Clinical signs and clinical pathology findings in horses with equine encephalosis at the Onderstepoort Veterinary Academic Hospital, South Africa

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List of Abbreviations:

AHS- African horse sickness
AHSV- African horse sickness virus
BTV- Blue tonguevirus
cells/L- Cells per litre
Ct- Cycle threshold
dsRNA- Doubled-stranded ribonucleic acid
EDTA- Ethylenediamine tetra-acetic acid
EE- Equine encephalosis
EEV- Equine encephalosis virus
GIT- Gastrointestinal tract
mRNA- Messenger ribonucleic acid
Nm- Nanometre
NSAID- non-steroidal anti-inflammatory drug
OVAH- Onderstepoort Veterinary Academic Hospital
PCR- Polymerase chain reaction
RNA- Ribonucleic acid
RT-PCR- Real time polymerase chain reaction
sd- standard deviation
TCID50- Median tissue culture infectious dose
VP- Viral structural protein

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Abstract

Clinical signs and clinical pathology findings in horses with equine encephalosis at the Onderstepoort Veterinary Academic Hospital, South Africa.

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Introduction:
Equine encephalosis is a systemic disease of horses caused by equine encephalosis virus. This virus is an *Orbivirus* that is transmitted by biting midges (*Culicoides* species). Equine encephalosis is often reported as a subclinical infection with a relatively limited number of clinical cases showing predominantly fever, with minimal morbidity and mortality. Rare instances of neurological disease have also been associated with the virus.

Clinical relevance:
Information regarding the clinical presentation of equine encephalosis is underrepresented in current scientific literature. This study aims to contribute to a more detailed conceptualization of the significance of the disease and its clinical impact.

Method:
A retrospective, descriptive, observational study was performed on data obtained from the University of Pretoria's clinical database for cases identified with equine encephalosis over the period 2013-2023. Data from the history, clinical signs and clinicopathological findings were analysed. The clinical presentation and clinicopathological findings are reported.

Results:
A total of 28 horses conformed to the study parameters as having clinical infection with equine encephalosis virus. Pyrexia was apparent in 89.2% of these cases. Other clinical findings included tachycardia (64.3%), tachypnoea (46.4%), colic (39.3%), neurological signs (21.4%), peripheral oedema (14.3%), and icterus (10.7%). Evaluation of the clinicopathological findings identified lymphopenia (86.7%), thrombocytopenia (76.0%), leukopenia (48.0%), immature neutrophilia (31.8%), and mature neutropenia (27.3%).

Conclusion:
Equine encephalosis can result in a wide variety of clinical signs in horses and is associated with changes in haematology variables. These haematological changes suggest a systemic response to the viral infection. Further research into the pathophysiology of equine encephalosis is required to better understand the disease and its clinical relevance.

Chapter 1: Introduction

1.1-Aim:

This study aimed to describe and identify clinical abnormalities as well as pertinent findings in the history of horses treated at a veterinary academic hospital for natural infection with equine encephalosis virus (EEV) using retrospective data. The study also aimed to describe if there are any notable changes in the clinicopathological presentation of these patients.

1.2-Benefits arising from the experiment:

Although EEV is not currently associated with a high mortality rate, it nevertheless leads to morbidity in the equine population of South Africa. The pyrexia and malaise caused by the disease can lead to considerable hinderance especially for competitive horses in both the racing and sports horse disciplines. This can then be associated with economic loss (unable to compete as well as veterinary costs). The similarities of EEV with the preliminary stages of African horse sickness (AHS) and other infectious diseases such as Equine Herpesvirus 1 and 4, also means infections can lead to confounding and apprehension for horse owners and veterinarians.

Equine encephalosis virus (EEV) is not extensively researched with regards to its disease presentation and clinical consequences. Literature indicates that the geographical isolation of EEV to Southern Africa is no longer an amorphism, with evidence of exposure to the virus in equine populations outside of this strict distribution including Ghana, Gambia, Ethiopia, and Israel (Mildenberg *et al.*, 2009; Aharonson-Raz *et al.*, 2011; Raz *et al.*, 2011; Wescott *et al.*, 2013; Yadav *et al.*, 2018).

As such, it is of value to attempt to investigate and describe the clinical consequences of the disease to develop a more detailed conceptualization of the significance of the disease and its impact on both the individual animal and the industry as a whole. This may lead to improvement in the management and treatment of the disease thereby reducing its morbidity.

1.3-Objectives:

The objectives of this study included identifying and describing the clinical signs in horses with naturally occurring EEV infection as well as the seasonality of the disease and other pertinent factors in the signalment and history. The objectives also included identifying and describing changes in the complete blood count and serum biochemistry of these patients; assessing the duration of fever in horses with EE; as well as the total duration of hospitalization in horses treated in a hospital setting.

Chapter 2: Literature review

2.1-In the confines of history

Equine encephalosis (EE) is a vector-borne viral disease that is endemic to the Southern part of Africa. The disease is postulated to appear in the scientific literature from as early as 1910, when Sir Arnold Theiler described a disease with similarities to African horse sickness, then referred to as "ephemeral fever"(Theiler, 1910). "Ephemeral fever" was described at this time as a viral disease that caused prominent fever but rarely resulted in mortality. This is contrasted to the high mortality associated with AHS.

It was not until the 1970s that equine encephalosis virus (EEV) was specifically isolated and identified in the scientific literature (Erasmus *et al.*, 1970). Erasmus et al (1970) described the identification and isolation of a viral disease termed equine encephalosis (EE) and caused by EEV. The virus was specifically isolated from a mare named "Cascara" that presented with unusual neurological signs and succumbed to the disease. The term encephalosis was coined in this publication due to the fact that horses showed neurological signs as well as signs of brain oedema without classic encephalitis (Erasmus *et al.*, 1970).

Erasmus et al (1978) described 3 viruses referred to as being serologically related to EEV: namely Gamil virus, Kaalplaas virus and Bryanston virus. It is now known that these viruses are in fact different serotypes of the EEV (Howell et al, 2002). At the time, Gamil virus was reported leading to signs of fever, inappetence and depression, Kaalplaas virus leading to signs of fever with severe conjunctivitis and chemosis but no supraorbital swelling and Bryanston virus leading to sudden death related to cardiac failure (Erasmus et al., 1978).

Additional scientific literature regarding the clinical presentation, pathological findings and pathogenesis of this disease then appears to be conspicuously absent until more recently when a new variant of EEV namely "Potchefstroom virus" was isolated (Gerdes and Pieterse, 1993). This literature provides scant novel information regarding the clinical presentation and pathogenesis of this disease.

2.2-Etiology

Equine Encephalosis is a systemic disease of horses caused by EEV. This virus is an Orbivirus of the family Sedoreoviridae (Walker *et al.*, 2022). As such the virus falls under a similar classification as African horse sickness virus (AHSV), although it is more closely related to Bluetongue virus (BTV) and epizootic haemorrhagic virus than AHSV itself (Viljoen and Huismans, 1989). The virus is considered an "arbovirus" in that it is transmitted by insect-vectors. At present, EEV has been characterised into 7 different serotypes (Steyn et al. 2021; Howell et al. 2002). The genome of EEV is classified as double stranded RNA that is encapsulated in a triple capsid protein layer. This triple capsid protein layer divides the viral particle into an inner core element surrounded by a double, outer capsid layer as seen in Figure 1 (Huismans *et al.*, 2004). Each viral particle can vary from 60-80 nm in diameter and takes the structure of a spherical particle with icosahedral symmetry (Gould and Hyatt, 1994). As is typical for Orbiviruses, EEV has seven structural proteins (designated VP-1 to VP-7) and 4 non-structural proteins (designated NS-1 to Ns-3 and Ns-3a) (Walker et al. 2022; Huismans et al. 2004; Gould & Hyatt 1994). The outer capsid layer is associated with the VP-2 and VP-5 structural proteins, which contribute to the processes of attachment of the virus to host cells and its subsequent penetration. The VP-2 protein is considered the major determinant of serotype for EEV. The inner core is made up of 2 major structural proteins namely VP-7 and VP-3 as well as 3 minor structural

proteins namely VP-1, VP-4 and VP-6 (Huismans *et al.*, 2004). These capsid layers then surround the viral genome made up of 10 dsRNA segments (Huismans *et al.*, 2004).

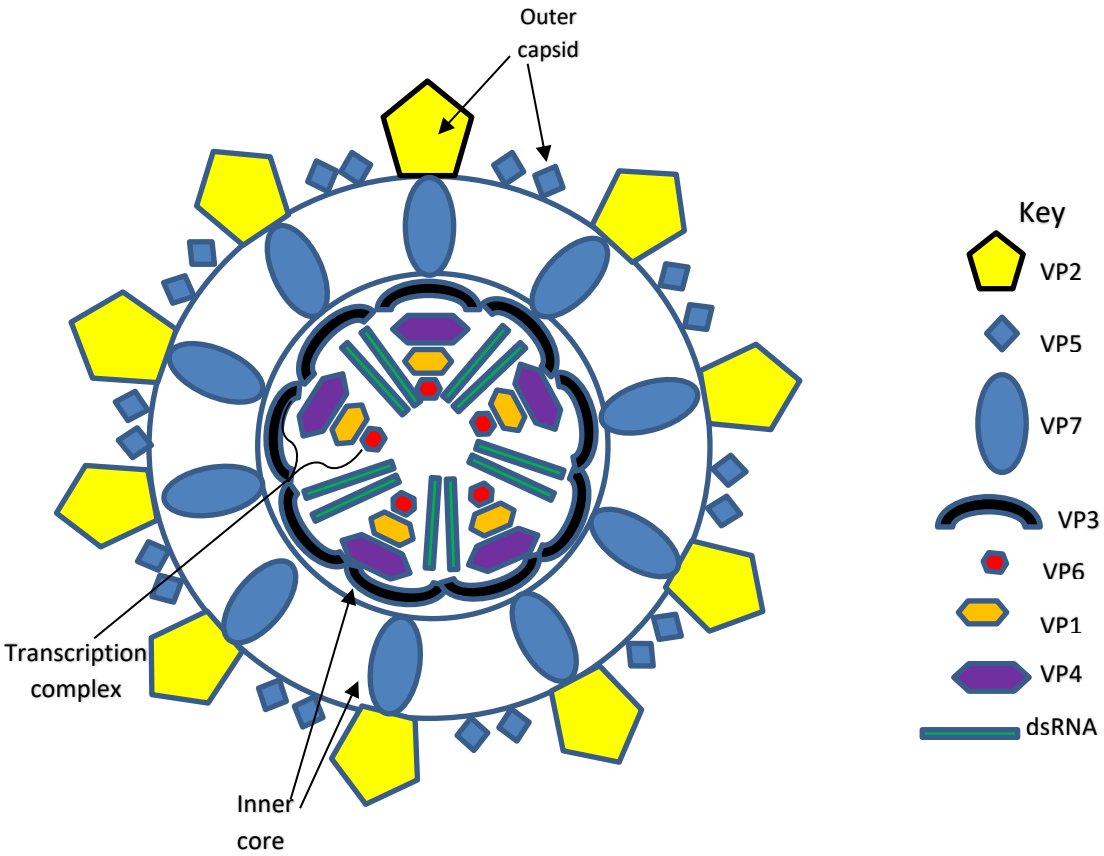

Figure 1: Adapted from Tirosh-Levy & Steinman (2022) and Gould & Hyatt (1994)-Simplified diagram of the EEV particle displaying its major structural component proteins.

2.3-Serotypes

Initial classification systems categorised EEV as well as several serologically related Orbiviruses (Erasmus, Boshoff and Pieterse, 1978). Howell et al. (2002) further examined these viral isolates and determined, using a western blot analysis, that these were all strains of EEV. Howell et al. (2002) then proposed a nomenclature in which the serotypes be assigned a numerical classification based on alphabetical order of the locations in which the reference strains were discovered. Based on these findings the serotypes are outlined below:

Table 1: Classification of the seven EEV serotypes (Howell et al., 2002; Steyn et al., 2021).

Original name	EEV serotype
Bryanston	1
Cascara	2
Gamil	3
Kaalplaas	4
Kyalami	5
Potchefstroom	6
Northrand	7

2.4-Epidemiology

Equine encephalosis virus is reported to be endemic to parts of Africa as well as the middle east with serological evidence of the virus in countries including South Africa, Zimbabwe, Ghana, Gambia, Ethiopia, and Israel (Gordon et al. 2017; Tirosh-Levy et al. 2017; Oura et al. 2012; Aharonson-Raz et al. 2011; Paweska & Venter 2004). Isolation of EEV has also occurred more recently from clinical cases in India (Yadav et al. 2018).

When considering the epidemiology of an arbovirus such as EEV, the disease cannot be discussed in isolation from the ecology of its vector. In the case of EEV, the associated vector is the *Culicoides* species of biting midges.

