecting the heart.

Change	(n-24)	Number of cases (%)
Interstitial oedema		18 (75%)
Haemorrhage		5 (21%)
Congestion		4 (17%)
Inflammatory cells		3 (13%)
No change		2 (8%)

4.2.3 Liver

The changes observed in the liver is shown in Table 9.

In all but two cases varying degrees of sinusoidal congestion could be observed. The congestion ranged from mild in 20%, moderate in 38% and severe in 42% of cases. Interstitial oedema could be observed in the periportal connective tissue of 21% of cases and was usually mild. Periacinar hepatocellular hydropic swelling was present in two cases (8%). One case (4%) revealed moderate hepatocellular necrosis affecting the periacinar and midzonal areas as well as moderate centrilobular fibrosis.

Table 9: Summary of changes affecting the liver.

Change	(n-24)	Number of cases (%)
Congestion		22 (92%)
Interstitial oedema		5 (21%)
Hepatocellular hydropic swelling		2 (8%)
Hepatocellular necrosis		1 (4%)
Centrilobular fibrosis		1 (4%)

4.2.4 Spleen

The changes observed in the spleen is shown in Table 10.

The most consistent finding observed in the spleen was mild to moderate congestion (expansion of the red pulp by accumulating erythrocytes) affecting 68% of cases. Mild to moderate follicular lymphoid hyperplasia was observed in 27% of cases while 9% of cases revealed follicular lymphoid depletion. Eighteen percent of spleens displayed moderate to severe contraction (depletion of erythrocytes from the red pulp). In 14% of spleens mild red pulp hyperplasia (characterised by monocytic infiltration) could be observed. One spleen (5%) displayed moderate haemosiderin accumulation within the red pulp.

Table 10: Summary of changes affecting the spleen.

Change	(n-22)	Number of cases (%)
Congestion		15 (68%)
Contracted		4 (18%)
Lymphoid hyperplasia		6 (27%)
Lymphoid depletion		2 (9%)
Red pulp hyperplasia		3 (14%)
Haemosiderin accumulation		1 (5%)
No change		1 (5%)

4.3 Immunohistochemistry

4.3.1 AHSV-specific NS4 protein

African horsesickness virus-specific NS4 IHC labelling was applied to 17 of the 29 cases due to a lack of tissue and/or anti-NS4 serum availability for additional sections from the remaining 12 cases. Positive labelling was clearly observed in the nuclei and cytoplasm of endothelial cells in all cases (Figure 58). In 12% of cases, positive labelling (also nuclear and cytoplasmic) was observed in monocytes/macrophages and in one case (6%) within fibroblasts. The nature of the nuclear labelling was generally diffuse, while that in the cytoplasm was finely granular. The degree of labelling in the lung tissue was determined by inspecting 10 high power fields (HPF – 400x magnification) and were found to vary from 1+ (≤1 clearly labelling cell per HPF) in 47% of cases, 2+ (2-5 clearly labelling cells per HPF) in 35% of cases and 3+ (≥ 6 clearly labelling cells per HPF) in 18% of cases. Neither of the control cases revealed any positive labelling for the anti-NS4 serum.

4.3.2 AHSV-specific VP7 protein

African horsesickness virus-specific VP7 IHC labelling was applied to all 29 cases. Characteristic positive labelling was clearly observed in the cytoplasm of endothelial cells in all cases (Figure 60). Monocytes/macrophages showed positive labelling in 62% of cases with one case (3%) exhibiting positive labelling in plasma cells. The nature of the labelling was small bead-like in all cases and in 17% of the cases fine granular staining could also be observed. The degree of labelling varied from 1+ in 52% of cases, 2+ in 28% of cases and 3+ in 21% of cases. The two control cases did not reveal any positive labelling.

4.3.3 BTV-4-specific VP7 protein

No BTV-4-specific IHC labelling could be observed in any of the cases including the two healthy controls (Figure 62).

