Non-suppurative encephalitis and encephalopathies of unknown origin in horses from Brazil

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Gisele Silva Boos

To my parents, my sister,
and my husband

"So the problem is not so much to see what nobody has yet seen, as to think what nobody has yet thought concerning that which everybody sees"

(Arthur Schopenhauer, Parerga und Paralipomena)

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Table of Contents

List of Figures

List of Figures

List of Figures

List of Tables

List of Tables

Abbreviations

AR	Antigen retrieval
BBB	Blood-brain barrier
BD	Borna disease
BoDV	Borna disease virus
BSL4	Biosafety level 4
cDNA	Complementary DNA
CDV	Canine distemper virus
CHIKV	Chikungunya virus
CNS	Central nervous system
CSF	Cerebrospinal fluid
DENV	Dengue virus
DICI	Degree of inflammatory cell infiltration
DIF	Direct immunofluorescence
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EEEV	Eastern equine encephalitis virus
EHM	Equine herpesviral myeloencephalopathy
EHV-1	Equine herpesvirus type 1
EIA	Equine infectious anaemia
ELISA	Enzyme-linked immunosorbent assay
EPgV	Equine pegivirus
EPM	Equine protozoal myeloencephalitis
FFPE	Formalin-fixed paraffin-embedded
GAPDH	Glyceraldehyde phosphate dehydrogenase
gDNA	Genomic DNA
GFAP	Glial fibrillary acid protein
HeV	Hendra virus
HIER	Heat induced epitope retrieval
HPF	High power field
Iba1	Ionized calcium-binding adapter molecule 1
IFA	Indirect immunofluorescence assay
IHC	Immunohistochemistry
ISH	In situ hybridization
ITS1	Internal transcribed spacer 1

Abbreviations

JE	Japanese encephalitis
JEV	Japanese encephalitis virus
LACV	LaCrosse virus
MADV	Madariaga virus
MAYV	Mayaro virus
mRNA	Messenger RNA
NGS	Next generation sequencing
NiV	Nipah henipavirus
nsRNA	Negative-stranded RNA
Nsp	Non-structural protein
NTC	Non-template control
OIE	World Organization for Animal Health
ORF	Open reading frames
PAP	Peroxidase-anti-peroxidase
PAS	Periodic acid-Schiff
qPCR	Quantitative real-time polymerase-chain reaction
qRT-PCR	Real-time quantitative reverse transcription polymerase-chain reaction
RABV	Rabies virus
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROCV	Rocio virus
RT-PCR	Reverse transcriptase polymerase-chain reaction
SLEV	Saint Louis virus
SuHV-1	Suid herpesvirus 1
TBEV	Tick-borne encephalitis virus
VEEV	Venezuelan equine encephalitis virus
VSBV-1	Variegated squirrel bornavirus 1
WEEV	Western equine encephalitis virus
WGS	Whole genome sequencing
WNV	West Nile virus
YFV	Yellow fever virus

1. Introduction

In Brazil, increasing invasion of forest and other natural areas destined to the pasture of approximately 218 million cattle and 5.8 million horses, as well as for human settlements have helped to spread diseases as well as to the discovery of exotic illnesses and/or the emergence of new vectors and hosts (Colwell et al., 2011; Confalonieri et al., 2014; Instituto Brasileiro de Geografia e Estatística, 2016; Vittor et al., 2006). Reports of serological surveys carried out in Brazil and in South America revealed that several neglected, emergent, and re-emergent agents are circulating among equine herds (Pauvolid-Corrêa et al., 2010; Pauvolid-Corrêa et al., 2014, 2017). This is also a reality in other regions, as the World Organization for Animal Health (OIE) releases annual lists of diseases, infections and infestations that are in force on a global scale; several of those are zoonotic and neuro-infective, like rabies and West Nile fever that occur regularly in Brazil (OIEh, 2019).

However, while most of the diagnostics tools are adjusted to fresh samples, formalin-fixed paraffin-embedded (FFPE) tissues are by far the most common sample type in pathology facilities (Bonin et al., 2003). Especially when autopsies of large animals are performed in the field, formalin fixation may be still the only alternative of safely acquiring material for investigation (Benavides et al. 2006). FFPE samples that could help understanding pathogen evolution and disease development, however, lose their quality over time due to over fixation and protein cross-link reactions, posing a challenge for histochemical, molecular biology, and metagenomics assays (Bhudevi & Weinstock, 2003; Lewis et al., 2001; Masuda et al., 1999; Webster et al., 2010).