Paweska & Venter (2004) identified that not all species of *Culicoides* are susceptible to oral infection with EEV and as such not all species can act as a competent vector. The species identified as susceptible to oral infection with EEV included *C. imicola* and *C. bolitinos* species, which are the most widely distributed and plentiful in South Africa, as well as three non-Avaritia Old World species, *C. leucostictus*, *C. magnus* and *C. zuluensis*. Paweska & Venter (2004) also identified a significant difference in the susceptibility of *C. imicola* and *C. bolitinos* to infection with 6 of the major serotypes of EEV. Considering this, the specific type of *Culicoides* and their individual differences in ecology and distribution can impact on the distribution of EEV as a whole as well as disease caused by specific serotypes (Paweska & Venter 2004).

Infection rates with EEV have been shown to display yearly fluctuations in several studies (Howell *et al.*, 2008; Aharonson-Raz *et al.*, 2011; Gordon *et al.*, 2017). These fluctuations are strongly associated with fluctuations in the climatic conditions experienced and its influence on vector numbers and distributions. In particular, seasonal droughts followed by heavy periods of rainfall have been associated with increased prevalence of disease caused by arboviruses although the exact reason for this has not yet been fully elucidated (Baylis, Mellor and Meiswinkel, 1999; Venter *et al.*, 2002; Howell *et al.*, 2008).

Although widely reported in horses, EEV has also been identified to infect other equid species including donkeys and zebra (Barnard and Paweska, 1993; Barnard, 1997; Lord *et al.*, 2002; Gordon *et al.*, 2017). EEV appears to be less clinically significant in donkeys due to a greater resistance to developing outright clinical infection (Howell et al. 2002). Zebras are thought to play an important role in the endemic nature of the disease in Southern Africa, providing a probable link to understand the persistence and over-wintering of the virus in the face of less favourable environmental conditions (Barnard and Paweska, 1993).
Serological evidence for EEV has also been reported in 4 elephants although the clinical and epidemiological relevance of this has not been determined (Barnard, 1997).

2.5-Pathogenesis

The cytopathological pathogenesis and replication of EEV is so far thought to be similar to that of other disease causing *Orbiviruses* (Lecatsas, Erasmus and Els, 1973). This involves several steps from the point of cellular attachment to viral replication and release from invaded cells. After inoculation into the host, the virus attaches to the host cell and penetrates its membrane through a process called receptor-mediated endocytosis (Gould & Hyatt 1994). This is mediated by the VP-2 protein. The virus then undergoes uncoating in order to release the transcriptionally active core particle into the cellular cytoplasm (Gould & Hyatt 1994). This uncoating takes place in endosomes and involves the VP-5 protein. The release of these particles is then followed by the formation of a matrix around them; ultimately forming a viral-inclusion body (Gould & Hyatt 1994). The matrix portion of these

inclusion bodies is composed of mRNA and translated virus-specific protein molecules. These viral inclusion bodies also contain various stages of viral particles i.e., sub-cores, cores, and virus-like particles within their matrix (Gould & Hyatt 1994; Lecatsas et al. 1973). Once the viral particles are matured (double shelled), they are released into the cytoplasm in vesicles. Viral tubules can also be seen to develop in the orbivirus-infected cells although the specific function of these tubules is not completely understood (Gould & Hyatt 1994; Lecatsas et al. 1973) The vesicles then travel to the cell membrane after which they are released by budding or extrusion (Gould & Hyatt 1994).
The exact sites of replication and specific cellular tropisms of EEV have yet to be thoroughly examined. Determination of these aspects of the pathogenesis would be a valuable step in further understanding the disease and its effects on infected individuals.

2.6-Immunology

As well as being the major serotype determinant, the VP-2 protein is primarily involved in the initiation of a neutralisation-specific antibody immune response (Huismans *et al.*, 2004). Serological studies have indicated that horses can be concurrently seropositive to multiple serotypes which is indicative of poor immunological cross-protection amongst the serotypes (Grewar, Thompson, Lourens & Guthrie 2015; Howell et al. 2002, 2008; Paweska & Venter 2004). Maternally acquired immunity does not appear to be protective against infection in foals primarily due to variability in the serotype specific antibody compositions as well as progressive waning of the maternal antibody levels approaching the high-risk periods of the year (Tirosh-Levy & Steinman 2022; Grewar et al. 2015; Howell et al. 2002, 2008). Genomic analysis has revealed widespread reassortment in EEV isolates with some evidence of the development of unique genome segments associated with geographic location (Steyn *et al.*, 2021; Tirosh-Levy and Steinman, 2022). This reassortment and variation may suggest genetic drift which can act as a mechanism of immunological evasion (Steyn *et al.*, 2021; Tirosh-Levy and Steinman, 2022).

2.7-Clinical signs

Theiler (1910) was the first to report the clinical signs now suspected to be associated with EEV (Guthrie, Pardini & Howell, 2009). Theiler (1910) described clinical signs observed in both naturally and experimentally induced cases of a disease referred to as “ephemeral fever” but did not indicate a specific number of cases over which these observations were made. He indicated fever as the predominant clinical feature of the disease process. Theiler (1910) also indicated that “ephemeral fever” could be associated with other clinical changes including tachycardia, mild tachypnoea, and changes in the mucous membranes of congestion or hyperaemia. Theiler (1910) observed that “ephemeral fever led to an abrupt onset of fever that terminated rapidly (usually after approximately 5 days). Horses were often tachycardic with heart rates of above 80 beats per minute and notable changes in pulse (hurried and soft). In contrast to the implication of the name equine encephalosis virus (Erasmus *et al.*, 1970), Theiler (1910) did not indicate any specific neurological associations with "ephemeral fever” although he did report that horses regularly appeared inappetant and lethargic. Theiler (1910) states that it was impossible to kill a horse during experimental infection with “ephemeral fever”.

Erasmus et al. (1970) appears to be the first in the literature to describe the initial isolation of EEV from a horse displaying neurological signs as well as an undisclosed number of horses displaying fever. This report described several horses displaying disease of similar clinical signs including fever, anxious facial expression and tight facial muscles, frenzy, and stupor as well as acute death. Ataxia is specifically mentioned as being absent in these cases. It is from one of these cases, a mare named *Cascara*, that the initial isolation of EEV is then reported. Experimental infection of 2 horses identified via serology as naïve to EEV exposure, was undertaken via inoculation with a 10%

suspension of liver and spleen obtained from *Cascara*. This reportedly resulted in isolated clinical signs of fever but did not reproduce the neurological signs or mortality seen in the naturally occurring cases (Erasmus *et al.*, 1970).
Later evidence, identified via serological investigations in a prospective study examining the serum of at least 42 horses after this outbreak, indicated that the majority of EE is subclinical with only a small portion developing clinical signs (Erasmus B. *et al.*, 1970). It was further concluded that horses expressing clinical signs of the disease would display signs of pyrexia and rarely, a peracute neurological syndrome and/or mortality. Mortality primarily occurred in horses older than 7 years of age (Erasmus *et al.*, 1970).

In a later report published by Erasmus et al. (1978), additional experimental infection of a single horse with EEV obtained after passage through suckling mice followed by 2 repeated passages in baby hamster kidney (BHK) cells led to similar disease findings as those described in natural cases by Erasmus B. et al. (1970). The horse initially developed pyrexia of 7 days duration as well as mild inappetence. Peracute neurological signs then developed on the tenth day with the horse displaying hyperexcitability and violent responses followed by coma and death. Necropsy findings also closely resembled the cases of natural infection (Erasmus et al. 1978).

Erasmus et al. (1978) also describe the clinical signs observed in association with 4 additional viruses described as serologically related to EEV. In this investigation, it was concluded that these viral isolates formed part of the EEV subgroup of Orbiviruses based on complement-fixation (CF) tests. These viral isolates have now been classified as serotypes of EEV and as such, the report describes additional clinical signs attributable to EEV. The first of these isolates was denoted as Gamil virus or M9/71. This virus was isolated from a single horse with naturally occurring disease displaying pyrexia, inappetence and depression of several days duration but ultimately resulting in clinical resolution of the disease (Erasmus, Boshoff and Pieterse, 1978).

The second of these isolates was denoted as Kaalplaas virus (7088) and was identified from a single mare that formed part of an AHS vaccine trial. The mare in question developed clinical signs, 17 days after experimental inoculation with a candidate AHS vaccine strain, which included severe conjunctivitis and eyelid swelling excluding the supraorbital fossae with concurrent fever. None of the other horses that formed part of the trial developed disease (Erasmus, Boshoff and Pieterse, 1978). Blood from this mare was then experimentally inoculated into a horse reportedly housed in vector protected stables for a period of 2 years. This horse developed fever of 5 days duration with signs of inappetence. Kaalplaas virus was identified in both these horse's blood samples during pyretic episodes.

The third viral isolate identified by Erasmus et al. (1978) was obtained from 1 out of 6 sampled cases of sudden deaths in horses due to cardiac failure. The isolate was designated Bryanston virus. Horses displayed signs of collapse under saddle or depression followed by acute death with necropsy findings indicative of acute cardiac failure. Later inoculation of this virus into several horses resulted in signs of fever, depression, and tachycardia but none of the signs of the cardiac failure displayed in the original case. Bryanston virus was however isolated from these experimental cases during pyretic episodes.

The final viral isolate identified was designated M8/76 which was isolated from aborted foetuses with the conclusion that, because of high viral concentrations in foetal tissue, the abortions likely occurred in association with active infection within the foetus (Erasmus, Boshoff and Pieterse, 1978).

Howell et al (2004) referred to signs of swelling of the face and supraorbital fossae, neurological signs including ataxia, stiffness, and reluctance to walk, changes in temperament and even

convulsions. They also reported signs of respiratory distress in association with clear foamy, clear or occasionally serosanguinous nasal discharge and mucous membrane petechiation. Rare reports of abortions have also been identified in association with EEV (Howell et al, 2004; Erasmus et al 1978).

Based on retrospective analysis of an outbreak of EEV in Israel starting in 2008, Mildenberg et al. (2009) identified clinical signs from horses that were noted to be PCR positive for EEV. No specific predilection for signalment could be identified with the duration of illness ranging from 7-30 days. These clinical signs included the commonly encountered signs of fever, tachycardia and tachypnoea along with unrest; inappetence; oedema of the neck, legs, lips and eyelids; and congested mucous membranes. This report identified a total of 60 affected stable yards although no specific mention is made of total case numbers. Morbidity in these individual yards was noted to be variable (2% to 100%) with no mortalities recorded. Aharonson-Raz et al. (2011) further expanded on this outbreak in a study in which EEV was isolated from EDTA blood samples in 3 out of 8 cases of pyrexia using Vero cell culture. These results were then confirmed using reverse transcription PCR of dsRNA genome segment 10. Clinical signs identified in these cases included fever, lack of appetite, colic, lethargy, congestion of the mucous membranes as well as rapid pulse (Aharonson-Raz *et al.*, 2011). Wescott et al. (2013) mentioned additional clinical signs described in this widespread disease outbreak in Israel including generalized weakness, myalgia, stiffness, conjunctivitis, nasal discharge, and coughing.

Most recently, a large study including 106 samples from horses identified to be EEV positive based on PCR, provided a better idea of the proportions of individuals that experience the clinical signs regularly associated with EEV (Steyn *et al.*, 2021). This retrospective study made use of the history and clinical information recorded on sample forms submitted as part of the diagnostic testing process, for horses with specific neurological signs. This study quantified that EEV was significantly associated with pyrexia, dyspnoea, and icterus. Additional clinical signs are summarized in Table 2. In this study, 47% of EEV cases presented with some form of neurological signs with an overall mortality of 9% (Steyn *et al.*, 2021). Other studies have also reported scant cases of mortality specifically ascribed to EEV (Erasmus et al, 1978; Yadav et al., 2018).

To date however no specific studies have been reported on data from horses presenting to a hospital facility due to EEV infection and no known studies report any clinicopathological changes in these infected horses.

Table 2: Clinical signs associated with EEV based on research by Steyn et al. (2021).

Clinical signs	Percentage of cases of EEV displaying the clinical sign
Pyrexia	72.6%
Icterus	18.8%
Dyspnoea	11.3%
Neurological	48.1%
Ataxia	25.5%
Recumbency	8.5%
Paralysis	5.7%
Paresis	14.1%
Depression	6.6%
Supraorbital swelling	1.9%
Swollen Limbs	0.9%
Abortions	0.9%

2.8-Necropsy findings

Initial necropsy evaluations from cases of peracute death associated with EEV revealed generalized venous congestion, fatty degeneration of the liver, catarrhal enteritis, and brain oedema (Erasmus, Boshoff and Pieterse, 1978). These abnormalities were reported to be identified macroscopically and corroborated using histopathological examination.