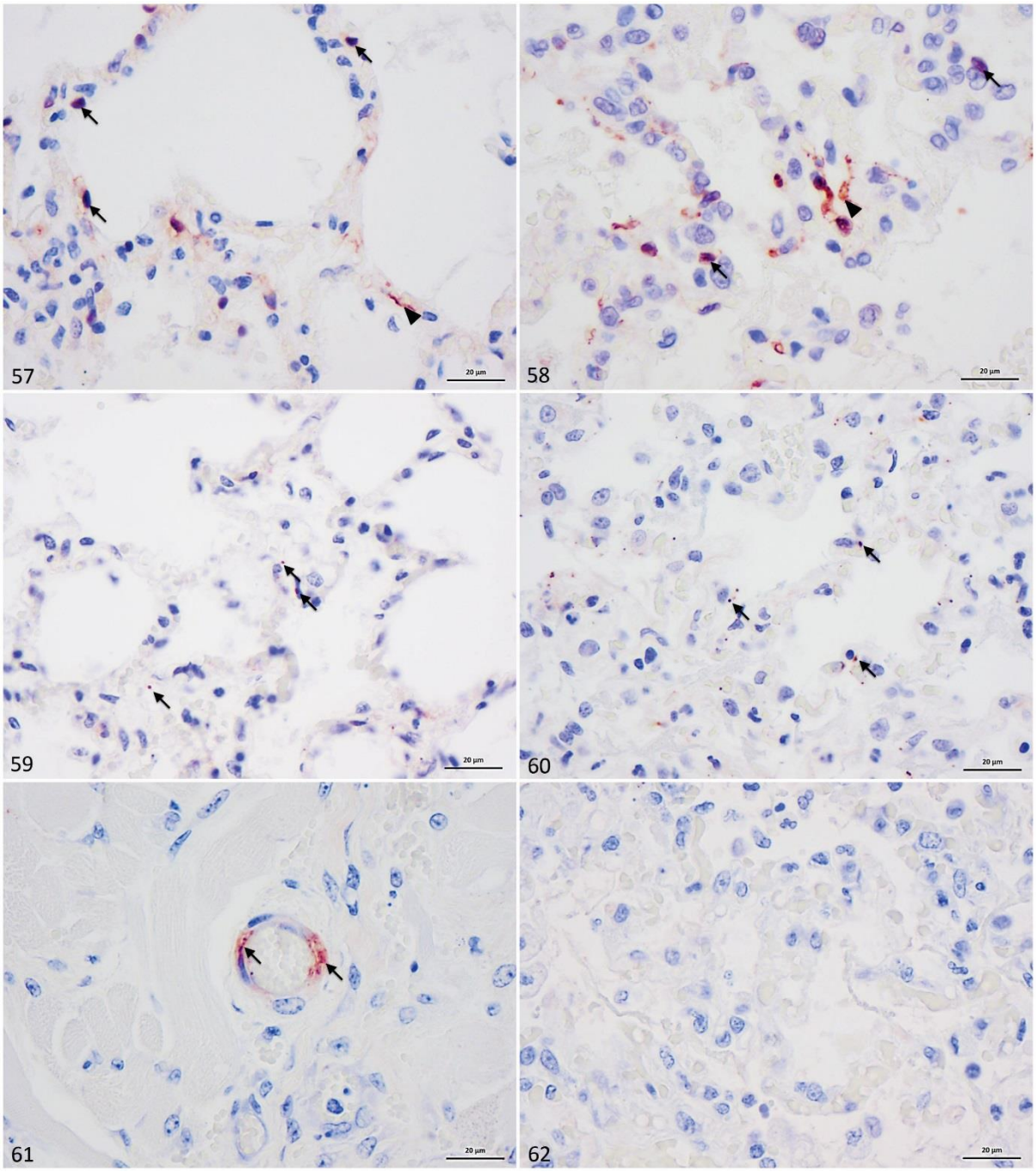

Figure 57: Positive control horse lung. Diffuse nuclear (*arrows*) and fine granular cytoplasmic (*arrow head*) positive immunohistochemical labelling (Anti-NS4 serum and NovaRED method, Mayer's haematoxylin counterstain, 400x magnification).

Figure 58: Positive dog lung. Diffuse nuclear (*arrows*) and fine granular cytoplasmic (*arrow head*) positive immunohistochemical labelling consistent with that of the positive control (see figure 57) (Anti-NS4 serum and NovaRED method, Mayer's haematoxylin counterstain, 400x magnification).

Figure 59: Positive control horse lung. Bead- and small granular cytoplasmic (*arrows*) positive immunohistochemical labelling (Anti-VP7 serum and NovaRED method, Mayer's haematoxylin counterstain, 400x magnification).

Figure 60: Positive dog lung. Bead- and fine granular cytoplasmic (*arrows*) positive immunohistochemical labelling consistent with that of the positive control (see figure 59) (Anti-NS4 serum and NovaRED method, Mayer's haematoxylin counterstain, 400x magnification).

Figure 61: Positive control sheep tongue. Fine- to coarse cytoplasmic granules in microvascular endothelial cells (*arrows*) (Anti-BTV-4 serum and NovaRED method, Mayer's haematoxylin counterstain, 400x magnification).

Figure 62: Negative dog lung. No positive immunohistochemical labelling present (Anti-BTV-4 serum and NovaRED method, Mayer's haematoxylin counterstain, 400x magnification).

4.4 Electron microscopy

Transmission electron microscopy was performed on the lung tissue of 4 of the 29 cases. Various changes could be observed affecting all of the four cases to some extent. Changes observed included severe congestion of alveolar capillaries (Figure 63), haemorrhage into alveolar lumens (Figure 64) and the presence of activated alveolar macrophages (Figure 65) and fibrin in the form of fibrillar fibres within alveolar lumens (Figure 65 and 66). The capillary basement membranes were thickened and displayed splitting in areas (Figure 67 and 70). Microthromi that consisted of conglomerates of platelets within a fibrin network (Figure 68 and 69) could be observed in areas where the basement membrane was denuded (Figure 70).

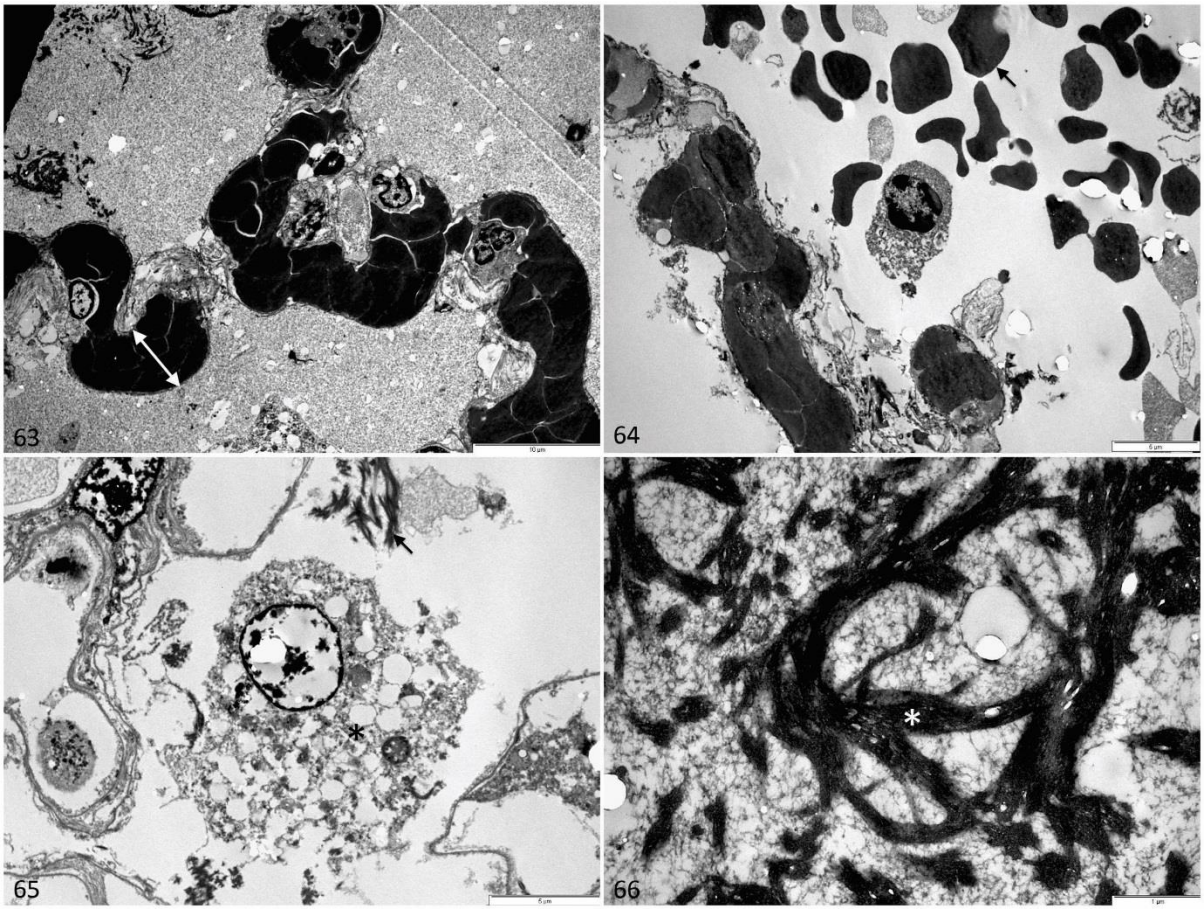

Figure 63: Severe congestion of alveolar capillaries, note distention by erythrocytes (*double arrow*).