Therefore, this study used FFPE fixed tissue from the central nervous system (CNS) of 35 horses from Brazil/South America suffering from non-suppurative encephalitis or encephalopathies as study model. The main aim was to provide adequate diagnostic tools adopted to FFPE tissues for morphologic and molecular detection as well as metagenomic screening of relevant pathogens. Equines were chosen since they have close contact to humans in Brazil and are susceptible to several similar zoonotic agents as humans so that they represent suitable sentinels for respective risk assessments (Bender & Tsukayama, 2004; Gossner et al., 2017; Timoney, 2000). The 35 horses were tested for nine infectious agents (five viruses, one bacteria, three protozoa) by immunohistochemistry (IHC) and newly developed reverse-transcriptase polymerase chain reaction (RT-PCR) or PCR assays as well as by metagenomics in order to provide evidence for an infectious aetiology Moreover, this study raises the awareness to emergent, re-emergent, and neglected diseases in Brazil.

2. Literature review

Central nervous system infections account for at least half of the viral zoonotic diseases, yet the cause remains unknown in approximately 50% of the cases in human and veterinary medicine (Griffin, 2010). Non-suppurative inflammatory and degenerative lesions of the CNS of horses, although uncommon, are usually devastating and frequently fatal (Seino & Long, 2014). There are few surveys on the causes of death in horses, but in general, fatal CNS disease is reported to occurr in 11% to 15.8% in cohorts from the United Kingdom, France, and Brazil, preceded only by diseases of the alimentary and locomotor systems (Baker & Ellis, 1981; Laugier et al., 2009; Pierezan et al., 2009). As infectious aetiology viruses, bacteria, rickettsia, protozoa, parasites, and fungi must be kept in mind, but the clinical outcome is frequently unspecific since several agents might produce similar clinical signs and lesions in the CNS. Also, non-infectious causes, like auto-immune disorders, trauma, and neoplasms might display comparable clinical signs (Radostits et al., 2007; Seino & Long, 2014). Furthermore, several studies have demonstrated the susceptibility of horses to several similar zoonotic agents as humans, so they represent quite suitable sentinels for risk assessments of infections caused by i.e. West Nile virus (WNV), Hendra virus (HeV), and Borna disease virus (BoDV) (Angenvoort et al., 2013; Bender & Tsukayama, 2004; Kumar et al., 2018; Timoney, 2000). Thus, on a global scale, dealing with these infections require the establishment of interdisciplinary measures and international programs that overlook political borders and that are accessible also to less equipped laboratories (McNerney, 2015).

2.1 Epidemiology of CNS diseases of horses

2.1.1 Sex, age, and breed

Sex, age, and breed predisposition might be influenced by geographical location, having representative individuals with determined characteristics for i.e. working or racing horses. Except for an association between *Neospora caninum* antibody in serum and foetal loss of seropositive mares, and neurological malformation in males, there seems to be no prevalence of CNS diseases regarding sex (Laugier et al., 2009; Tyler et al., 1993; Villalobos et al., 2006). Age on the other hand, is a predisposition for the development of certain conditions. Baker and Ellis (1981) found a large number of cases of grass sickness affecting young adult horses, from 2 to 4 years of age. Tyler et al. (1993) observed that in young horses, with up to 2 years, head injury as the most frequent cause of neurological disease. Bacterial infections tend to be uncommon in adult horses, while it might represent up to 50% of the cases of neurologic disease in weanlings (Laugier et al. 2009; Morresey et al., 2011; Tyler et al. 1993; Viu et al. 2012) due to failure of passive immunogens, resulting in sepsis after pneumonia, omphalophlebitis,

meningoencephalitis, arthritis, or new-teeth wounds (Koterba et al., 1984; Morresey et al., 2011; Wilkins et al., 2000). Concurrently, viral and parasitic encephalitis tend to occur in adult and aging animals, potentially as a signal of extended exposure time, lack of appropriate sanitary management and/or defficient vaccination programs (Ehizibolo et al., 2012; Tyler et al., 1993).

2.1.2 Seasonality

Seasonality of infections of the CNS are usually attributed to vector-borne diseases, i.e. presence of mosquitos. An increase in arboviruses infections might be noticed when migratory birds serve as reservoir hosts in the transmission cycle, like in Eastern equine encephalitis virus (EEEV), or WNV. These birds land naïve to an infected area, and carry the viruses on the way back (Long & Gibbs, 2014; Timoney, 2000). These factors are more easily recognizable in temperate climate regions, like southern Brazil, where disease occurrence is linked to the late summer season and early fall. However, this could be misleading, since in (sub) tropical regions temperatures do not decrease sufficiently to interfere in the life cycle of the mosquitos, and therefore, vector-borne diseases like dengue and yellow fever occur all year round (Delcambre & Long, 2014; Dupuis et al., 2003; Long & Gibbs, 2014). Equine herpesvirus type 1 (EHV-1) infections on the other hand might reflect not only a seasonality towards colder seasons, when most animals are stabled, but might also indicate herd management failures since it is more easily transmitted from horse-to-horse (Dunowska, 2014). Thus seasonality should be understood as a predicting tool, allied to the knowledge that each region might have different inherent or adapted environmental conditions (Fanany, 2012; Lima-Camara, 2016; Pereira et al., 2014).