Later cases of disease, caused by the Bryanston serotype(serotype 1), revealed signs of acute heart failure composed of a "flabby" appearance to the heart (most prevalent in the right ventricle) with grey-white mottling or streaky appearance of the myocardium (Erasmus, Boshoff and Pieterse, 1978). These cases showed focal areas of fibrosis in the myocardium or in some cases, degenerative changes of the myocytes with associated haemorrhage (Erasmus, Boshoff and Pieterse, 1978).

2.9-Diagnosis

A variety of diagnostic methodologies have been developed and applied to the identification of infections due to EEV. These methods include viral isolation on a variety of cell lines, serology as well as molecular assays (Tirosh-Levy and Steinman, 2022).

Currently the most practical and rapid diagnostic modalities for determination of a clinical diagnosis of EEV appear to be the real-time PCR assays (Tirosh-Levy and Steinman, 2022). Rathogwa et al. (2014) describe the development of the first real-time RT-PCR assay. This assay makes use of a TaqMan® minor groove binder (MGBTM) hydrolysis probe with the assay designed to target the conserved 5' terminal end sequence of the segment 7 (S7) gene (Rathogwa *et al.*, 2014). This assay was noted to be specific for all 7 serotypes of EEV without detecting AHS or BTV. It also displayed reportedly appropriate sensitivity (though the specific value is not indicated) with a 95% limit of detection of $10^{2.9}$ $TCID_{50}$/ml blood (95% confidence interval: $10^{2.7}$ to $10^{3.3}$). The PCR efficiency for this assay was also determined to be 81% (Rathogwa *et al.*, 2014). Maan, Belaganahalli, Maan, Potgieter & Mertens (2019) further developed a species-specific RT-PCR assay targeting the segment 9 (S9) portion of EEV as well as a serotype-specific RT-PCR assay targeting segment 2 (S2) of the EEV genome. These assays were also noted to be specific with no detection of other Orbiviruses. Efficiency of the segment 9 assay was identified as 91.3% (Maan *et al.*, 2019).

2.10-Treatment, prevention, and control

No specific treatment strategies for EE are currently available. Treatment is based on symptomatic management of clinical signs such as fever. There is also no currently available vaccine for EEV (Aharonson-Raz *et al.*, 2011; Dhama *et al.*, 2014; Tirosh-Levy and Steinman, 2022). Considering this, management practices focusing on prevention of the disease are indicated and valuable. Prevention strategies should focus mainly on the reduction of vectors in the environment as well as reduction in contact with these vectors i.e., *Culicoides* midges. Insect repellents and insecticides applied in the stable environment and directly to horses at risk can serve as a valuable prevention approach. Stabling of horses from before sunset and until after sunrise is also recommended as these are reportedly the times of greatest vector activity (van der Rijt *et al.*, 2008; Venter, Boikanyo and de Beer, 2019). Applying vector protection such as screens to the entry points of the stables i.e., windows and doors, can also assist in prevention of the disease (Tirosh-Levy and Steinman, 2022).

Chapter 3: Materials and Methods

3.1-Study Design

This study was a retrospective, (descriptive) observational case series.

3.2-Study population

Medical records of horses that were examined by the Onderstepoort Veterinary Academic Hospital (OVAH) of the University of Pretoria and diagnosed with EEV from 2013 to February 2023 were reviewed. Horses were included in the dataset if they met the following inclusion criteria:

1) The horse tested positive for EEV based on a PCR performed on whole blood with a cycle threshold (CT) count less than 35. The cut-off point is considered by the laboratory to be the point above which the PCR is unlikely to identify viral genetic material even if it is present (Rathogwa *et al.*, 2014).
2) The horse had no evidence of piroplasmosis on peripheral blood smear.
3) The horse was negative for AHSV based on PCR results.
4) The horse was preferably negative for Equine Herpes virus based on PCR results or could be reasonably excluded based on signalment and history.
5) *Streptococcus equi* subs *equi* infection was ruled out based on history, clinical signs, and where applicable, appropriate bacterial culture.
6) Other infectious causes of pneumonia were ruled out based on clinical exam, thoracic ultrasound, respiratory sampling, cytology, and culture.
7) Peritonitis was ruled out based on abdominal ultrasound and/or abdominocentesis.
8) Enterocolitis was ruled out based on clinical signs, abdominal ultrasound, and haematology.
9) The horse was preferably tested for infection with any of the neurological arboviruses (West Nile Virus, Shuni, Sindbis and Middleburg Virus) and was negative based on PCR of whole blood and/or serology.
10) Other obvious concurrent disease was excluded based on a standard diagnostic approach and evaluation including clinical examination, routine haematology, and biochemistry (complete blood count, serum biochemistry and venous blood gas), thoracic and abdominal ultrasound and abdominocentesis.

3.3-Sampling procedure:

Data from individual horses that met the inclusion criteria was analysed retrospectively. Information obtained from the medical records included age, breed, sex, weight, time of the year at evaluation, primary presenting complaint at the time of evaluation and previous treatments prior to evaluation. Results of the physical examination on presentation, diagnostic tests performed within 24 hours of admission including RT-PCR for EEV and Ct count, complete blood count, venous blood gas analysis and biochemistry and where available in subsequent examinations were recorded. Abnormalities on the follow-up clinical examination were recorded once for each individual regardless of how many follow-up examinations revealed these abnormalities. Duration of illness prior to presentation, total number of days of pyrexia from when it was first identified, total length of hospitalization and patient outcome were also recorded.

3.4- Data analysis:

Qualitative (nominal) data was summarised in the form of a frequency table and then expressed in the form of a percentage.

Quantitative data was checked for normality using the Shapiro wilk test. Normally distributed data was then summarized as mean ± standard deviation (sd). Non-parametric data was summarized in the form of median ± range.

When assessing the clinical parameters and haematology, abnormalities were defined based on the normal reference range for the specific age categories, the reference ranges supplied by the appropriate laboratory or as can be found in the literature when not available for the laboratory in question. Due to the lack of cases <12 months of age, descriptive statistics for quantitative data was only calculated for cases which could be categorised as >12 months.

Chapter 4: Results

4.1: Study population

Analysis of the patient records between the periods of January 2013 and March 2023 revealed a total number of 37 patients with positive test results on Rt-PCR for EEV.
Of these 37 positive cases, 32 cases (86.49%) met the inclusion criteria. Of the 5 cases that tested positive for EEV but were excluded from this group, 1 horse had concurrent infection with AHSV, one horse had concurrent surgical site infection of an exploratory laparotomy site, one horse had fungal pneumonia caused by a Mucor species, one horse had bacterial pneumonia due to *Streptococcus equi* subspecies *zooepidemicus* and one horse had a large colon torsion. These cases were excluded from data acquisition in all instances where it was deemed that the results could logically be biased due to the concurrent disease process. None of the EE cases were excluded based on the Ct count obtained from PCR testing for EEV.

4.2: Signalment

The sex distribution of the study population can be found in Table 3 while the breed distribution is displayed in Table 4. The ages ranged from 3 months to 20 years old with a median age of 9 years and 6 months. Weight data was available for 27/32 (84.4%) cases with a median of 507 kg and a range of 95 to 700 kg.

Table 3: Sex distribution of cases of EE.

Sex	Number	Percentage (%)
Stallion	6	18.8
Gelding	13	40.6
Mare	13	40.6
Total	32	

Table 4: Breed distribution for cases of EE.

Breed	Number	Percentage (%)
Thoroughbred	11	34.4
Warmblood	10	31.3
Friesian	4	12.5
Boerperd	2	6.3
Nooitgedacht	2	6.3
Nordic	1	3.1
Trakehner	1	3.1
Percheron	1	3.1
Total	32	

4.3: History

Between the years of 2013 and 2023, the highest number of horses testing positive for EEV in this data set was seen in 2014, and 2020, with no cases seen in 2018. Each year during this period had a median of 2 cases (range 0-7). EEV cases were reported to occur between the months of December and April with an isolated case in June. The distribution of these cases is presented in Table 5.

Table 5: Distribution of EEV cases according to the months of the year.

Month	Number of cases	Percentage (%)
January	2	6.2
February	9	28.1
March	9	28.1
April	10	31.2
May	0	0
June	1	3.1
July	0	0
August	0	0
September	0	0
October	0	0
November	0	0
December	1	3.1
Total	32	

Of the total cases determined to be positive for EEV, 29/32 (90.6%) of cases had pyrexia (rectal temperature >38.5 degrees Celsius) identified as the primary presenting complaint. The remaining horses that did not have fever as the primary presenting complaint (9.3%) were examined due to colic (3.1%), supraorbital swelling (3.1%) and collapse (3.1%). Fever was reported as the only presenting complaint in 40.6% of cases. In addition to the primary complaint of fever, presenting complaints for several horses included colic (21.9%), neurological signs (9.4%), inappetence (12.5%), lethargy (3.1%), supraorbital swelling (3.1%) and coughing (3.1%). The neurological signs reported in these cases included ataxia, stupor, and agitation.

History on prior treatments was recorded in 28/32 (87.5%) of cases. Horses received non-steroidal anti-inflammatories (NSAID) including flunixin meglumine and phenylbutazone prior to presentation in 12/28 (42.9%) cases. Horses received a combination of NSAIDS and dexamethasone (corticosteroid) prior to presentation in 3/28 (10.7%) cases. A combination of NSAIDS and dipyrone was administered prior to presentation in 2/28 (7.1%) cases and dipyrone was administered without concurrent NSAIDS in 1/28 (3.6%) cases prior to presentation.

4.4: Results of clinical examination

Of the 32 cases determined to have illness due to EEV, 4 cases had no specific clinical examination data recorded other than the Ct values and diagnosis. These cases were excluded from the analysis of clinical signs as well as clinicopathological findings.

Horses suffering from EE displayed a variety of different clinical abnormalities on presentation (Figure 2) and throughout the illness (Figure 3). Of the 15/28 (53.6%) cases that did not have pyrexia on presentation, only 3/28 (10.7%) had not received antipyretic medications prior to clinical examination.