Figure 64: Haemorrhage into alveolar lumens, note extravasated erythrocytes (*arrow*).

Figure 65: Presence of activated alveolar macrophage (*asterisk*) and fibrin (*arrow*).

Figure 66: Fibrilar fibres typical for fibrin (*asterisk*) in alveolar lumen.

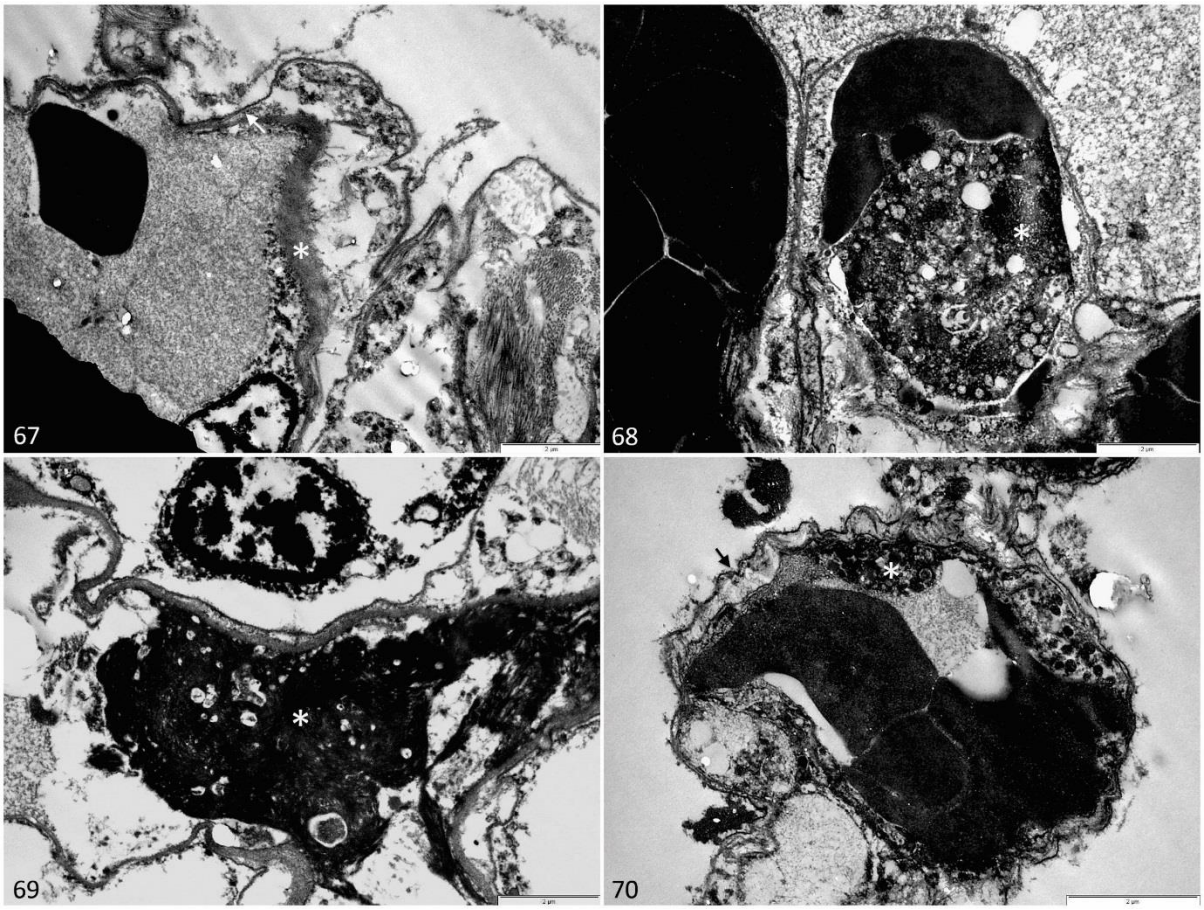

Figure 67: Thickened basement membrane (*asterisk*) with splitting (*arrow*).

Figure 68: Conglomerate of platelets (*asterisk*) within a capillary lumen.

Figure 69: Fibrin network (*asterisk*) typical for a microthrombus within a capillary lumen.

Figure 70: Platelet (*asterisk*) attached to a denuded basement membrane that also display splitting (*arrow*).

4.5 Molecular testing

The RT-qPCR results are shown in Table 11.

The 12 cases on which PCR was performed, all tested positive for AHS virus. The serotyping or typing results were available for 10 of the 12 cases of which all but one (serotype 2) were identified as serotype 6. The CT (cycle threshold)-values for eight of the cases submitted for PCR were determined and consistently ranged between 20.0 and 31.7, with an average value of 24.5. In all cases the CT-values for the spleen were consistently lower (average 22.3) than that of the lung (average 25.1), and the lung CT-values were consistently lower than that of the liver (average 26.8).

Table 11: Summary of RT-qPCR results.

Case number	PCR result	Lung CT-value	Spleen CT-value	Liver CT-value	Serotype/type
1	+	-	-	-	6
8	+	-	-	-	6
10	+	-	-	-	-
11	+	-	-	-	6
21	+	24.9	22.6	24.4	6
22	+	23.8	22.2	25.4	6
23	+	25.0	24.4	27.5	6
24	+	30.7	20.0	-	6
25	+	22.5	22.2	-	6
27	+	24.6	22.2	26.7	2
28	+	24.4	22.6	25.0	-
29	+	24.8	22.1	31.7	6
Total average		25.1	22.3	26.8	

CHAPTER 5

DISCUSSION

In all the cases described in this study, exposure to AHSV through ingestion of horse meat could be excluded. Epidemiologically, this is extremely noteworthy, since according to all the literature preceding this study, except for one case reported by Van Sittert *et al.* in 2013, dogs can only contract the disease by means of experimental infection or the ingestion of infected horse meat.[70] This apparent misconception was supported by studies indicating that the arthropod vectors associated with AHSV transmission (*Culicoides* spp. midges) did not prefer dogs as hosts .[12,43] Recently however, a number of studies have shown that any type of mammal in the vicinity of the midges may serve as hosts, and this include domestic dogs.[6,39,63] The clinical and critical epidemiological findings supporting vector borne transmission of AHS to dogs were described a recent publication that included the cases used for this study. [51]

From the 29 cases included in this study, complete post mortems were performed by the authors in only 14 cases. The other 15 cases comprised tissues submitted in formalin by private practitioners for diagnostic histopathology.