2.1.3 Brazilian environment as a model for infections in horses

Brazil, due to its proportions, possesses many climatic zones, resulting in a wide spectrum of ecological variations, forming six distinct *biomes* harbouring the fauna and flora from almost half of South America (**Fig. 1**). Meanwhile, socioeconomic disparities, regional limitations, and susceptibility to certain diseases might reflect the (un)success of public politics that are relatable to other regions of the world (Botelho, 2010):

Fig. 1: Biomes in the Brazilian territory

Modified from Botelho (2010), Brazilian Institute of Geography and Statistics (IBGE).

The *Pampas* are extensive grass-rich areas in south Brazil, where the temperate climate offers suitable conditions to infections caused by parasites, arboviruses, and plant intoxications during the warmer months, when animals can daily graze (Hammerschmitt et al., 2017; Lorenzett et al., 2018; Ministry of the Environment, n.d.-b; Pierezan et al., 2009). In colder periods, horses are kept stabled, which predisposes to horse-to-horse infections, allergies, and infectious diseases of the respiratory tract (Elfman et al., 2009; Friday et al., 2000; Goehring, 2015; Kristiansen & Lahoz, 1991; Saastamoinen et al., 2015; Studdert et al., 2003; Wolny-Koładka, 2018).

The *Atlantic Forest*, also called the "Brazilian spine", is responsible for the structure of the national territory since the Portuguese colonization. Metropoles like São Paulo and Rio de Janeiro are located there, influencing the 87% of forest that is anthroposised. The critical decrease in biological diversity, especially the extinction of sentinel animals species, has led to a higher exposure of humans and domestic animals to infections once contained by the forest environment (Khalil et al., 2016).

The permanently flooded areas of *Pantanal* house numerous fauna and flora species, and harbours plentiful migratory birds species (Donatelli et al., 2017; Ministry of the Environment, n.d.-a). The region is also exceptionally favourable to mosquito breeding, with a high proliferative potential of vectors infected with arboviruses, rarely seen in other regions

(Borges et al., 2013; Palaniyandi et al., 2017; Pauvolid-Corrêa et al., 2010; Pauvolid-Corrêa et al., 2011, 2014, 2017; Pauvolid-Corrêa et al., 2015). Risk factors of equine infection also concern tick infestation and internal parasites, as well as the proximity of livestock herding to wild life sites (Cançado et al., 2017).

The *Cerrado* biome in Midwest Brazil, is the richest savannah of the world and is where the capital city, Brasília, locates. Although the climatic variance in this region is not that drastic as in the south, there are disparities in equine health, mostly due to socioeconomic factors. Because of poor investments in human resources and increase in the sub-employed population, attention is drowned to the urban cartwright-pooling animals. Most reported are alterations in muscle-skeletal system, poor nutritional status, and cutaneous lesions (Guirro et al., 2011; Schade et al., 2013).

The *Amazônia* biome is considered the biggest biologically diverse reserve, harbouring at least one third of all alive species on the planet (Botelho, 2010). Albeit concentrating vast natural resources, high pluviosity and humidity added to the regional low socioeconomic index and increasing disorganized urbanization are undoubtedly risk factors (Franco-Luiz et al., 2016).

The *Caatinga* is one of the least preserved biomes, yet, harbours a rich biodiversity and is characterized by seasonal forests. During the winter, the hot and dry weather represent a decrease in the already hard to obtain foliage for animal feeding. Extreme high animal mortality rates due to starvation are quite frequent during this period. While in the summer, during the extremely hot and rainy season, the vegetation starts to grow back, and the streams begin to flow again. However, during this period several outbreaks, specially of mosquito-borne diseases, reach the highest levels of occurrence (Lima-Camara, 2016).

Finally, environmental features that suit all regions in Brazil, would offer plenty of suitable hideouts for the hosts involved in the rabies virus chain. Recently, breakouts of the disease among humans and domestic animals are alleged by the increasing invasion of natural bat-housing areas by pasture. This condition only increases the narrow contact with other wild life species, leading to another endless list of infections (Khalil et al., 2016; Wood et al., 2014; Young et al., 2017). Allied to outdated public health politics, and the lack of permanent and comprehensive projects aiming regional development, self-sustaining initiatives, and conservation, recent restrains in scientific budget in Brazil might lead to even more catastrophic events for global health (Overbeck et al., 2018).