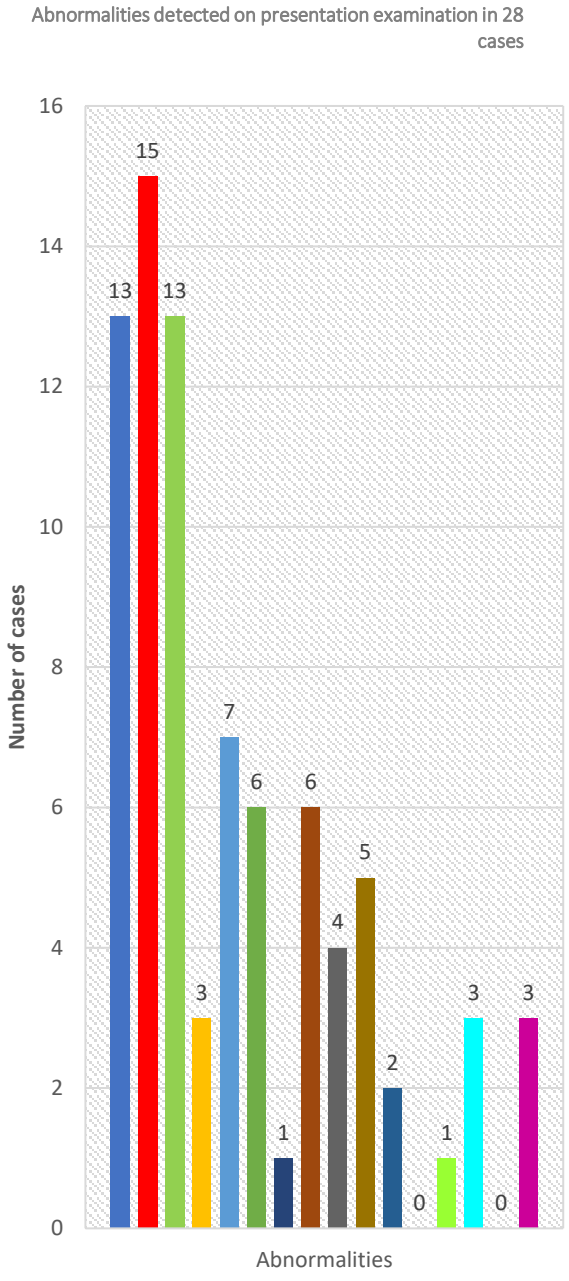

Figure 2: Clinical abnormalities detected on presentation in horses with EEV

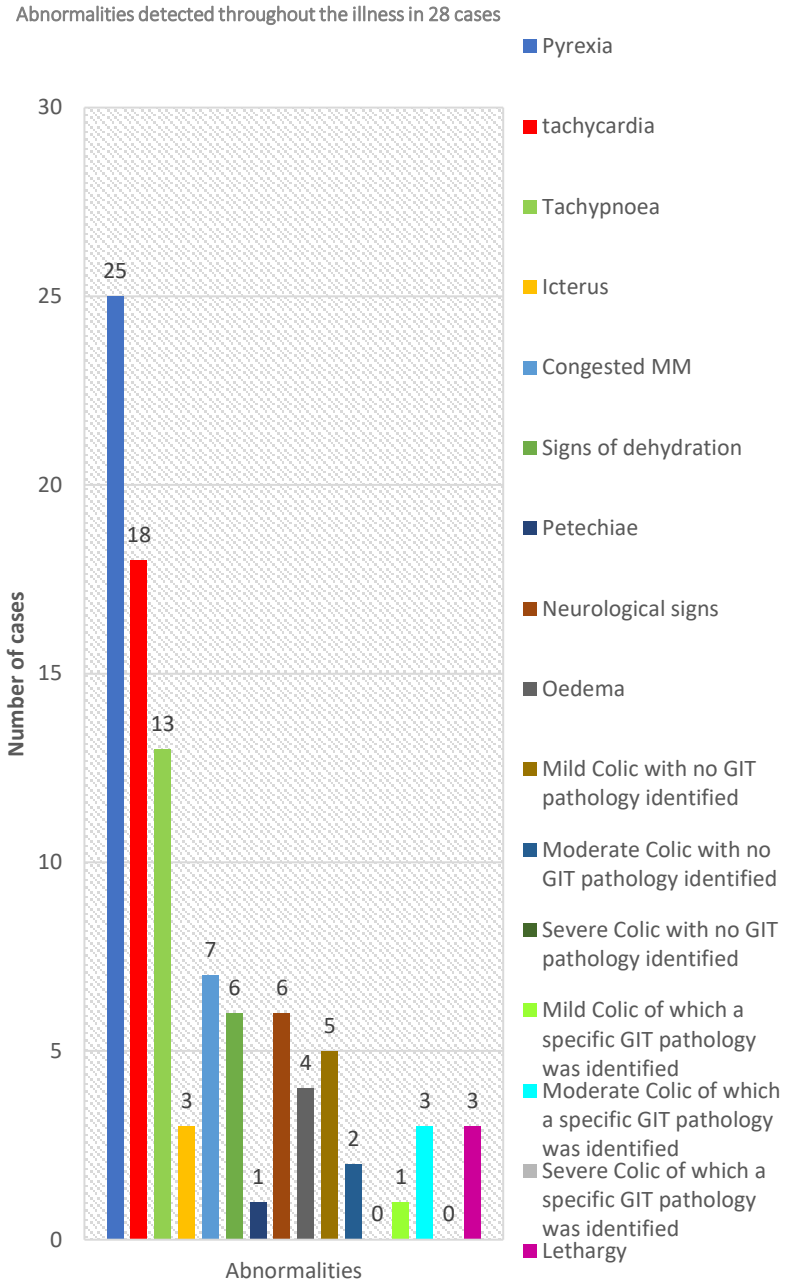

Figure 3: Clinical abnormalities detected on follow-up examinations in horses with EEV

In this data set, 3/28 (10.7%) cases were noted to be less than 12 months of age with each case part of an age category with different reference range clinical parameters. Due to this fact, descriptive statistics were only calculated for horses >12 months which amounted to 25/28 (89.3%). Horses presenting with EE that had temperatures recorded, had an average temperature of 38.5 °C (sd ±0.93^{o}C). Horses presenting with EE that had heart rates recorded, had a median heart rate of 48 beats per minute (range: 36;100). Horses presenting with EE that had respiratory rates recorded, had a median respiratory rate of 24 (range: 12; 60).
Throughout the illness, horses with EE had an average recorded maximum temperature of 39.3 °C (sd ±0.86^{o}C), a median maximum recorded heart rate of 52 beats per minute (range: 36;100)

including during times of pyrexia, and a median maximum recorded respiratory rate of 24 (range 12; 60) including during times of pyrexia. The descriptive statistics are visually represented in Figures 4-6 and compared in table 6.
Throughout the illness, 18/28 (64.3%) cases had tachycardia at times concurrent with fever. In addition, 15/28 (53.6 %) of the cases displayed evidence of tachycardia at times without concurrent evidence of fever (i.e., that could not be attributed to the influence of fever at the time). These cases had a median maximum heart rate of 46 beats per minute (range: 36;100) when not displaying clinical pyrexia. In this data set, 2/15 cases had tachycardia that was never associated with fever.

Table 6: Comparison of admission values and maximum values during illness for clinical parameters.

Clinical exam parameter	Admission (mean±sd)	Admission (Median (Range))	Maximum value during illness (mean±sd)	Maximum value during illness (Median (Range))
Rectal temperature (°C)	38.5±0.93	-	39,3±0.86	-
Heart rate (beats per minute)	-	38 (36;100)	-	52 (36;100)
Respiratory rate (breaths per minute)	-	24 (12;60)	-	24 (12;60)

Only 4/18 (22.2%) of the presented EEV cases with tachycardia, had complete electrocardiographic evaluations performed. One horse had recorded evidence of significant pathological arrhythmias which included accelerated idioventricular rhythm as well as isolated ventricular premature complexes. This horse also had an echocardiogram performed and displayed evidence of subjectively poor ventricular diastolic function, hyper-echogenicity of the left papillary muscle as well as subjective thickening of the left ventricular free wall and interventricular septum. This horse also displayed colic signs with no specific cause identified. The other three horses displayed sinus tachycardia with no other recorded arrhythmias. One of these cases of sinus tachycardia also had echocardiography performed with reported subjectively weak and uncoordinated ventricular contraction.

Thoracic ultrasound indicated mild bilateral ring-down artifacts in 8/28 (28.6%) cases and moderate bilateral ring down artefacts over the pleural surface in 1/28 cases (3.57%).

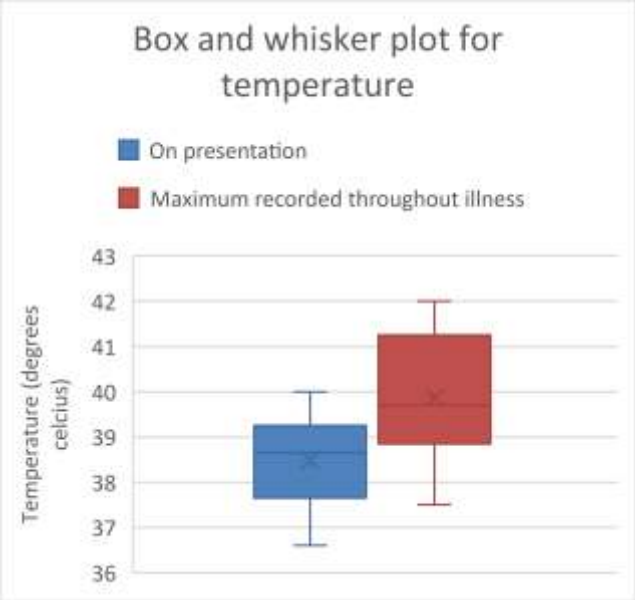

Figure 4: Descriptive statistics for maximum temperature throughout illness.

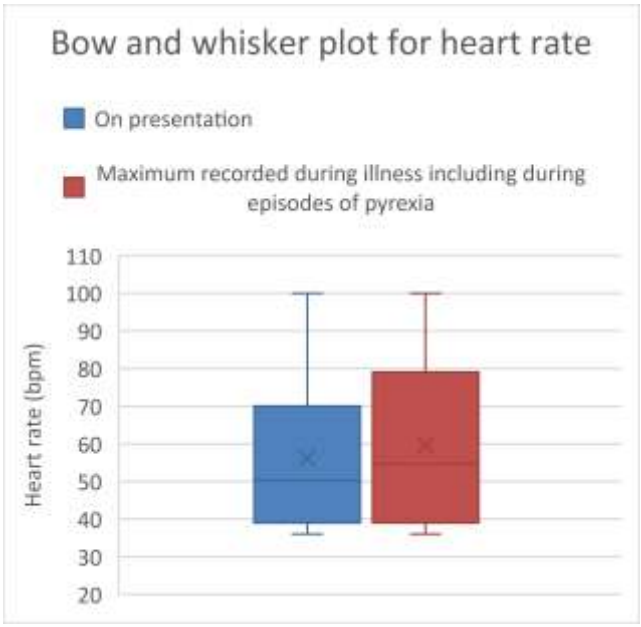

Figure 5: Descriptive statistics for maximum heart rate including during episodes of pyrexia.

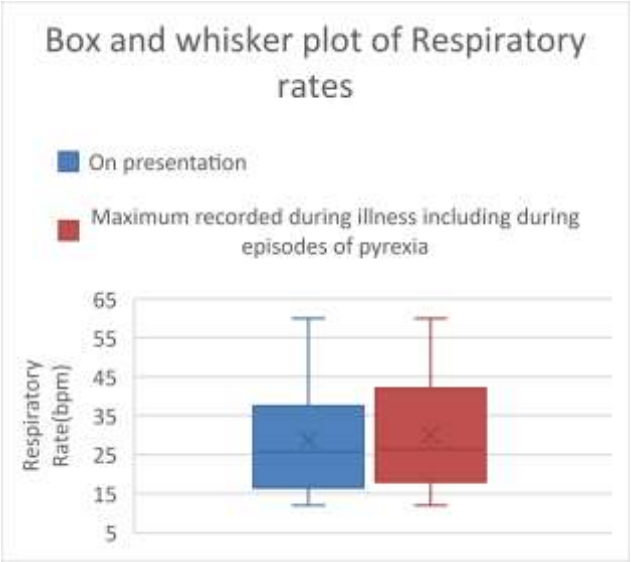

Figure 6: Descriptive statistics for maximum respiratory rate including during episodes of pyrexia.

4.5: Laboratory findings

Of the 32 positive cases that met the inclusion criteria only 1 case did not have a recorded Ct value. The mean Ct value for all positive cases was 29.8 (sd ±2.99). Based on the history, the Ct count was measured on samples taken on the day that illness was detected in 16 cases (50%). The average Ct count from the day clinical signs were first detected was 30.03 (sd ± 2.9).

Clinicopathological data was collated and analysed for horses where it was available for within 24 hours of admission. Descriptive statistics were calculated for horses >12 months of age (this excluded one case based on age related differences) and are summarized in Table 7. Total white blood cell counts were available for 25/28 (89.3%) cases of which 1/28 (3.6%) was < 12 months of age. Complete blood counts were available for 22/28 (78.6%) cases all of which were >12 months of age. No data was available to assess the leucogram in 3/28 cases (10.71%). Assessment of the total white blood cell count was performed within 24 hours of onset of disease signs in 14/25 (56%) cases. Of these cases, 6/14 (42.9%) showed leucopenia within 24 hours of the onset of disease signs.

Venous blood gas assessment was performed in 11/28 (39.3%) of EE cases. Values encompassed in the venous blood gas analysis included: pH, partial pressure of carbon dioxide, bicarbonate, sodium, chloride, calcium, and potassium. A summary of the findings with regards to the abnormalities detected on clinical pathology for all 28 cases is presented in Figure 7. Additional graphical representation of the haematological findings can be found in Figures 8-12 with a graphical representation of total protein findings in Appendix 2.