On thoracic dissection, the lungs showed severe oedema (foam in trachea) in association with variable, diffuse to caudo-dorsal congestion and increased parenchymal consistency that supported the presence of interstitial pneumonia. This finding predicted suboptimal oxygen transfer across the alveolar blood-air barrier resulting in systemic hypoxia and metabolic acidosis, hence the presence of mucous membrane cyanosis observed at necropsy. On the surface of the lungs fibrinous exudation was commonly observed and in addition to protein-

rich oedema observed in the pleural space and mediastinum suggested an increased vascular permeability mechanism with a significant inflammatory component. Haemorrhage was present within the lung parenchyma and in some cases subcutaneous petechiation was observed. These changes are indicative of significant vascular injury, which is the hallmark of Orbiviral infections.[31] Significant visceral congestion especially affecting the liver and to a lesser extent the spleen was observed in most cases. This finding suggests that the terminal cause of death in these animals was most probably acute cardiac failure. The pathogenesis of the cardiac failure is suspected to be two-fold in these cases (see below).

In the experience of the author, the combination of thoracic and pulmonary lesions described may be regarded as very specific for AHSV-infection in dogs and does not resemble any other canine disease at a macroscopic level.[51]

For most of the cases submitted in formalin by private practitioners, lung was only sampled from a single location. Therefore, representative examination of the total lung volume was limited to the cases where a full range of regions were sampled. In these cases, the changes were relatively consistent in all lung sections regardless of the location, thereby confirming the diffuse nature of the lesion. Considering this, even when a single lung sample is received for histological examination in these cases, the histological changes may be deemed representative of the entire lung.

Both control cases displayed very mild protein-poor oedema and congestion, but this was regarded to have increased hydrostatic pressure as underlying mechanism following pentobarbitone-induced circulatory failure with euthanasia.[55]

The overall histological changes in the lung were consistent in most cases and only differed slightly in the degree of inflammation and haemorrhage. Protein-rich oedema (indicated by

the eosinophilia of the oedema fluid)[75] and fibrin exudation was a consistent finding in all cases supporting the pathogenesis of vascular endothelial injury.[31]

The location within the lung on which most of the inflammation was centred was consistently found to be the alveolar walls and their associated alveolar lumens. This finding in association with the diffuse distribution of the lesions throughout the alveolar interstitium indicates that the injurious insult is haematogenous in origin.[75] This is also the area most important for the exchange of oxygen and therefore the severity of the clinical picture.

The alveolar walls were characterised by mild to moderate inflammatory cell infiltration, predominated by monocyte-macrophages, lymphocytes and neutrophils with fewer plasma cells. Mild to moderate apoptosis/necrosis appeared to affect endothelial cells as well as some mononuclear leukocytes. In the absence of specific immuno-labeling of the respective cells it was not possible accurately determine the cell type; however, considering the pathogenesis of AHS as a disease and the described tropism of the virus, the apoptotic cells were most likely endothelial cells and mononuclear leukocytes. Difficulty in determining intra- or extravascular location of monocytes in the alveolar wall location necessitated the use of the term "monocyte-macrophages" for the identification of the large mononuclear leukocytic inflammatory cell component. Mild to moderate microvascular endothelial cell hypertrophy was observed in all cases. This supports the endothelial alterations observed by Gómez-Villamandos *et al.* (1999) associated with AHSV infection in horses.[26]

The alveolar lumen was characterised by mild to moderate inflammatory cell infiltration, consisting of mostly macrophages with fewer lymphocytes, plasma cells and neutrophils. Fibrin exudation and haemorrhage was mild to moderate with moderate to severe oedema. The protein content of the oedema fluid is judged by the degree of eosinophilia of the fluid

histologically, using H&E staining.[75] The oedema affecting the alveolar lumens displayed a moderate to severe degree of eosinophilia, indicating an increased protein content. This is suggestive of increased vascular permeability (discussed below). Although the histopathologic detection of pulmonary oedema is neither sensitive nor specific (since protein leaching from the exudate may occur during processing and thereby falsely reducing its detection, or autolysis may result in eosinophilic material filling the alveoli making it appear more prominent), the degree of oedema we observed histologically could be correlated to the macroscopic picture, which is the more accurate indicator of the severity of oedema.[42] Autolysis did not play a significant role in these cases.

The alveolar wall and lumens were the areas most severely affected in all cases. This finding is consistent with the known fact about the main target cell of the AHSV which is the microvascular endothelial cell.

The perivascular interstitium was characterised by mild to moderate inflammatory cell infiltration, predominated by macrophages and lymphocytes, and associated with mostly mild fibrin exudation, mild to moderate haemorrhage and moderate oedema. The reaction is typical for what has been described for orbiviral infections.[31] A characteristic observation was that the adventitial region of smaller blood vessels (arterioles and venules) appeared to be affected more by inflammatory cell infiltration, while the larger blood vessels (arteries and veins) seemed to be mostly affected by oedema, fibrin-exudation and haemorrhage. The latter finding raised suspicion about a possible underlying haemodynamic mechanism (hydrostatic pressure and congestion) associated with terminal decompensating heart failure, resulting in increased intravascular pressure in the larger vasculature with leakage of large amounts of fluid and even fibrin and/or erythrocytes from areas with endothelial injury. The venous/arteriolar endothelial cells displayed mild hypertrophy (for similar reasons

discussed for the microvascular endothelial cells). Mild lymphatic distention draining excess interstitial exudate was present in most cases.

The sub-pleural interstitium was characterised by mild inflammatory cell infiltration, consisting of mostly macrophages, and was associated with mild fibrin exudation, mild haemorrhage and mild to moderate oedema. What appeared to be pleural mesothelial activation could be observed in many cases (cuboidal to tomb-stoning appearance). This is as a result of the acute inflammatory response that causes the pleural capillaries to become more permeable to proteins and cells. In response to inflammatory mediators the mesothelial cells round up and separate from each other to allow cells and proteins to pass into the pleural space.[30]

The peribronchiolar interstitium was characterised by mild inflammatory cell infiltration, predominated by macrophages and lymphocytes, and was associated with mild oedema. Hyperplasia of the bronchiolar-associated lymphoid tissue was not typical a feature and when present was only associated, to a mild degree, with severe anthracosis in three cases. This supports the fact that the pathogenic agent, AHSV in this case, is not aerogenous in origin and thus not responsible for significant, if any bronchiolar injury.