2.1.4 Sanitary status and disease-control policies

In Brazil, there are two federal programmes linked to equine health, the National Programme for Equine Health and the National Programme of Control of Herbivore's Rabies (Ministério da Agricultura Pecuária e Abastecimento, 2017b, 2017a). Even though the OIE provided a thorough report on Brazilian veterinary services in 2014, there is no mention whatsoever of both equine health programmes, being rabies virus control cited as being carried out and implemented by municipal authorities for companion animals. Meanwhile, only bovine rabies vaccination was described, and promoted voluntarily by the veterinarian services (Schneider et al., 2014). Rocha et al. (2015), emphasised the predominant attention of rabies virus control in Brazil towards canine disease, rather than an acting investigative tool that could assess the country's current epidemiological trend.

This has been well documented in the last decade, unravelling new bats species involved in the aerial and rural cycles of rabies, as well as the increasing participation of wild life on the spread of the disease (Castilho et al. 2017; Itou et al. 2016; Rocha et al. 2015; Rosa et al. 2011). In consonance with these statements, the OIE report reiterates that there are deficiencies in respect of developing clear, adapted, and compulsory strategies of control and eradication of major diseases, citing bovine tuberculosis and brucellosis as examples. In some cases, veterinarians are considered unimportant/expensive for tasks like vaccination, which breaks the sanitary surveillance success (Schneider et al., 2014). The lack of continued public health education might have led to the increasing refusal of vaccination against preventable diseases, resulting in recent outbreaks of long-time controlled diseases. In Brazil, the "Situação sanitária das doenças de animais terrestres" (Sanitary situation of disease from terrestrial animals) report from 2016, officially recognized as confirmed equine diseases the equine infectious anaemia, equine influenza, glanders, equine babesiosis, and equine rhino pneumonia. Diseases affecting multiple animal species are rabies, myiasis, and leishmaniosis (Ministério da Agricultura Pecuária e Abastecimento, 2016). On a global scale, the OIE-listed diseases, infections and infestations in force for the years 2019/2020 highlights several diseases that similarly infect humans and horses, like rabies, West Nile fever, Equine encephalomyelitis, and Japanese encephalitis (JE) (OIE, 2019f), which are also circulating and/or causing disease in horses in Brazil. These data clearly indicate the necessity of adequate, affordable diagnostic tools for infectious and zoonotic diseases.

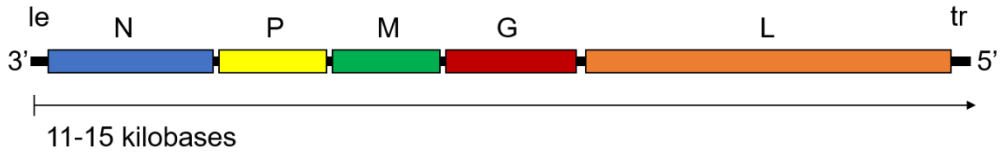
2.2 Infectious causes of non-suppurative encephalitis and encephalopathies in horses

2.2.1 Viral encephalitis

2.2.1.1 Rabies

Rabies is caused by viruses assigned to the order *Mononegavirales*, family *Rhabdoviridae*, and genus *Lyssavirus*. Rabies virus (RABV) is the prototype, structured as enveloped, bullet-shaped virions, containing a non-segmented, negative-sensed single-stranded RNA genome of approximately 11-15kb in length. The viral genome is composed of five genes that encode the correspondent structural proteins 3'-N-P/C-M-G-L-5' (**Fig. 2**), vastly used as targets for diagnostics (Lyles et al., 2013). The coding regions for the nucleocapsid (N), phosphoprotein (P), and viral polymerase (L) consist on the ribonucleocapsid. The matrix protein gene (M) is associated to the ribonucleoprotein (RNP) production, and the glycoprotein gene (G) encodes the respective protein for the viral envelope (Lyles et al., 2013).