Table 7: Descriptive statistics for clinicopathological parameters

Variable	Unit	Reference range	Number of cases tested <12 months old	Median	Range	Mean ± Standard deviation	Number of cases above reference range (%)	Number of cases below reference range (%)
Leucocytes	x 10^9 cells/L	*6-12	24	5.44	2,08-18.07		2 (8.3%)	15 (62.5%)
Mature neutrophils	x 10^9 cells/L	*3.54-7.08	22	3.69	1.09-12.79		2 (9.1%)	10 (45.4%)
Immature Neutrophils	x 10^9 cells/L	*0-0.24	22	0.08	0-1.32		7 (31.8%)	0
Lymphocytes	x 10^9 cells/L	*1.8-3.6	22	1.17	0.15-9.21		2 (9.1%)	16 (72.7%)
Monocytes	x 10^9 cells/L	*0-0.72	22	0.18	0-1.26		3 (13.6%)	0
Thrombocytes	x 10^9 cells/L	**>100	24	67.5	3-303		0	16 (66.7%)
Red blood cell count	x 10^{12} cells/L	*5.5-9.5	24	7.75	5.53-12.34		2 (8.3%)	0
Haematocrit	l/l	*0.24-0.44	24	0.34	0.24-0.54		2 (8.3%)	0
Gamma-glutamyl transferase	U/L	*2-25	9	15	0-73		1 (11.1%)	0
Glutamate dehydrogenase	U/L	*1-8	9	3	0-11		1 (11.1%)	0
Albumin	g/L	*28-39	10			30.99 ±2.64	0	2 (20%)
Globulin	g/L	*28-44	10			28.91 ±7.07	0	5 (50%)
Total Serum protein	g/L	*66-78	13			61.85±6.88	0	10 (76.9%)

Creatinine	umol/L	*105-170	8			105.00±20.81	0	4 (50%)
Urea	mmol/L	*3.8-7.7	5			3.92±0.63	0	1 (20%)
Total bilirubin	mmol/l	*8.3-30.2	5			63.96±15.61	5 (100%)	0
Serum Amyloid A	mg/l	*0-24	6	386.35	5.2-1817.1		5 (83.3%)	0
Blood lactate	mmol/l	**<2	6	1.2	0.47-4.1		1 (16.7%)	0
Sodium	mmol/L	*136-144	12			133.67±3.65	0	8 (66.7%)
Potassium	mmol/L	*2.1-5.1	12			3.42±0.45	0	0
Chloride	mmol/L	*96-110	7	103	92-104		0	1 (14.3%)
Ionised calcium	mmol/L	***1.48-1.65	11			1.5±0.068	0	0
PH		*7.32-7.44	12			7.43±0.04	4 (33.3%)	0
Bicarbonate	mEq/L	***27.06-32.94	12			24.56±1.81	0	0
Partial pressure of Carbon dioxide	mmHg	**37-43	12			38.57±4.18	0	1 (8.3%)

* Reference range obtained from University of Pretoria Faculty of Veterinary Science Clinical Pathology Laboratory

** Reference range obtained from Bayly, Sellon & Reed (2009)

*** Reference range obtained from Lascola et al. (2017)

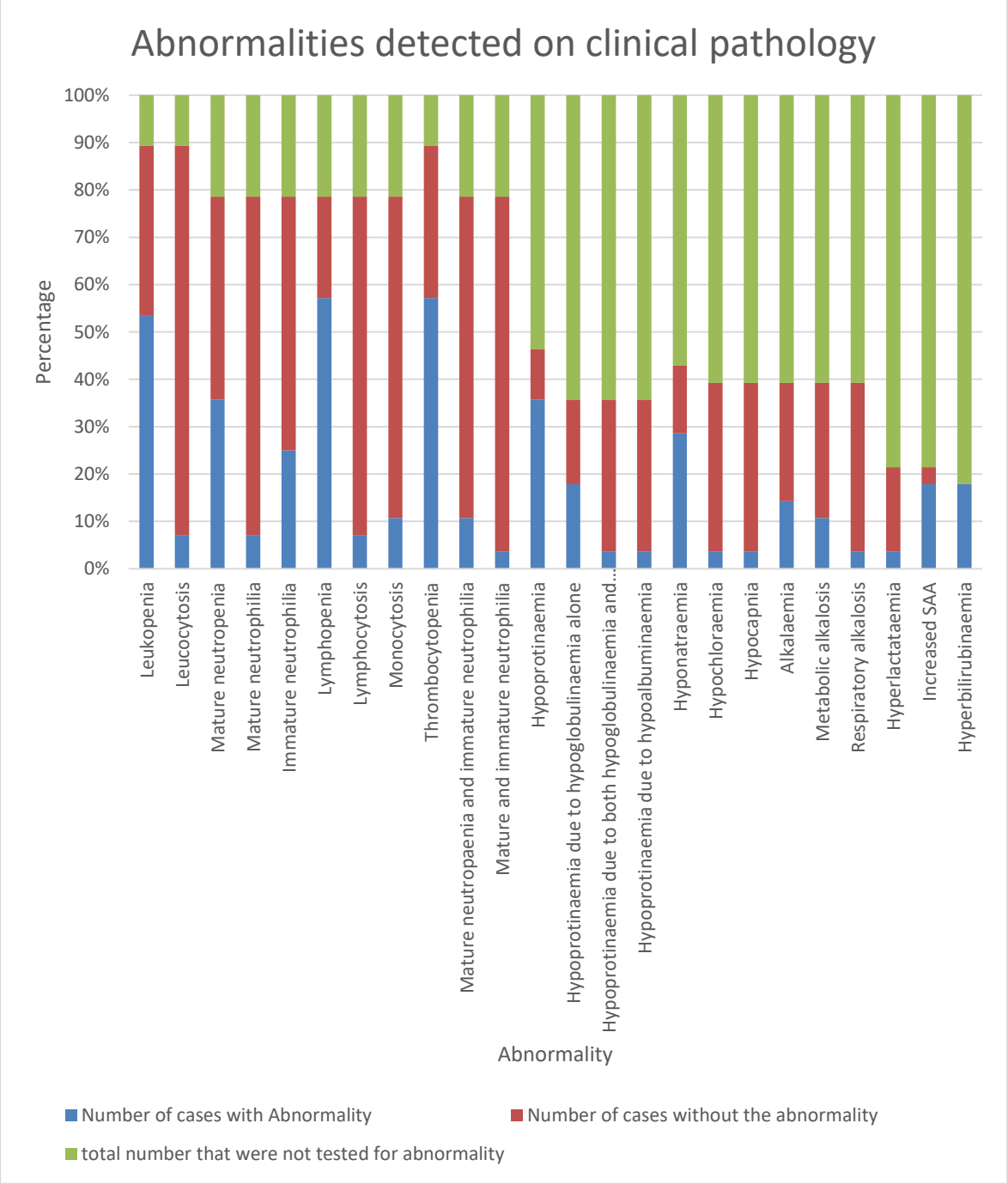

Figure 7: Percentage of cases that developed abnormalities as a representation of the total number of cases.

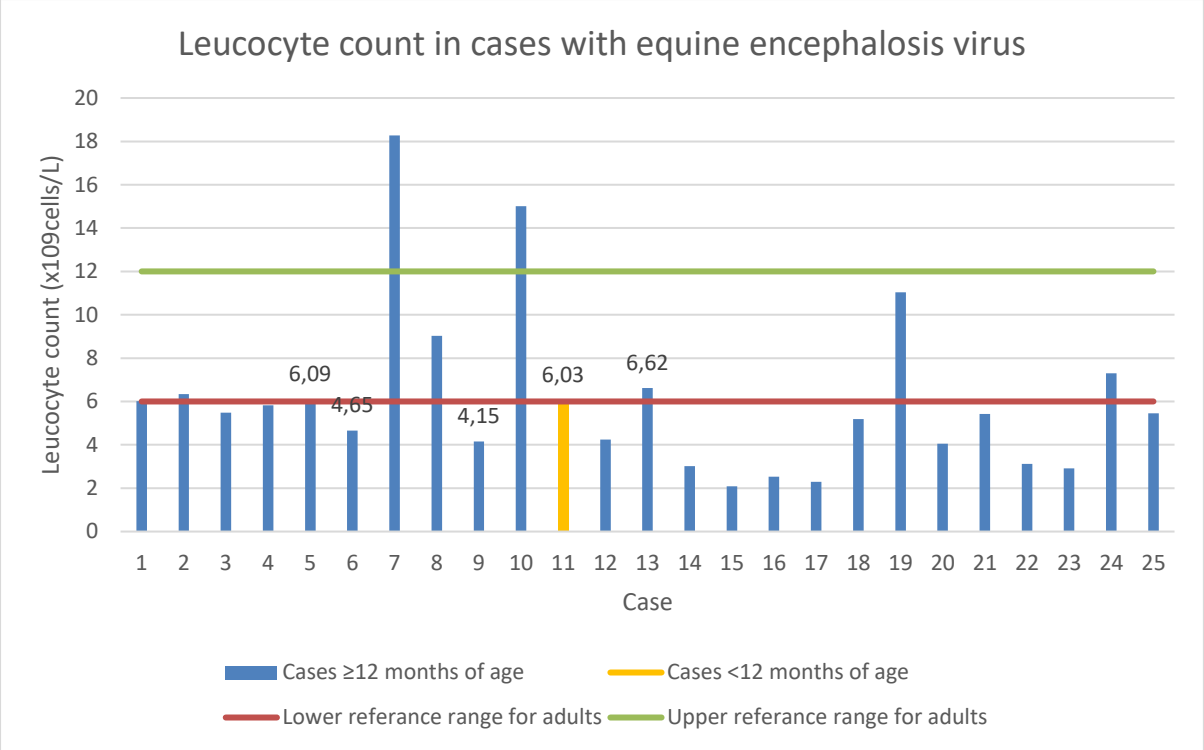

Figure 8: Leucocyte count in cases of EE determined within 24 hours of presentation to the Onderstepoort Equine Clinic.

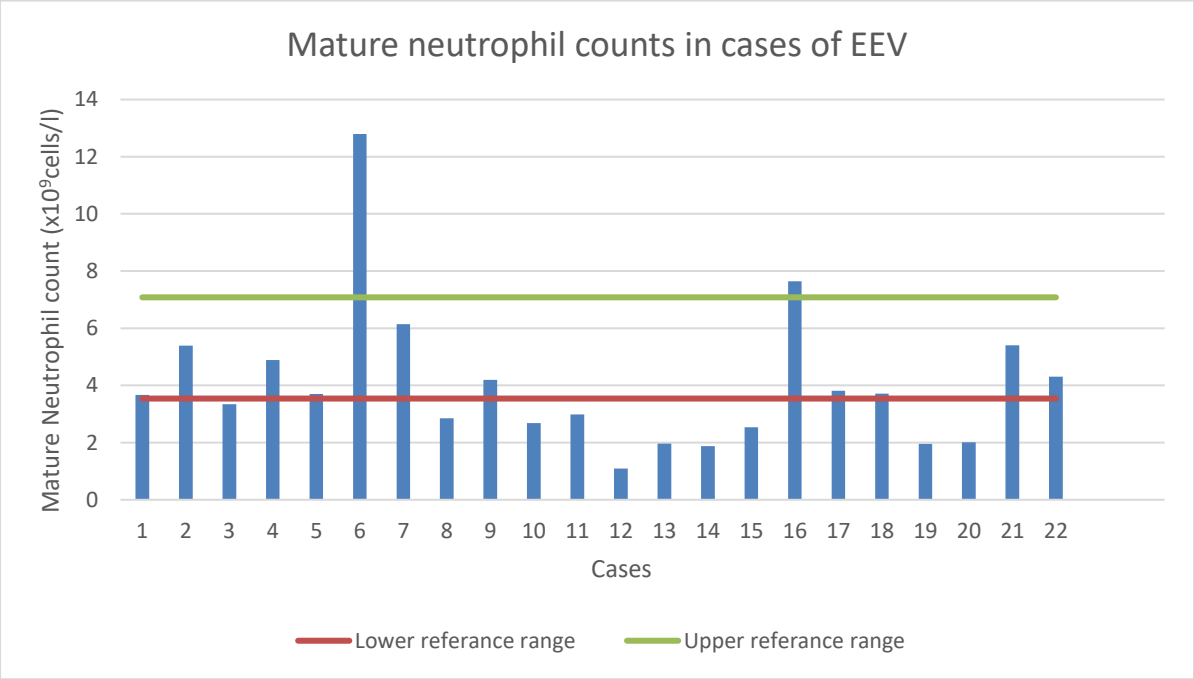

Figure 9: Mature neutrophil count in cases of EE determined within 24 hours of presentation to the Onderstepoort Equine Clinic

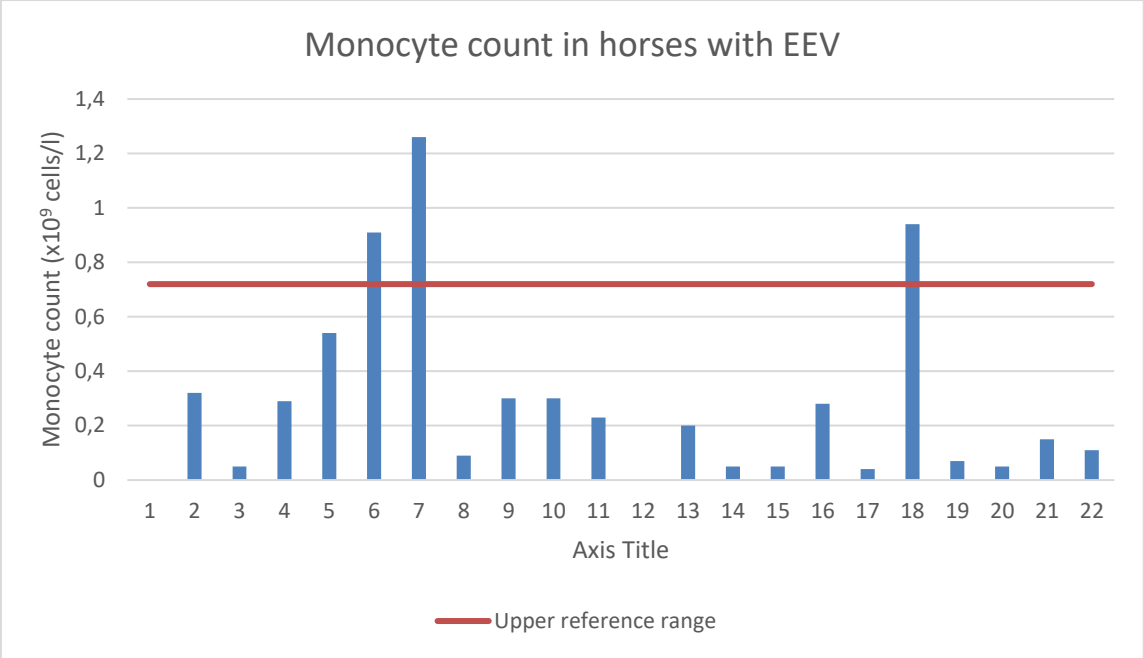

Figure 11: Monocyte count in cases of EE determined within 24 hours of presentation to the Onderstepoort Equine Clinic