The bronchiolar lumen was characterised by mild inflammatory cell exudation in only a few cases, with mild fibrin exudation, mild haemorrhage and mild to moderate oedema. The degree of oedema was consistently lower than that observed in the alveolar lumens, suggesting that the oedema observed in the bronchiolar lumens is most likely as a result of retrograde flow from the alveolar lumens and would vary in degree dependant on extent of capillary injury and the rate of fluid leakage from the microvasculature.

The bronchiolar or airway epithelial cells were unaffected in all cases. This is an important feature to distinguish AHS from other canine viral infections that affect the lung, such as

canine distemper virus, canine herpesvirus, canine parainfluenza virus 2, canine adenovirus and canine parvovirus.[41]

Histological changes are not considered to be helpful in the diagnosis or the understanding of the pathogenesis of AHS in the horse.[42] The histopathological findings in dogs, as in horses infected with AHSV, are not pathognomonic; however, the changes in dogs are highly suggestive of a condition that affects the integrity of the microvascular endothelium. In dogs there are a number of aetiologies with overlapping changes suggestive of endothelial injury e.g. endotoxaemia, toxic gas inhalation, thermal injury and near drowning[41], but when gross pathology and epidemiology is considered in conjunction to the histopathology, AHSV should be an important differential diagnoses.

The histopathological changes observed in the hearts of AHSV-infected horses are usually characterised by microvascular injury with the associated consequences, as well as foci of myocardial necrosis.[19,50] In the hearts of the dogs included in this study however no major lesions were consistently present. Three of the dogs displayed some inflammatory cell infiltrate, but in all cases this was very mild and not considered a distinctive AHSV-associated lesion.

No virus-associated changes could be observed in the liver, similar to horses with AHS. Severe congestion of the hepatic sinusoids, especially affecting the centrilobular areas, was a consistent finding in most cases. This is considered to be suggestive of terminal acute heart failure attributable to metabolic acidosis and hypoxia as a result of interstitial pneumonic respiratory failure, as mentioned above and discussed below. The one case that revealed moderate hepatocellular necrosis and fibrosis was considered incidental and probably suffered from pre-existing right AV-valvular insufficiency or a similar condition. Cardiac failure in canine AHS cases is likely attributable.

No consistent splenic lesions could be observed. The only significant finding was that of congestion, thought to be due to terminal heart failure similar to the congestion in the liver.

The purpose of the IHC labelling in this study was to comparatively determine the presence of AHS specific viral antigen in the lung tissue associated with lesions typical for what has been described for horses.[20]

Initially when the first dog was diagnosed in 2006 up until 2014 the AHSV-specific anti-VP7 serum was the only antibody available at the VHL, Section of Pathology, DPS, FVS, UP. Thereafter, the antibody was replaced by AHSV-specific anti-NS4 serum as a result of the lack of availability of the original antibody. Many of the initial cases on which the anti-VP7 serum was used did not have sufficient wax-imbedded tissue left for additional sections, therefore the reason why the anti-NS4 serum was used on only 17 of the 29 cases (cases for which sufficient tissue was left and those received post 2014). In 2018 new anti-VP7 serum was obtained and the authors were able to utilise this for all the cases on which the anti-VP7 serum was not previously used. Fortunately all the remaining cases had sufficient tissue available for additional sections, therefore the labelling of all 29 cases with VP7.

The labelling characteristics for both AHSV-specific antibodies were identical in this study to what has been described for the lung tissue of infected horses.[21,77]

The degree of positive labelling showed no significant relation to the severity of the pulmonary lesion. Some severely affected cases displayed scant labelling, while some mildly affected cases displayed widespread labelling. Cases where the opposite is true were also found. This finding highlights the effect of the immune response as an important consideration in the development of the lesion, rather than direct viral injury alone (see below).

Bluetongue virus, which is an orbivirus closely related to AHSV has been shown to cause a similar clinical disease in ruminants as well as dogs fed the meat of infected ruminants.[64] Significant levels of antibodies have also been found in dogs in Morocco that did not have access to infected ruminant meat. For this reason BTV-4-specific IHC was performed on cases to exclude the possibility of BTV as the cause of disease in the current study. All cases included in this study were negative for BTV.[53] These negative results also ruled out the possibility of cross reactive labelling with AHS virus.

The ultrastructural changes observed were comparable with that found in horses with AHS.[26] A more detailed study evaluating the virus-associated endothelial cell changes is still necessary. In the current study, virus particles could not be visualised, but haemodynamic changes and those affecting the blood-air barrier could be described.

Even though thrombosis could not be observed in the alveolar capillaries with light microscopy, microthrombosis and platelet plugging was evident with TEM. This supports a pathogenesis of endothelial injury and loss followed by haemostasis and early vascular repair.

All 12 of the cases from this study that was submitted for molecular analysis (rtPCR) tested positive for AHS virus. This supports the validity of the IHC test results in these and also the cases where PCR was not performed. Serotyping/typing results were available for 10 of the 12 cases on which PCR was performed. Nine of the 10 cases was identified as serotype 6 and only one of the 10 cases was identified as serotype 2. From this it is clear that serotype 6, and to a lesser extent, serotype 2 is of importance in dogs, however; further investigation is required to determine the effect of other AHSV strains on dogs. Work by Aklilu *et al.* (2012) found no correlation between the form of the disease and the AHSV serotype in horses[2], while

Laegried *et al.* (1993) suggests a strong correlation between the severity of lesions and the virulence of the AHSV biotype.[34] Due to the low incidence of AHS in dogs we can only speculate that serotype 6 may be more virulent to dogs due to the overrepresentation (90%) of this serotype in this study. An important factor to consider when analysing the serotypes is the geographical origin of the cases. It would therefore be meaningful to compare the serotypes isolated from horses in the same year and geographical location from that isolated from the dogs. If the serotypes are comparable between the horses and dogs, geographical location is probably the determining factor, while if the serotypes differ between the horses and dogs it may indicate that serotype 6, or at least a specific biotype within the serotype 6 group, is more virulent to dogs.

The CT (cycle threshold)-values for eight of the cases submitted for PCR was determined. The average CT-values for the different organs (lung, spleen and liver) differed with the spleen having the lowest value, followed by the lung and then the liver. The low number of cases for which the CT value was determined precludes its use in statistical analysis. However, in the event of availability of larger number of cases, the CT values may be used in conjunction with IHC labelling to determine the terminal tropism of the virus in dogs.