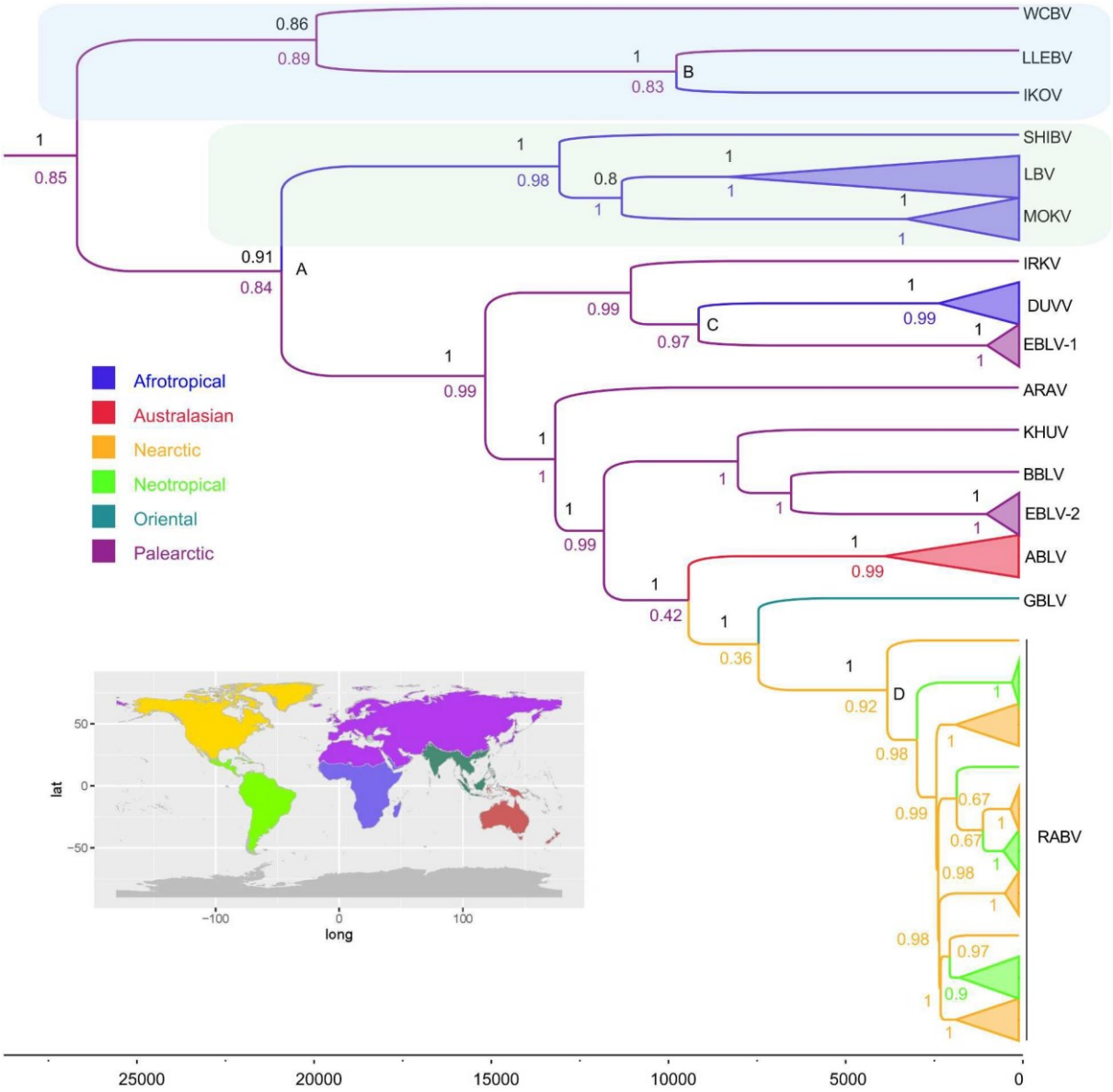
Fig. 2: Organization of rabies virus genome



The genome is composed of a linear molecule of RNA with negative polarity, represented by the continuous line. Each box represents separately the five encoded proteins by their correspondent genes. Nucleoprotein (N), phosphoprotein (P), matrix protein (M), envelope glycoprotein (G), and viral polymerase, or large protein (L), with intergenic regions between them. le and tr represent the leader and trailer sequences. Modified from Lyles et al., 2013.

Based on antigenic properties and phylogenetic relationships, viruses in the genus *Lyssavirus* are subdivided into two phylogroups, and other three most divergent viruses are unassigned (Botvinkin et al., 2003; Ceballos et al., 2013; Freuling et al., 2011; Gunawardena et al., 2016; Kuzmin et al., 2003, 2005, 2010; Marston et al., 2012). Hayman et al. (2016) generated a time-scale phylogeny from 153 nucleoprotein gene sequences from lyssaviruses to show their global distribution (**Fig. 3**). Although viruses from different species have a specific geographical circulation, countries like England, Germany, Ireland, Japan, and Nordic countries, were successful to eradicate rabies transmission to humans and domestic dogs (Fooks et al., 2017).