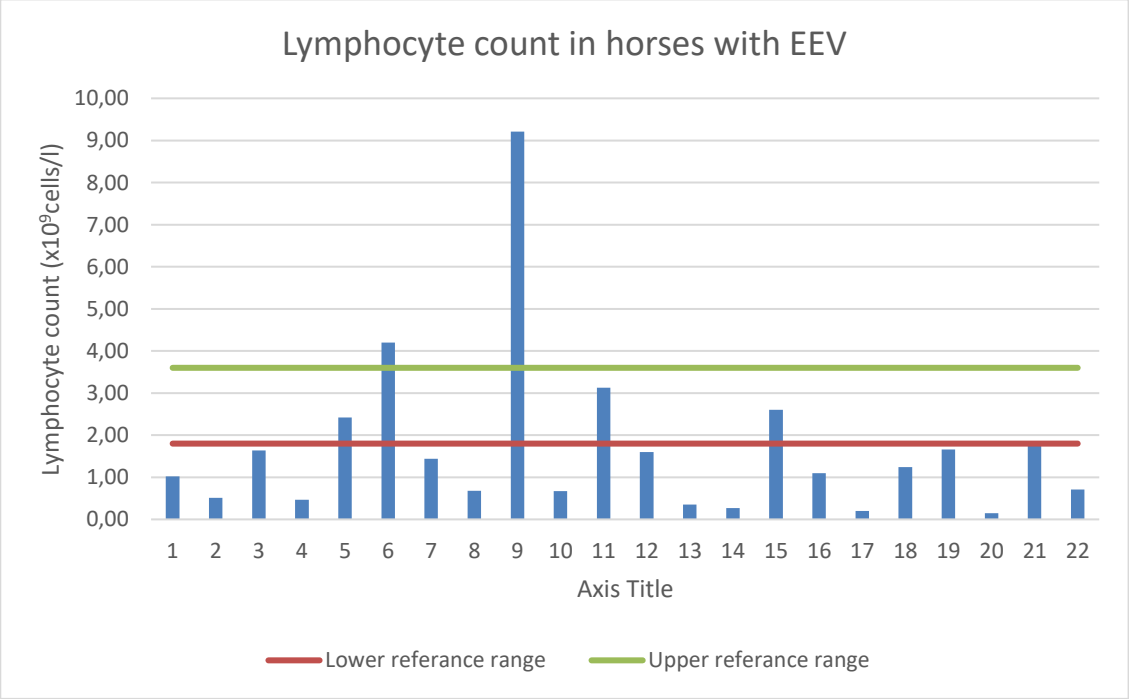

Figure 10: Lymphocyte count in cases of EE determined within 24 hours of presentation to the Onderstepoort Equine Clinic.

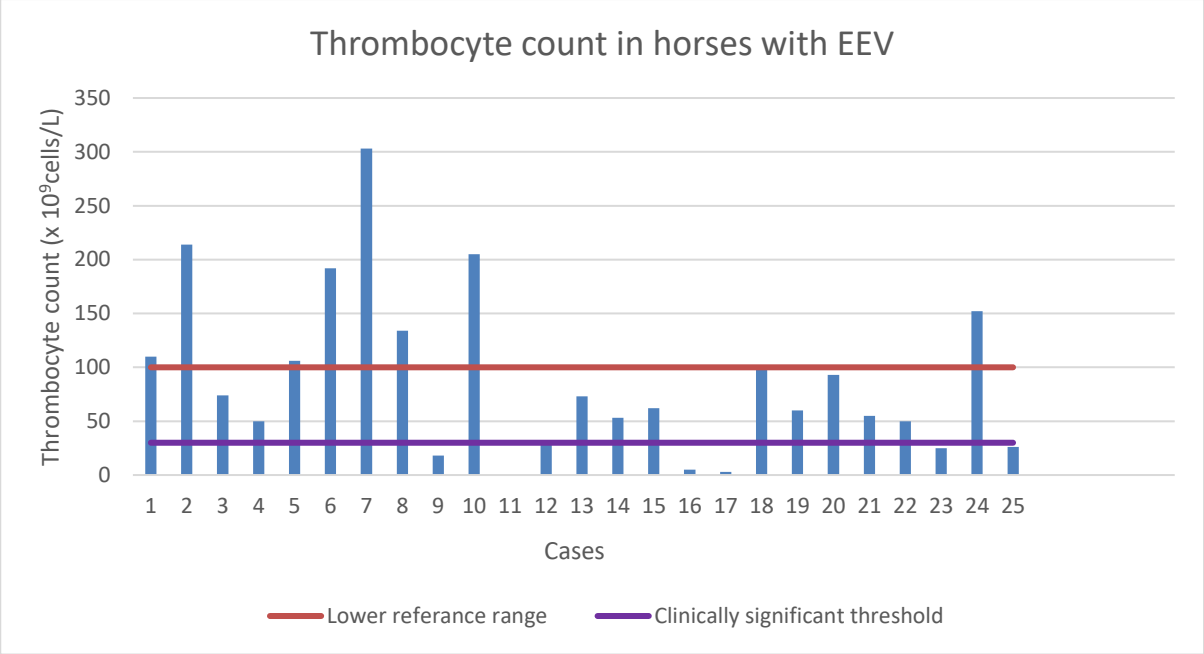

Figure 12: Thrombocyte count in cases of EE determined within 24 hours of presentation to the Onderstepoort Equine Clinic

4.6: Results of hospitalization

In addition to the presence of pyrexia on initial evaluation, 24/28 (85.71%) cases had repeated measurements of their rectal temperatures recorded. EE cases had a median of 3 (range:1;7) days of fever despite the regular administration of NSAIDS in all cases reported.

EE cases underwent veterinary examination by the OVAH on the day clinical signs were identified by the owner in 17/28 (60.7%) of cases. In 1/28 (3.6%) cases, no reference to the date of initial onset of clinical signs could be identified while the remaining 10/28 (35.7%) cases had an average of 2.4 days of illness (sd ±1.5 days) before they were presented to the OVAH for evaluation with a maximum of 5 days prior to presentation. The number of days prior to referral can be seen in Table 8.

Table 8: Number of days of illness prior to presentation

Days of illness prior to presentation	Number of cases
0	17
1	4
2	2
3	1
4	2
5	1
unknown	1

Horses were hospitalized in 24/28 (85.7%) of the EE cases examined by the OVAH over this period with 21/24 (87.5%) of the hospitalized cases examined on a referral basis (as opposed to first opinion). The remaining 5/28 (17.9%) cases were treated on an outpatient basis by the OVAH. The total number of days of hospitalization had a wide range due to cases developing complications while in hospital. The median number of days that horses were hospitalized (including cases with complications) was 6 days (range 2;27) while the median number of days that horses were hospitalised without substantial complications was 5 days (range: 2;15).

During hospitalization 3/24 (12.5%) of the cases developed complications. Complications in these cases included jugular vein thrombophlebitis associated with the jugular vein used for intravenous catheter placement (n=2), one of which also developed bacterial bronchopneumonia due to *Streptococcus equi* subsp. *zooepidemicus*. Another horse developed subsolar abscess in the right hind hoof as well as acute laminitis in all hooves. A 3-month-old filly died because of EE within 24h of the development of clinical signs, the rest were discharged from veterinary care. Horses were identified as clinically healthy at the time of discharge or at the end of the treatment period in 26/28 (92.9%) cases. One horse was reported to have a grade 3 ataxia still present at the time of discharge. Follow-up evaluation in this horse indicated a grade 1 ataxia after a period of 9 weeks.

Non-steroidal anti-inflammatories were administered in 26/28 (92.9%) cases of EE with antipyretic efficacy observed in all the cases. Non-steroidal anti-inflammatory drugs in these cases included phenylbutazone (oral administration), flunixin meglumine (intravenous and oral administration) and meloxicam (intravenous and oral administration). Corticosteroids in the form of dexamethasone and prednisolone was administered to 5/28 (17.9%) cases of EE. Antimicrobials were administered in 8/28 (28.6%) of EE cases with 4 cases receiving trimethoprim sulfamethoxazole, 1 case receiving trimethoprim sulfamethoxazole initially followed by procaine penicillin and gentamicin, and 3 cases receiving procaine penicillin combined with gentamicin. Intravenous crystalloid fluid therapy was administered in 11/28 (39.3%) cases of EE with enteral fluid therapy (isotonic fluids) administered in

3/28 (10.7%) cases. A lignocaine constant rate infusion was used in 3/28 (10.7%) cases and digital cryotherapy in 3/28 (10.7%) cases. Additional medications administered in individual cases included synthetic hydroxyethyl starch-based colloids (Voluven®), low molecular weight heparin (enoxaparin), hypertonic saline (5%), aspirin, furosemide, dobutamine, and acyclovir.

Chapter 5: Discussion

5.1-Clinical and economic relevance

EE is widely purported to be a viral disease of limited clinical importance and minor economic relevance (Howell *et al.*, 2008; Tirosh-Levy and Steinman, 2022). The driving factors behind this impression appear to stem from the wide seroprevalence of the disease in Southern Africa with a disproportionately smaller number of individual animals reported to display severe debilitating disease or mortality (Paweska & Venter, 2004; Snyman et al., 2021). Although data from study indicates an almost insubstantial mortality rate of 3.6% with no adult horses succumbing to the disease, the morbidity and economic relevance attributable to this disease is a more complicated factor when appraising whether the disease should be confined to the category of an inconvenient distractor with limited clinical relevance.

Considering its close clinical resemblance to some cases of AHS as well as the similarities in epidemiology, EE should be considered as an important "imitator" for AHS. As reported here, EE can lead to a variety of clinical signs and as such act as a "confuser" for a multitude of other disease processes. This coupled with the possibilities of co-infection means these cases often require multiple diagnostic modalities to ensure complete definitive diagnosis, a fact already highlighted by Sir Arnold Theiler (Theiler, 1910). This contributes not only to the economic aspects of these cases but also to the need for a diverse array of diagnostic capabilities. This is particularly the case when dealing with clinical cases showing a variety of clinical signs over and above fever.

Considering the widespread nature of the disease in Southern Africa: the multiple diagnostic modalities needed to rule out different differential diagnosis; the management practices required for prevention; and the fact that a large portion of the horses presented in this study were hospitalized for treatment for several days suggests that the economic relevance of EEV should not be discounted. The total duration of fever in horses in this data set is on average similar to that reported for "ephemeral fever"(Theiler, 1910). There are, however, some cases that display longer periods of fever with some cases displaying up to 6-7 days of fever despite non-steroidal anti-inflammatory therapy. This equates to financial expenses with regards to diagnostics, symptomatic treatment and additionally contributes to the stressors associated with disease for the owner and veterinary management team.

The clinical relevance of the disease for the individual patient is more difficult to define with the current level of research. Although not frequently reported, more severe clinical signs associated with EEV have been identified in the literature including neurological disease and sudden death (Erasmus *et al.*, 1970; Erasmus, Boshoff and Pieterse, 1978; Steyn *et al.*, 2021) . This combined with the several clinical signs such as ventricular arrythmias identified in this research suggest that further research into the pathogenesis, with more detailed monitoring of physiological disturbances is required before a definitive statement in this regard can be made.

5.2-Epidemiology and clinical signs

The specific reasons for the years during the study period with the highest number of cases were not investigated here as they were beyond the scope of this project. These years do not correspond with higher average rainfall in the Gauteng province or significant deviations from the average temperatures when historical records of weather (South African Weather Service, 2023) are evaluated in conjunction with our data, which would be the obvious causes for changes in disease numbers. The number of cases seen on a referral basis during these years does however not necessarily reflect the total number of cases over the country or even the local area. Factors such as

the absence of an ambulatory service offered by the OVAH until 2019 could be influential in determining the case load seen in this clinic rather than disease prevalence in specific years.