CT-values below 29 indicates the presence of abundant target nucleic acid in the tissue, CT-values of 30-27 suggests the presence of moderate amounts, while CT-values of 38-40 indicates minimal amounts which could indicate environmental contamination.[29,35,59] Considering this it is clear that in all cases (average CT-value of 24.5) abundant AHSV was present in the respective tissues. The averages were 25.1, 22.3 and 26.8 for the lung, spleen and liver respectively.

Unlike in horses infected with AHSV that display four different syndromes of the disease [20,22,40,42,48], the macroscopic changes observed in all the dogs in this study were consistent with the peracute pulmonary syndrome observed in horses. In the peracute pulmonary form the course of the disease is extremely rapid and mainly affects the pulmonary system. The other three syndromes of the disease observed in horses, namely the sub-acute cardiac form, the chronic AHS fever form and the mixed form did not feature significantly in this study. One may however argue that there may be some characteristics of the mixed form, but not to the extent that is observed in horses.

African horse sickness virus has a profound effect on dogs that become clinically infected. All the dogs involved in this study presented clinically with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) as defined by the Dorothy Russell Havemeyer Working Group on ALI and ARDS in Veterinary Medicine.[72] The defining characteristics according to the group are: Acute onset of respiratory distress; the presence of a known risk factor (such as viral-associated inflammation); evidence of protein-rich oedema leaking from pulmonary capillaries; insufficient oxygenation of the blood and finally inflammation. The outcome of ALI that progresses to ARDS is usually fatal.

The pathogenesis of orbiviral disease is sparked by the transmission of the virus to a susceptible animal from the bite of a virus-infected arthropod vector. In this case, the vector is likely to be a *Cullicoides* sp. The dendritic macrophages in the area of the bite become infected and transport the virus to the regional lymph node where primary replication of the virus occurs. From there the virus is disseminated to the target organs and replicates secondarily in endothelial cells and mononuclear phagocytes.[31,76] This result in the release of inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α) as well as vasoactive mediators such as nitric oxide (NO) by the infected mononuclear cells

and endothelium. These cytokines that form part of the acute inflammatory response then result in vasodilation and increased vascular permeability leading to exudation of fluid into the interstitial spaces. As the disease progress, endothelial cell necrosis occurs resulting in detachment from the vascular basement membrane with association of protein exudation, haemorrhage and microthromboses.[31] This sequence of events could clearly be observed histologically, and supported ultrastructurally in the pulmonary tissue of this study. Protein-poor oedema was consistently associated with mild inflammation and apoptosis, suggesting early infection, while the cases with moderate to severe inflammation and apoptosis was usually associated with protein-rich oedema and occasional haemorrhage, suggestive of more advanced disease. Zientara *et al.* (2015) suggested that the oedema and effusion is the result of direct viral injury to endothelial cells but that indirect effects of the immune response cannot be excluded. Analysing the results in this study, it would appear that the initial activation of the endothelial cells and mononuclear cells that initiates the inflammatory response is related to the direct effect of the virus, however; the progression of the lesion in most cases is most likely mediated by the inflammatory response. This conclusion is supported by IHC labelling for the virus. The viral concentration observed with IHC could not be positively correlated to the severity of the histological lesion. This suggests that the severity of the pulmonary injury is not necessarily related to the direct effect and presence of the virus, but to a large extent is the result of the virus induced immune response.

Although most of the histologic changes observed in the lungs ranged from mild to moderate in the specific anatomical locations, the sum of all the changes that affected the entire lung had a profound effect on the pulmonary function.

Studies by Carrasco *et al.* (1999) on horses showed that pulmonary intravascular macrophages (PIMs) play a central role in the pathogenesis of vascular permeability through

the action of inflammatory mediators released by them.[17,19,26] Winkler *et al.* (1988) however suggested that PIMs do not appear to perform a major function in the lungs of dogs.[60,74] In this study the presence of significant inflammation induced vascular injury raises renewed concern about the significance and presence of PIMs in dogs.

As mentioned earlier, the pathogenesis of the suspected terminal cardiac failure, which is supported by the protein-poor oedema surrounding the larger blood vessels as well as severe visceral congestion is suspected to be two-fold. Firstly, the hypoxic state inducing metabolic acidosis[62] in these animals may result in significant cardiac myocyte degeneration and death that will ultimately contribute to terminal cardiac dysfunction.[33] Even though no histologic features of cardiac myofibre injury could be identified in any of the histologic sections, the sudden onset and short duration of the cardiac insult may have precluded histological detection, since histological lesions typical for cardiac myofibre necrosis only become detectible with routine H&E staining around 12 hours after the initial insult.[42] Secondly, acute cor pulmonale (ACP), which can be defined as acute right heart failure resulting from a sudden increase in the pulmonary resistance to blood flow. In human medicine ACP is often observed as a complication of ARDS. The severe inflammation, oedema and microthrombosis of the alveoli that occur as a result of infection by the AHS virus will result in increased pulmonary vascular resistance and predispose the animal to terminal ACP associated with ARDS.[9,32]

Originally dogs were regarded as epidemiological bottlenecks for orbiviral infection[76] and therefore unimportant as reservoirs of the virus. As suspected since the first diagnosis in 2006, and recently suggested by Chris Oura in a research comment, viraemic dogs may play a role in the spread of the virus and may pose a major risk of introducing the virus to non-

endemic countries where a competent vector is present.[52] Needless to say this may have major implications on international canine transport and investigations to determine the viraemic period and ability to transmit the virus should be prioritised in further studies. Dogs are regarded as dead-end hosts[18] in the transmission of AHS; however, the unlikelihood that all dogs infected by the virus develop clinical illness and die and that many may only show a transient fever reaction[69] should raise concern for the development of a carrier state. This concern is supported by the finding of Van Sittert *et al.* (2013) that in a single dog colony the prevalence of positive AHS antibody titres was as high as 43%.[70]

Further studies that will add valuable insight to the pathogenesis of canine AHS are necessary. Among others, these investigations should include the deficiencies identified in the current study such as determination of tissue tropism in canine AHS and a detailed immunohistochemical characterisation of the pulmonary inflammatory reaction.

The question remains why dogs are the only other species to become fatally infected by AHSV. With infection in closely related orbiviruses such as BTV, the initial release of Interferon-1 (IFN-1) by multiple cell types plays an important role in the development of disease. In animals such as bovines where the release of IFN-1 is rapid, severe disease is less likely to occur, while in animals where the release is delayed (sheep) or absent (IFN-1 receptor deficient mice) severe disease usually follows.[16,24,31] It is unclear whether IFN-1 plays an important role in the development of AHS, therefore; further investigation into the release of IFN-1 and the pathogenesis of AHS, specifically in dogs, may be valuable.