Fig. 3: Evolutionary relationships between lyssaviruses

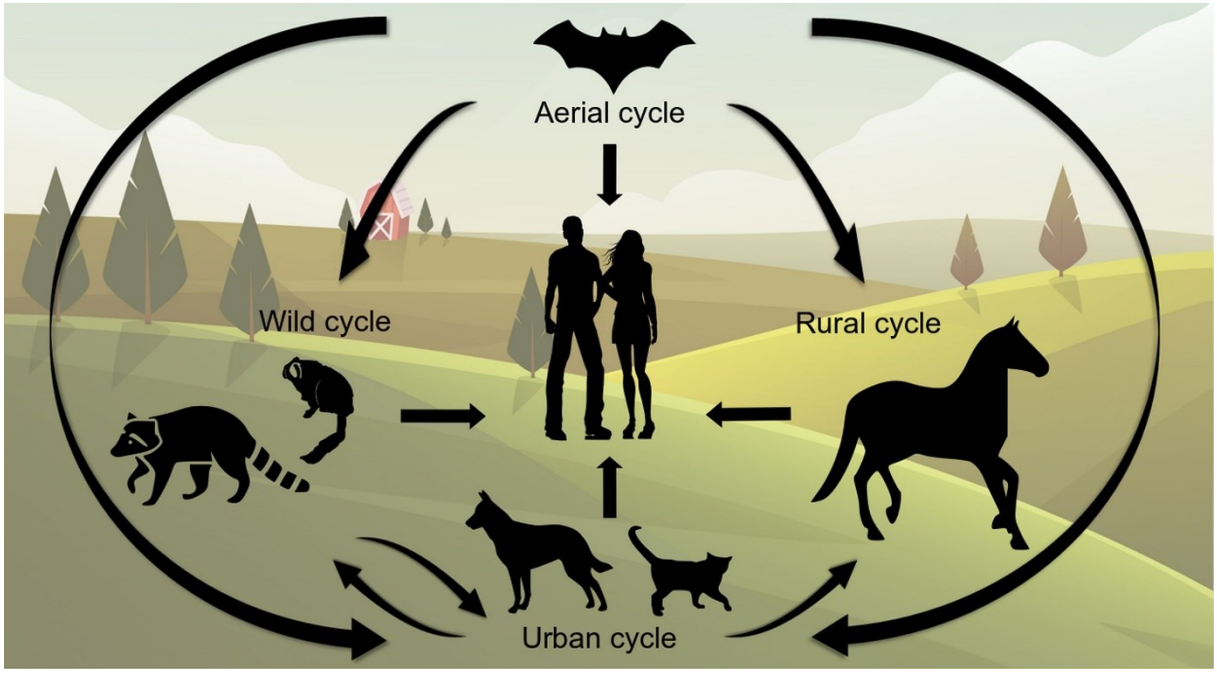


Hayman et al. (2016) generated a time-scale phylogeny from 153 nucleoprotein gene sequences from lyssaviruses to show their global distribution, where branch colours correspond to ecozones on the inset map. Viruses in the Phylogroup 1: *Rabies lyssavirus* (RABV), *Australian bat lyssavirus* (ABLV), *Duvenhage lyssavirus* (DUVV), *European bat lyssavirus 1* (EBLV-1), *European bat lyssavirus 2* (EBLV-2), *Aravan* (ARAV), *Khujand* (KHUV), *Irkut lyssavirus* (IRKV), *Gannoruwa bat lyssavirus* (GBLV), *Bokeloh bat lyssavirus* (BBLV). Phylogroup 2: *Lagos bat lyssavirus* (LBV), *Mokola lyssavirus* (MOKV), *Shimoni bat lyssavirus* (SHIBV). Unassigned: *West Caucasian bat lyssavirus* (WCBV), *Ikoma lyssavirus* (IKOV), *Lleida bat lyssavirus* (LLEBV).

RABV is maintained by cycles that employs specific hosts (**Fig. 4**) (Kotait et al., 2009). In Latin America, dogs are still the main transmitters of rabies to humans, involving an urban cycle (Ito, 2005; Millien et al., 2015; Rocha et al., 2015). However, transmission through bats is increasing and responsible for the maintenance of the virus in all cycles (Castilho et al., 2017; Cordeiro et al., 2016; Moutinho et al., 2015). The wild cycle is maintained by wild dogs (*Cerdocyon thous*), marmosets (*Callithrix jacchus*), crab-eating raccoons (*Procyon cancrivorus*), wild-cat oncilla (*Leopardus tigrinus*) and the rodent agouti (*Dasyprocta* spp.).

Herbivores are mainly infected by vampire bats in the rural cycle, and the aerial cycle is maintained by bats of the *Phyllostomidae* family, including the hematophagous *Desmodus rotundus*, and also by the *Mollosidae* and *Vespertilionidae* families (Sodré et al., 2010; Wada et al., 2011). These animals are susceptible to infection with bat lyssaviruses, present clinical signs and die (da Rosa et al. 2011).

Fig. 4: Rabies virus transmission cycles



The transmission may occur in four distinct cycles: urban, wild, aerial, and rural. Illustration prepared on Inkscape© with VectorStock® royalty free icons.