Infections in this data set were noted to be most common from February to April each year, corresponding to the late summer and early autumn period in Southern Africa. Similar findings have been reported for AHS (Schliewert *et al.*, 2022) as well as BTV regarding the times of peak infections (Steyn *et al.*, 2016). This is likely a function of the epidemiology of the virus and its vector transmission. *Culicoides* midge numbers start to increase co-inciding with the warmer temperatures and wetter weather typical for the Southern African climate during the rainy season (Nevill, 1967). In areas that receive frost, *Culicoides* numbers slowly start to rise from spring, peaking in late summer. A rise in the ratio of parous female *Culicoides* can also be seen as the summer season progresses. Virus can only be transmitted by parous females and as such, an increase in the ratio of parous females corresponds with a greater vector potential (Venter, Nevill and Van Der Linde, 1997). The lack of cases in the winter months correlate with the colder temperatures and reduced rainfall seen over much of South Africa during this period. Lower temperatures reduce vector activity and maturation, and decreased rainfall reduces the number of suitable habitats for vector breeding (Venter et al., 1997).

No specific information is available for the influences of temperature on the infection rates and virogenesis of vectors with EEV. Infection rates of vectors with AHS and its virogenesis are reported to be temperature dependent with higher environmental temperatures leading to increases in both of these factors (Mellor and Hamblin, 2004). Further research is required to determine if similar temperature responses are seen with EEV. Considering the similarities in vector transmission and seasonal occurrence, EEV could be considered as a valuable indicator that horses in the vicinity of positive EE cases are at high risk of exposure to or development of AHS. Positive cases of EE in a stable yard should serve to heighten the vigilance and management interventions aimed to prevent AHS and other arboviral diseases.

A large portion of the cases identified as positive for EEV displayed episodes of pyrexia. This is supported by the findings described by Snyman et al. (2021) indicating that there was a significant association between pyrexia and infection with EEV although the actual proportion of cases that were positive for EEV and had pyrexia was greater in the present study. It must be considered that due to its close clinical associations with fever (Erasmus B. *et al.*, 1970), horses presenting without fever are less likely to be tested for EEV, which could lead to confounding relating to the true case prevalence of fever in this study. As can be seen by the clinical cases presented, rectal temperatures of 40 ^{o}C and above can be seen in cases of EE. This corresponds with findings from confirmed cases of EE in the literature (Erasmus et al, 1978; Aharonson-Raz et al., 2011; Wescott et al., 2013).

Additional common clinical signs identified on clinical examination included tachycardia as well as tachypnoea. This coincides with the findings reported by Theiler (1910) for the disease he referred to as equine “ephemeral fever” as well as findings from Erasmus et al. (1978) and Aharonson-Raz *et al.*, 2011 regarding tachycardia, and Mildenberg et al. (2009) regarding tachypnoea. The exact cause for the episodes of tachycardia and tachypnoea have not been intuited. Although pyrexia is a known cause for sinus tachycardia (Bayly, Sellon and Reed, 2009), a large portion of the horses had tachycardia without the concurrent presence of fever. This may represent the inaccuracies of using rectal temperature as a proxy for core body temperature, although could also suggest other mechanisms leading to tachycardia. Considering the fact that tachyarrhythmias can have a multitude of underlying mechanisms besides sinus tachycardia, thorough evaluation of the origins of tachycardia would require electrocardiography. Further investigation of the tachycardias using electrocardiography was only performed in a small number of cases but indicated that 25% of cases that underwent ECG had evidence of other abnormal rhythms. This further raises the question of

whether more of the cases had pathological arrythmias that were not identified because no ECG was performed. To the authors knowledge, no other studies have examined ECG findings in cases of EE. Additionally, both cases that had echocardiography performed had identifiable abnormalities. Combining this information and considering the literature reports that cases of infection with the Bryanston serotype (serotype 1) of EEV had histopathological evidence of myocardial degeneration, fibrosis and haemorrhage (Erasmus, Boshoff and Pieterse, 1978), a more thorough evaluation of the cardiovascular system including echocardiography and electrocardiography may have the potential to contribute valuable information to understanding the pathogenesis and cardiovascular consequences of this disease.

Tachypnoea was observed as a common clinical finding in EE similar to AHS. The finding by Steyn et al. (2021) of an association between EE and dyspnoea seems to support the idea of EE leading to pulmonary pathology. Additional pulmonary evaluations in the present data set generally only revealed subjectively minor clinical indicators of pathology and as such a definitive cause for this clinical sign remains hypothetical. In particular, the severe pulmonary oedema and pleural effusions often present in cases of AHS (Long and Guthrie, 2014) were conspicuously absent in the present cases of EE. Further evaluation of the pulmonary parenchyma at the histopathological level should be considered in cases of EE to better understand the target tissues and complex interplay between virus and host tissue.

Colic was reported in 39.29% of the clinical cases in this data set with 27.02% of these cases presenting or being referred to a hospital setting for this reason in conjunction with fever. Specific gastrointestinal tract (GIT) lesions were only identified in 10.71% of the cases and included large colon impaction, large colon displacement and gastric ulceration. The remaining cases had no specific GIT pathology identified on routine GIT evaluation. Colic signs have been reported previously in cases of EE (Aharonson-Raz *et al.*, 2011) but this literature does not expand on reasons for these signs in association with the disease. The lack of specific GIT lesions identified on routine evaluation of multiple cases means that more detailed research into the cause of this clinical sign is required. These findings may however suggest that the colic may not be solely due to the development of secondary GIT lesions such as large colon impactions but rather related to direct effects of EEV on the GIT. Scant reports in the literature of segmental areas of catarrhal enteritis (Erasmus, Boshoff and Pieterse, 1978; Tirosh-Levy and Steinman, 2022) lend credence to the idea of a primary lesions caused by EEV in the GIT.

Neurological signs were recorded as an infrequent clinical sign in these cases. Neurological abnormalities recorded included ataxia, stupor, anxiety and hyperaesthesia, abnormal head position with the head lowered, head tilt, tongue paresis, seizure like activity, and weakness. This small number of cases with neurological signs is in contrast to the findings discussed by Steyn et al. (2021), who reported 47% of EEV cases in their study having neurological signs. Several other arboviral diseases including West Nile Virus and Middelburg virus are present in Southern Africa and also lead to neurological signs. Considering that infection with these neuro-arboviruses were not excluded in all cases in this study as well as the fact that co-infections with some of these viruses and EEV have been reported (Steyn *et al.*, 2021), a definitive link between EEV and the neurological signs cannot be concluded. Despite this, EEV continues to be associated with neurological disease in horses and cannot be eliminated from the differential diagnosis list in these cases. BTV has been associated with clinical signs including weakness, abnormal head and neck position (torticollis) and paresis with the specific pathophysiology relating to skeletal muscle necrosis (Saminathan *et al.*, 2020). It is plausible that some of the clinical signs in this research as well as reported in the literature are due to a similar muscular pathology rather than a primary neurological cause although this statement requires significant substantiation by future research before it can be considered fact.

Several other non-specific clinical signs including lethargy, congested mucous membranes and dehydration were identified. These signs can arise due to a multitude of reasons and as such may or may not be directly ascribed to EEV. Pyrexia can provide a logical explanation as the primary cause of several other clinical signs identified in these cases including tachycardia, tachypnoea, congested mucous membranes, lethargy, and dehydration. Likewise, gastrointestinal pain and or inflammation and neurological disease could result in similar findings. Further research into the pathophysiology of EEV and its specific cellular tropisms is required to better understand the clinical signs directly associated with the pathogenesis.

Although the specific serotypes responsible for the cases of EE and therefore the specific clinical signs identified in this data set were not evaluated, review of the literature does suggest specific clinical signs ascribed to certain serotypes of EEV (Erasmus, Boshoff and Pieterse, 1978). Considering this, it is plausible to hypothesise that the presence of specific clinical signs in a population can be influenced by the serotype of EEV causing the infection with the prevalence of these clinical signs correlating to the prevalence of specific serotypes in the region. Further research regarding the association between clinical signs and specific serotypes of EEV is warranted.

5.3-Clinical pathology and diagnostic testing:

The veterinary literature is sparse regarding the description of clinical cases of EE with no specific publications examining clinicopathological data. As such, this if the first study, to the authors' knowledge, that directly describes clinicopathological findings in cases naturally infected with EEV. In this study, 68% of cases that had haematology performed showed total white blood cell counts outside of the laboratory reference ranges.

The vast majority of these changes were reflected by leucopenia. The most pronounced haematological abnormality comprising these cases of leucopenia was lymphopenia followed by mature neutropenia. Additionally, immature neutrophilia was also seen. Findings of leucopenia, neutropenia with a left shift and lymphopenia have been reported in cases of AHS following experimental infection (Skowronek *et al.*, 1995). The specific cause of the changes in the leukogram in cases of EE are currently not known. Skowronek et al. (1995) hypothesised that similar leucopenia characterized by lymphopenia, neutropenia, and left shift, seen in cases of AHS, may be due to increased leucocyte margination. This leucocyte margination is due to the increased expression of adhesion molecules by endothelial cells which can be induced by viral infections and inflammation (Skowronek *et al.*, 1995). Skowronek et al (1995) also suggest that lymphopenia may result from stress induced endogenous corticosteroid release. Similar mechanisms may explain the findings in cases of EE. Schliewert et al. (2022) reported that although decreases in the leucocyte count were observed in their data set for horses experimentally infected with AHS over time, none of the cases displayed clinically significant leucopenia (which was defined as WCC<4700cell/ul). In cases of EE examined in the present study however, 10 (40% of the cases that had white cell counts measured) showed leucocyte counts below this level. Bluetongue Virus, another Orbivirus, is also not associated with consistent findings of leucopenia (Jaynudin et al. 2019). It is interesting that EE, a disease generally considered significantly milder than AHS or BTV in their susceptible mammalian species, should then show notable changes in the leucogram. One explanation for the lack of consistent leucocyte changes in both BTV and AHS is suggested to be the down-regulation of the hosts immune response due to viral escape mechanisms (Jaynudin *et al.*, 2019; Schliewert *et al.*, 2022). The more prominent changes in the leucocyte count in cases of EE may as such suggest that this virus lacks the same abilities at immunological evasion hypothesized in these related viruses. This lack of immune-evasion could also explain the reduced severity of disease associated with EEV, with individuals able to initiate and mount a counterbalancing immunological response to mitigate the disease. Leucopenia was identified in 42.86% of cases that had white cell counts assessed within 24 hours of

onset of disease signs. This suggests a rapid leucocyte response from the onset of clinical disease. Hypothetically, the rapid response of leucocytes in these cases may indicate prompt initiation of protective mechanisms and recognition of infection which would lend further support as to the basis of mild clinical disease. Horses with clinically significant leucopenia especially associated with neutropenia are also at increased risk of complications relating to secondary infections (Sheats, 2019). These complications may then compound the severity of the disease and result in prolongation of recovery as well as increased costs associated with additional treatments and care. Although this study describes abnormalities in the leucogram of horses with EEV, a prospective case-control experimental study following haematology over time should be considered to corroborate the significance of these findings. Despite the need for further research, the findings in this study demonstrate that horses with the suspicion of EE should have haematology performed as part of their diagnostic assessment.

Thrombocytopenia was also a common clinicopathological finding in cases that underwent haematology. Although all haematology was conducted on blood with ethylenediamine tetra-acetic acid (EDTA) as an anti-coagulant, all cases with thrombocytopenia were confirmed to have a low platelet count on manual assessment of central blood smear. The cause for this thrombocytopenia is currently not known in these cases. Thrombocytopaneia can be caused by several mechanisms including decreased production from progenitor cells, sequestration in organs such as the spleen, or consumptive and/or destructive processes (Sellon and Wise, 2010) . Thrombocytopenia has also been reported in AHS and in these cases is thought to be linked to platelet activation and consumption via damaged endothelial cells. No current studies have directly ascertained a similar endothelial tropism for EEV. Considering the endothelial tropisms of viruses in a similar class such as AHSV (Clift and Penrith, 2010) and BTV (Russell *et al.*, 1996), as well as some similarities in clinical signs such as oedema development; endothelial injury is a possible initiator of platelet consumption and thereby thrombocytopenia in these cases of EE. Although a large portion of horses show evidence of thrombocytopenia, a considerably smaller number (6/25; 24%) showed thrombocytopenia below the threshold suggested to be associated with clinical signs of petechiae i.e., $<30 \times 10^9$ cells/l (Sellon, 1998). This appears to corroborate the small number of cases noted to have petechiation on clinical presentation.

Hypoproteinaemia was identified in a portion of the horses infected with EEV although a specific determination of whether this was due to changes in the albumin of globulin components of the total protein was only specified in 3 cases. The hypoproteinaemia was mild in all the reported cases although not all cases had protein levels monitored repeatedly over time. Only one of the cases of hypoproteinaemia had evidence of peripheral oedema and as such no meaningful association could be determined.