Furthermore, intensive research is needed to establish the epidemiology of the virus in dogs, especially regarding the suspected transmission by arthropod-vectors as well as the effect of different serotypes on dogs.

CONCLUSION

Acute lung injury presenting as acute respiratory distress syndrome clinically is the hallmark of AHS in dogs. This is supported by characteristic macroscopic and histologic pathology typical for the peracute pulmonary form of AHS demonstrated by this study.

The pathological findings associated with AHS in dogs are characterised by acute mononuclear interstitial pneumonia, and fatal pulmonary vascular injury.

With this study the authors were able to report on 29 canine cases of AHS where no contact with horses or their tissues were documented. This finding supports the suspicion of vector-transmission of the virus to dogs.

In areas where the *Cullicoides*-vector exist, AHS should be considered as an important differential diagnosis for dogs presenting with acute respiratory distress.

AHS in dogs should be considered as an emerging disease, especially considering the effect global warming may have on vector ecology.

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APPENDIX 1

Table 12: Score sheet used for histological evaluation.

Criteria	S-number:						Comments
Signalment							
PM findings							
RT-qPCR results and serotype							
Lung histopathology							
Autolysis			Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
Lesion distribution			Focal ☐	Multifocal ☐	Coalescing ☐	Diffuse ☐	
Congestion			Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
Alveolar wall	Cellular infiltration	Monocyte-macrophages	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Lymphocytes	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Plasma cells	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Neutrophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Eosinophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Apoptosis		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
Microvascular endothelial cells	Nuclear hypertrophy/activation		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
Thrombosis					Present ☐	Absent ☐	
Alveolar lumen	Cellular infiltration	Monocyte-macrophages	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Lymphocytes	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Plasma cells	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Neutrophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Eosinophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Fibrin (MSB)		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Haemorrhage		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
			Focal ☐	Multifocal ☐	Coalescing ☐	Diffuse ☐	
	Oedema		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
Perivascular interstitium	Cellular infiltration	Monocyte-macrophages	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Lymphocytes	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Plasma cells	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Neutrophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Eosinophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Fibrin (MSB)		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	

	Haemorrhage		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
			Focal ☐	Multifocal ☐	Coalescing ☐	Diffuse ☐	
	Oedema		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
				Mild eosinophilia ☐	Moderate eosinophilia ☐	Severe eosinophilia ☐	
Venous/arteriolar endothelial cells	Nuclear hypertrophy/activation		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
Lymphatic vessels	Distention		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Content		Oedema ☐	Haemorrhage ☐	Fibrin ☐	Leukocytes ☐	
Sub-pleural interstitium	Cellular infiltration	Monocyte-macrophages	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Lymphocytes	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Plasma cells	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Neutrophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Eosinophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Fibrin (MSB)		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Haemorrhage		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
			Focal ☐	Multifocal ☐	Coalescing ☐	Diffuse ☐	
	Oedema		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
				Mild eosinophilia ☐	Moderate eosinophilia ☐	Severe eosinophilia ☐	
Pleural mesothelium	Hypertrophy/activation				Present ☐	Absent ☐	
Peribronchiolar interstitium	Cellular infiltration	Monocyte-macrophages	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Lymphocytes	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Plasma cells	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Neutrophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Eosinophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Fibrin (MSB)		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Haemorrhage		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
			Focal ☐	Multifocal ☐	Coalescing ☐	Diffuse ☐	
	Oedema		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
				Mild eosinophilia ☐	Moderate eosinophilia ☐	Severe eosinophilia ☐	
	Anthracosis				Present ☐	Absent ☐	
	BALT activity				Hyperplastic ☐	Within normal limits ☐	
Bronchiolar lumen	Cellular infiltration	Monocyte-macrophages	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Lymphocytes	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Plasma cells	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	

		Neutrophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
		Eosinophils	Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Fibrin (MSB)		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
	Haemorrhage		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
			Focal ☐	Multifocal ☐	Coalescing ☐	Diffuse ☐	
	Oedema		Absent ☐	Mild ☐	Moderate ☐	Severe ☐	
				Mild eosinophilia ☐	Moderate eosinophilia ☐	Severe eosinophilia ☐	
				Mild eosinophilia ☐	Moderate eosinophilia ☐	Severe eosinophilia ☐	
Immunohistochemistry							
AHS IMP (NS4)	Cells	Epithelial cells ☐	Endothelial cells ☐	Monocyte-macrophages ☐	Lymphocytes ☐	Fibroblasts ☐	
	Location			Nuclear ☐	Cytoplasmic ☐	Inclusions ☐	
	Degree		0 ☐	1+ ☐	2+ ☐	3+ ☐	
	Nature		Diffuse ☐	Fine granules ☐	Beads ☐	Linear punctate dots ☐	
AHS IMP (VP7)	Cells	Epithelial cells ☐	Endothelial cells ☐	Monocyte-macrophages ☐	Lymphocytes ☐	Fibroblasts ☐	
	Location			Nuclear ☐	Cytoplasmic ☐	Inclusions ☐	
	Degree		0 ☐	1+ ☐	2+ ☐	3+ ☐	
	Nature		Diffuse ☐	Fine granules ☐	Beads ☐	Linear punctate dots ☐	
BTV IMP labelling					Present ☐	Absent ☐	
Other organs histopathology							
General comments							

MSB: Martius scarlet blue stain; AHS: African horsesickness; IMP: Immunoperoxidase; BTV: Bluetongue virus.

APPENDIX 2

Table 13: Grading criteria for histological lesions.