Transmission of RABV occurs through the bite of an infected animal that inoculates saliva containing virus into tissues of a receptive animal (Long, 2015b; Wilkins & Del Piero, 2014). Other routes of transmission have additionally been documented, as in organ transplant (Srinivasan et al., 2005; Vora et al., 2013), trans-placental, contamination of recent wounds with saliva or infected material, mucosa contamination (eyes, nose, and mouth), and aerosol transmission (Riet-Correa et al., 2001). The virus replicates initially in muscle tissue at the site of inoculation and is believed to remain near this site for most of the incubation period, usually 14 days to 12 weeks (Wilkins and Del Piero 2014). However, a 25-year incubation period has been reported in a human patient (Shankar et al., 2012). The incubation period is variable after natural infections and many factors may be involved in a prolonged incubation: virus strain, location of the bite (the closest to the CNS, the quickest the virus transport), the load of virus inoculated, susceptibility of the exposed species, and immunity of the bitten animal. After initial virus replication, a second multiplication phase occurs when the virus binds to nicotinic

acetylcholine receptors at the neuromuscular junction and enters motor and sensory neurons. The virus then travels ascendingly through the spinal cord to the brain or from the cranial nerves directly to the brainstem (Long, 2015b). With neuron entry, rabies virus ribonucleoprotein is released from its envelope and travels towards the CNS by retrograde intra-axonal transportation into the cell body of the infected neuron. That is the period when the dumb and paralytic form of the disease is manifested, and most horses will be euthanized during this time (Long, 2015b). In the late course of infection, the third phase, centrifugal or descending spread, rabies virus is spread into highly vascularized organs including adrenal cortex, pancreas, and, most importantly, the salivary glands (MacLachlan & Dubovi, 2011). The presence of virus in the saliva facilitates its spread and excretion into the environment and transmission to a new host (Long, 2015b).

The clinical presentation of rabies in horses can be truly diverse. Although unspecific, initial clinical signs are inclined to the paralytic form of the disease, and have been reported as lameness or unilateral limb weakness, muzzle tremors, lethargy, severe ataxia, behavioural disorders, seizures, and even colic (Bassuino et al., 2016; Hamir et al., 1992; Radostits et al., 2007). The spinal cord is most commonly affected in horses, and perhaps reflects the limbs as the most common site for inoculation (Bassuino et al. 2016; Hamir et al. 1992). Paraesthesia at the bite site may develop which results in rubbing, biting, or self-mutilation (MacLachlan & Dubovi, 2011). Involvement of the brainstem is seen less frequently, followed by rare cerebral infection. Most encountered clinical signs are therefore ascending ataxia and hindquarters paresis/paralysis combined with loss of tail and anal sphincter tone, loss of sensory perception of the limbs, and muscle tremors (Radostits et al., 2007). In addition, but also independently, brainstem signs are described including cranial nerve dysfunction such as blindness, abnormal vocalisation, drooling, and dysphagia. The “furious” or cerebral form is rare in the horse, and clinical signs such as convulsions, aggressiveness, photophobia and hydrophobia, tenesmus, circling, and hyperesthesia are attributed to this form (Long, 2015b).

Typically, only a single horse out of a group is affected. Once expressed clinically as neurologic disease, rabies is almost invariably fatal; recovery with or without neurologic deficit is quite rare but has been observed in several species following experimental exposure (Cantile & Youssef, 2016). It takes usually between four (Bassuino et al., 2016) and 10 days until death occurs or euthanasia is performed (Long, 2015b). Its clinical course may be prolonged in cases of intensive nursing, which increases the risk of transmission to humans, because it becomes more likely that the virus has spread to the salivary glands (Long, 2015b).

There are no characteristic macroscopic lesions in animals that die of rabies (MacLachlan & Dubovi, 2011). In brain and/or cervical spinal cord lesions may consist of focal to multifocal, mild to moderate haemorrhages. Self-mutilation or aspiration pneumonia may also be seen (Wilkins & Del Piero, 2014). The histologic lesions are typical non-suppurative encephalomyelitis, ganglioneuritis and parotid adenitis (Cantile & Youssef, 2016). In the brain, lesions are mainly observed in the grey matter of the brainstem and hippocampus. They are characterized by mild to severe lymphocytic perivascular cuffing combined with macrophages and plasma cells, and the formation of microglial nodules (Cantile & Youssef, 2016; Lima et al., 2006; Wilkins & Del Piero, 2014). There is neuronal vacuolization (rabies-induced spongiform encephalopathy), and less Negri body formation in horses when compared to cattle (Lentz et al., 1982). Negri bodies consist of round eosinophilic intracytoplasmic inclusions, most commonly observed in the Purkinje cells and in the pyramidal cells of the hippocampus (Cantile & Youssef, 2016; Wilkins & Del Piero, 2014). The virus forms fine to large granules and variable amounts of micron inclusion bodies that are distributed homogenously within the cytoplasm (Wilkins & Del Piero, 2014). Examination of the spinal cord could reveal extensive congestion and acute perivascular haemorrhages in the dorsal grey matter. Additionally, there is moderate to marked mononuclear perivascular cuffing, nodular to diffuse gliosis, neuronophagia, and eventually prominent malacia (Bassuino et al., 2016). In the trigeminal ganglion similar inflammation occurs as in the brain and acute degeneration of ganglion cells, proliferation of capsule cells, and microglial nodules are also present (Hamir et al., 1992; Lima et al., 2006; MacLachlan & Dubovi, 2011).