Oedema development was identified in a small number of cases of EE (14.3%) with reported areas including the supraorbital fossae, lips, and distal limbs. This has been previously reported in the literature with several other sites of oedema also identified (Erasmus, Boshoff and Pieterse, 1978; Mildenberg *et al.*, 2009; Steyn *et al.*, 2021). When reported in the literature, the occurrence of oedema in cases of EE was also low (Steyn *et al.*, 2021). Oedema is a prominent sign of AHS (Coetzer and Tustin, 2004) although cases with oedema of the more ventral extremities such as the distal limbs are less typical for AHS and are more often associated with diseases such as purpura hemorrhagica and equine viral arteritis (Long and Guthrie, 2014). Oedema formation can however also occur in horses due to reduced activity as seen in the distal limbs of sick, stabled horses or of the head in severely depressed horses with abnormal lowered head position. These specific causes of oedema cannot be completely excluded as contributing factors in cases of EE.

Serum Amyloid A has been determined to be a major acute phase protein in horses and increases in association with inflammatory pathology. It is accepted that SAA measurements are higher when associated with infectious aetiologies, especially with bacterial infections(Viner *et al.*, 2017; Witkowska-Piłaszewicz *et al.*, 2019). In general, viral infections have been reported to produce a less marked response although patterns show greater variation between individuals and SAA does not accurately predict between bacterial and viral respiratory infections(Viner *et al.*, 2017; Witkowska-Piłaszewicz *et al.*, 2019). Although Serum Amyloid A was only assessed in 6 cases, the findings here suggest that EE may be associated with systemic inflammatory responses and increases in acute phase proteins. This association between EE and systemic inflammation is further supported by changes in the haematology including neutrophilia and monocytosis seen in some cases. SAA values showed a wide range in the current data set with the maximum value of 1817.1mg/l. Values for SAA greater than 1000mg/l were considered to be "very high" (Witkowska-Piłaszewicz et al. 2019) although have been reported to increase to well above 5000mg/l in cases of substantial inflammation (Viner *et al.*, 2017). Further testing is required to assess the significance of increases in SAA during EEV infection as well as to determine if the measurement of this inflammatory marker holds any value for prognostication.

Some cases of EE did display metabolic disturbances although these were considered clinically mild. Mild hyponatraemia was a commonly identified electrolyte abnormality in EE cases.
Icterus was only identified in 10.7% of cases as opposed to 18.8% identified by Steyn et al. (2021)**.** Only one of these cases displaying clinical icterus had corresponding biochemistry results available for assessment (results were indicative of hyperbilirubinaemia). No definitive cause for the icterus in these cases was identified. Icterus can be related to anorexia caused by the virus leading to increases in total circulating bilirubin (Kaneko, Harvey and Bruss, 1997). Increases in bilirubin have been reported to occur within 12 hours in cases of complete anorexia (Cole, 1986; Bayly, Sellon and Reed, 2009) . Hyperbilirubinaemia was identified in cases of EE although increases were noted to be mild. This further corresponds with the likelihood of the development of hyperbilirubinaemia / icterus due to fasting (Bayly, Sellon and Reed, 2009).

The cycle threshold or Ct count is a marker used during PCR testing. This value relates to the amplification of pathogen nucleic acids as part of the testing process. The Ct is defined by the number of amplification cycles that need to occur for a specific threshold of fluorescence to be achieved, in order to confirm the presence of the nuclei acid of interest (Association of Public Health Laboratories, 2021). This thereby gives an indirect assessment of the degree of viraemia in cases of EE when tested on whole blood. Although a Ct value was determined for the majority of cases, the horses in this study were all tested at different stages of the disease process and as such a direct correlation between Ct value and disease severity or clinical signs could not be evaluated. Further research into the usefulness of the Ct count for association with disease severity and presence of clinical signs is warranted.

5.4-Treatment and Outcome:

No specific treatments for EE are available and as such treatment in this data set reflected a symptomatic approach. Medications aimed at controlling pyrexia, predominantly non-steroidal anti-inflammatories, were commonly administered. Intravenous and enteral fluid therapy was also frequently administered. Antimicrobial usage was identified in cases of EE and likely reflect delays in determining the definitive diagnosis, concerns over patient susceptibility to secondary infection or treatment of complications in these cases.

Due to the low mortality rate and general indications for a favourable outcome, the duration of pyrexia as well as total duration of hospitalization were assessed as a crude determinant of disease

severity. These variables can be considered for use in further research examining correlations between clinical findings and disease severity. A large number of horses in this data set were hospitalized although this likely reflects the specific case distributions of a referral hospital rather than a true propensity toward needing treatment on a hospitalized basis. This information does however reflect that some cases of EE do require hospitalization and intensive care and monitoring. Prolonged periods of hospitalization were identified in some cases of EE, especially when associated with the development of secondary complications. Although not examined statistically in this research, the relationship between total duration of fever and the total duration of hospitalization should be further examined. Due to the strong association between EE and pyrexia, it is likely that the presence of fever is influential in the determination of when cases have recovered sufficiently to be discharged.

Mortality in cases of EE was low, with no adult horses succumbing to the disease and only 1 case of mortality in a foal. This corresponds with the low levels of mortality described in the literature. The majority of horses were reported to make a complete recovery with 92.9% noted to be clinically healthy after treatment. One case showed prolonged evidence of ataxia over a period of several weeks after discharge.

Chapter 6: Study Limitations

Several limitations can be identified with regards to the study design. The retrospective nature of the study results in an innate set of limitations. Retrospective studies rely heavily on a complete and thorough set of clinical and diagnostic records in order to present an accurate and thorough data set. Inconsistencies and omissions in the records directly influence the quality of data available and thereby can greatly impact the research results.

Selection bias is a limitation of retrospective studies including the study in question, as cases were selected based on their known positive test results for EEV. Because all included cases were known to be positive for EEV with no associated case-control designations, direct conclusions regarding the association between the disease and other findings are constrained. The total number of cases included was noted to be relatively small and likely only represents a small number of the total cases present in South Africa over this time period.

Recall bias is another innate limitation for retrospective studies, however, is minimised in this research as all data was collated from the records written at the time of evaluation of patients with no data obtained based on individual memory.

Although all horses included in the data set were noted to be PCR positive for EEV and had the majority of other potential infectious causes for clinical signs excluded, an exhaustive exclusion of potential causes and/or co-infections was impractical especially considering the retrospective nature of the research. This can lead to substantial confounding in associations. Not all cases presenting with specific neurological signs were tested for the multitude of neurological arboviruses present in Southern Africa which may lead to confounding when attributing neurological signs to EEV infection.

Due to the fact that many of the cases were examined on a referral basis, some cases were evaluated at differing stages of the disease process. This means that many of the cases could have had abnormalities that had resolved prior to referral. Referral of cases at different stages of the disease process also means that the total duration of hospitalization could be influenced.

Many of the clinicopathological parameters were not tested in a serial fashion, with some only having isolated clinicopathological data generally from the initial diagnostic process. This can lead to underreporting of clinical signs and clinicopathological abnormalities and does not allow the progression of disease to be analysed.

None of the positive cases underwent retesting to assess continued presence of EEV in the blood once the initial diagnosis had been made.

Chapter 7: Conclusion

Equine encephalosis is a relevant differential diagnosis for other infectious diseases in horses with similarities identified in the epidemiology and clinical signs specifically relating to AHS. Pyrexia, tachycardia, and tachypnoea remain the most common clinical signs associated with EE. However, this study shows that EE can be associated with a variety of clinical signs suggestive of the involvement of multiple organ systems in the currently poorly elaborated pathogenesis and pathophysiology of the disease. Equine encephalosis is associated with leukopenia, lymphopenia, and thrombocytopenia. Further research into the pathogenesis and pathophysiology is vital to better describe these haematological changes and the reasons for their development and to quantify the clinical significance of EE.

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Ethical considerations:
The project is retrospective and as such no animals were presently involved in the research. This study was assessed and approved by the University of Pretoria, Research Ethics committee, reference number REC123-22.

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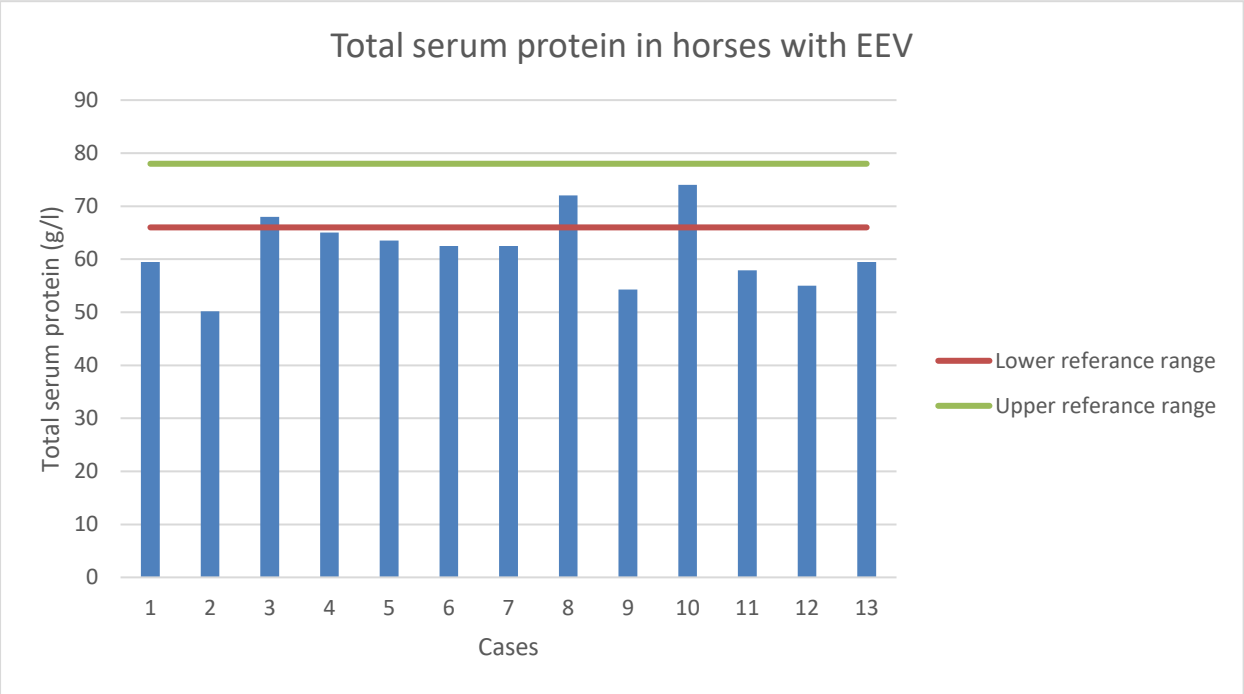
Chapter 9: Appendices

Appendix 1: Table showing the results for distribution of data sets.

Tests of Normality				
	Shapiro-Wilk			
	Statistic	df	Sig.	Distribution
Temperature	0.931	22	0.128	Normal
Heart rates	0.894	22	0.023	Non-Normal
Respiratory rates	0.883	22	0.014	Non-Normal
Leucocyte count	0.792	22	0.000	Non-Normal
Mature Neutrophil count	0.804	22	0.001	Non-Normal
Immature Neutrophil count	0.631	22	0.000	Non-Normal
Lymphocyte count	0.677	22	0.000	Non-Normal
Monocyte count	0.749	22	0.000	Non-Normal
Thrombocyte count	0.883	22	0.014	Non-Normal
Ct value for all cases	0.944	22	0.241	Normal
Ct value for cases with EEV only	0.940	22	0.200	Normal
SAA	0.731	6	0.013	non-normal
Total Bilirubin	0.978	5	0.924	normal
Days of Fever	0.821	24	0.001	non-normal
Days hospitalized	0.759	23	0.000	non-normal
Sodium	0.911	12	0.220	normal
GGT	0.691	9	0.001	non-normal
GLDH	0.751	9	0.006	non-normal
albumin	0.851	10	0.060	normal
glob	0.936	10	0.513	normal
TSP	0.979	13	0.976	normal
Days prior to presentation	0.853	10	0.062	normal
Age	0.914	28	0.025	non-normal
potassium	0.920	12	0.290	normal
chloride	0.573	7	<0.001	non-normal
Calcium (ionised)	0.970	11	0.883	normal
pH	0.913	12	0.233	normal
Bicarbonate	0.925	12	0.334	normal
PCO_2	0.947	12	0.595	normal

Appendix 2: Graphical representation of total protein levels determined within 24 hours of presentation to the Onderstepoort Equine Clinic.



Appendix 3: Ethics Approval Document.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

14 October 2022

LETTER OF APPROVAL

Ethics Reference No	**REC123-22**
Protocol Title	**Retrospective analysis of clinical signs and clinical pathology in horses with Equine Encephalosis**
Principal Investigator	**Dr GB Piketh**
Supervisors	**Dr C Eberhardt**

Dear Dr GB Piketh,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC123-22) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals**: FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Approved

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS

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