Parameter	Grading	Criteria
Autolysis		
Severity	Mild	Anatomical architecture easily distinguishable, cellular morphology well-defined
	Moderate	Anatomical architecture still easily distinguishable, cellular morphology not well-defined, red blood cells still preserved, small numbers of putrefactive (Clostridial) bacteria may be present in some fields
	Severe	Cellular morphology cannot be defined, anatomical architecture barely distinguishable, red blood cells lysed and difficult to identify, numerous putrefactive (Clostridial) bacteria present
Histopathology	(All changes in comparison with normal control lung **[Figure 9 and 10])**	
Lesion distribution	Focal	Single well circumscribed area affected
	Multifocal	Multiple non-overlapping areas affected
	Coalescing	Multiple overlapping areas affected
	Diffuse	Entire lung tissue OR all specific anatomical sites affected **[Figure 11 and 12]**
Hyperaemia/congestion	Mild	Single layer of erythrocytes in capillaries with open spaces in between **[Figure 13]**
	Moderate	Single layer of erythrocytes in capillaries with no open spaces
	Severe	Double layer or more of erythrocytes in capillaries with no open spaces **[Figure 14]**
Inflammatory cell infiltrate* (monocyte-macrophages, lymphocytes, plasma cells, neutrophils, eosinophils) - **perivascular interstitium** - **peribronchiolar interstitium** - **sub-pleural interstitium**	Mild	Single leukocytes present without notable expansion of the interstitial anatomy **[Figure 29, 43 and 49]**
	Moderate	Two to three layers of leukocytes present resulting in slight expansion of the interstitial anatomy
	Severe	More than three layers of leukocytes present resulting in marked expansion of the interstitial anatomy **[Figure 30]**
Inflammatory cell infiltrate* (monocyte-macrophages, lymphocytes, plasma cells, neutrophils, eosinophils) - **alveolar wall**	Mild	Single extravascular leukocytes without notable expansion of the alveolar wall **[Figure 15]**
	Moderate	Slight expansion of the alveolar wall by extravascular leukocytes (double normal thickness) **[Figure 16]**
	Severe	Marked expansion of the alveolar wall by extravascular leukocytes (more than double normal thickness) **[Figure 17]**
Inflammatory cell infiltrate* (monocyte-macrophages, lymphocytes, plasma cells, neutrophils, eosinophils) - **alveolar lumen** - **bronchiolar lumen**	Mild	≤1 leukocytes observed in >50% of the alveolar/bronchiolar lumens **[Figure 21]**
	Moderate	2-5 leukocytes observed in >50% of the alveolar/bronchiolar lumens
	Severe	≥6 leukocytes observed in >50% of the alveolar/bronchiolar lumens **[Figure 22]**
Fibrin	Mild	Affecting less than a third of the anatomic location **[Figure 31, 53 and 54]**
	Moderate	Affecting more than a third to two thirds of the anatomic location **[Figure 24]**
	Severe	Affecting more that more than two thirds of the anatomic location **[Figure 32]**

Haemorrhage* - **perivascular interstitium** - **peribronchiolar interstitium** - **sub-pleural interstitium**	Mild	Single extravasated erythrocytes present without notable expansion of the interstitial anatomy **[Figure 33 and 50]**
	Moderate	Slight expansion of the interstitial anatomy by extravasated erythrocytes (double normal thickness) **[Figure 34 and 44]**
	Severe	Marked expansion of the interstitial anatomy by extravasated erythrocytes (more than double normal thickness) **[Figure 35]**
Haemorrhage* - **bronchiolar lumen** - **alveolar lumen**	Mild	Small numbers of extravasated erythrocytes present in less than 50% of alveolar/bronchiolar lumens **[Figure 25]**
	Moderate	Small numbers of extravasated erythrocytes present in more than 50% of alveolar/bronchiolar lumens OR less than 50% of alveolar/bronchiolar lumens are filled with extravasated erythrocytes multifocally **[Figure 26]**
	Severe	>50% of alveolar/bronchiolar lumens are filled with extravasated erythrocytes **[Figure 27]**
Oedema* **(amorphous clear to eosinophilic extravascular fluid)** - **perivascular interstitium** - **peribronchiolar interstitium** - **sub-pleural interstitium**	Mild	Slight expansion (1x) of the interstitium by oedema fluid **[Figure 36, 45 and 51]**
	Moderate	Moderate expansion (2-3x) of the interstitium by oedema fluid **[Figure 37 and 46]**
	Severe	Marked expansion (>3x) of the interstitium by oedema fluid **[Figure 38 and 47]**
Oedema* **(amorphous clear to eosinophilic extravascular fluid)** - **bronchiolar lumen** - **alveolar lumen**	Mild	Affecting less than a third of alveolar/bronchiolar lumens
	Moderate	Affecting a third to two thirds of alveolar/bronchiolar lumens
	Severe	Affecting more than two thirds of alveolar/bronchiolar lumens **[Figure 28 and 56]**
Microvascular endothelial cell activation (endothelial cell nucleus thickness is double or more the normal thickness)	Mild	Less than a third of endothelial cells display nuclear activation/hypertrophy
	Moderate	A third to two thirds of endothelial cells display nuclear activation/hypertrophy
	Severe	More than two thirds of endothelial cells display nuclear activation/hypertrophy **[Figure 20 and 41]**
Lymphatic vessel distention	Mild	Distension of less than a third of lymphatic vessels
	Moderate	Distension of a third to two thirds of lymphatic vessels **[Figure 39]**
	Severe	Distension of more than a third of lymphatic vessels
Apoptosis **(clusters of closely aggregated dark purple nuclear fragments [Figure 19])** - **alveolar wall**	Mild	≤1 apoptotic cells per HPF
	Moderate	2-5 apoptotic cells per HPF
	Severe	≥6 apoptotic cells per HPF **[Figure 18]**
Immunohistochemistry		
Degree of labelling	1+	≤1 clearly labelling cells per HPF
	2+	2-5 clearly labelling cells per HPF
	3+	≥6 clearly labelling cells per HPF **[Figure 58 and 60]**

* Criteria for the evaluation of inflammatory cell infiltrate, haemorrhage and oedema varied dependent on anatomical location.

HPF: High power field (400x magnification)

APPENDIX 3

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Pathology in dogs that died following natural infection by African horse sickness virus**
PROJECT NUMBER	**V069-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. N O'Dell**

STUDENT NUMBER (where applicable)	**UP_23034379**
DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

ANIMAL SPECIES	**n/a**	
NUMBER OF ANIMALS	**n/a**	
Approval period to use animals for research/testing purposes		**June 2016 –June 2017**
SUPERVISOR	**Dr. JCA Steyl**	

KINDLY NOTE:

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

APPROVED	Date 27 June 2016
CHAIRMAN: UP Animal Ethics Committee	Signature 

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Animal Ethics Committee

Extension No.1

PROJECT TITLE	**Pathology in dogs that died following natural infection by African horse sickness virus**
PROJECT NUMBER	**V069-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. N O'Dell**

STUDENT NUMBER (where applicable)	**UP_23034379**
DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

ANIMAL SAMPLES	**n/a**	
NUMBER OF ANIMALS	**n/a**	
Approval period to use animals for research/testing purposes		**January 2017-January 2018**
SUPERVISOR	**Dr. JCA Steyl**	

KINDLY NOTE:

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

APPROVED	Date 2 February 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15