Medulla, cerebellum, and hippocampus from suspect animals must be submitted to a laboratory approved by the respective state health department for rabies testing (MacLachlan & Dubovi, 2011). Post-mortem diagnosis is usually made with direct immunofluorescence (DIF) using monoclonal antibodies (Wilkins & Del Piero, 2014). In some cases, the brain tissue may be negative for the RABV using this standard diagnostic technique, but immunohistochemistry (IHC) may detect the presence of the antigen (Brookes et al., 2005). In this case, RABV is detected as granular positivity of the neuropil of cerebral and cerebellar cortices, thalamus, and grey matter of brainstem and spinal cord. Other ancillary tests, including enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR, also when fresh tissue is not available), are reliable and may be also used as research tools (Wilkins & Del Piero, 2014). For FFPE tissues in situ hybridization (ISH) (Jackson & Wunner, 1991; Jackson, 1992; Junior & Ventura, 2006) and the in situ PCR (Warner et al., 1997; Praveena et al., 2007) have been described. More recently, high-throughput

sequencing (HTS) by RABV RNA enrichment has been successfully implemented as an alternative for highly contaminated samples, like the ones retrieved from decomposed animals (Orłowska et al., 2019). Antemortem diagnosis on the other hand, is only attempted in suspected human rabies cases, using either RT-PCR of saliva specimens, immunofluorescence staining of skin biopsy or corneal impression (MacLachlan & Dubovi, 2011).

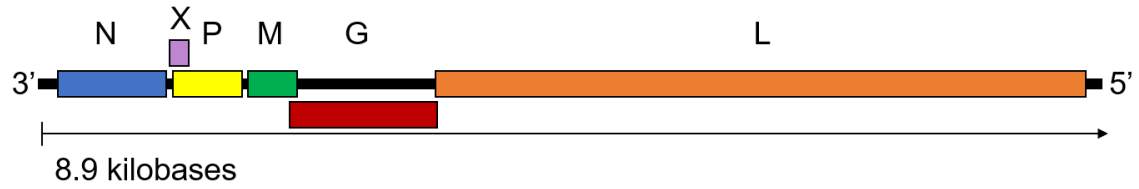
No treatment should be attempted after clinical signs are evident. If the bite is seen, immediately after exposure, irrigation of the wound with 20% soft soap solution or a solution of benzalkonium chloride may prevent the establishment of the infection (Radostits et al., 2007). Once the infection is already set, the horse presenting clinical signs should be isolated (Wilkins & Del Piero, 2014). Exposed and vaccinated horses may be revaccinated and quarantined for 45 days. However, it is recommended to euthanize the truly exposed (observed bitten by rabid animal) and unvaccinated horses (Long, 2015b). Vaccination is highly effective, but protection is not absolute. The main ways of preventing further infections are vaccination and to avoid exposure to diseased animals (Wilkins & Del Piero, 2014).

The control of rabies in different regions of the world poses very different problems, depending on which reservoir hosts are present and the level of infection in such hosts (MacLachlan & Dubovi, 2011). Rabies cases have declined 95% in the Americas since the 1980's, due to vaccination programmes (Linn et al., 2012). In Brazil, the large number of species of chiropterans, the impossibility of vaccinating these animals and their different eating habits and ecological niches highlight the importance of this order in the maintenance of different epidemiological cycles of rabies (Castilho et al., 2017).

2.2.1.2 Borna disease

Borna disease is caused by viruses of the order *Mononegavirales* within the *Bornaviridae* family. Among the three genera, two species of the *Orthobornavirus* genus are known to affect mammals, the *Mammalian 1 orthobornavirus* and the *Mammalian 2 orthobornavirus* (Amarasinghe et al., 2019; Korn et al., 2018). They are structured as enveloped, spherical virions, composed of a negative-sense, single-stranded, non-segmented RNA lengthening around 8.9kb (Herden et al., 2013). The viral genome is composed of six protein-encoding genes (3'-N-P/X-M-G-L-5'), which lack specific intergenic regions and instead, have mostly overlapping open reading frames (ORFs) (**Fig. 5**) (Herden et al., 2013). There are three transcription units in the genome, the first unit encodes the nucleocapsid protein (N), while the viral phosphoprotein (P, p23) and the regulatory X protein (X, p10) are encoded by the second unit, and the matrix protein (M,p16), the type I surface glycoprotein (G, p57), and the L-polymerase (L, p190) are encoded by the third unit (Herden et al., 2013).

Fig. 5: Borna disease virus genome structure

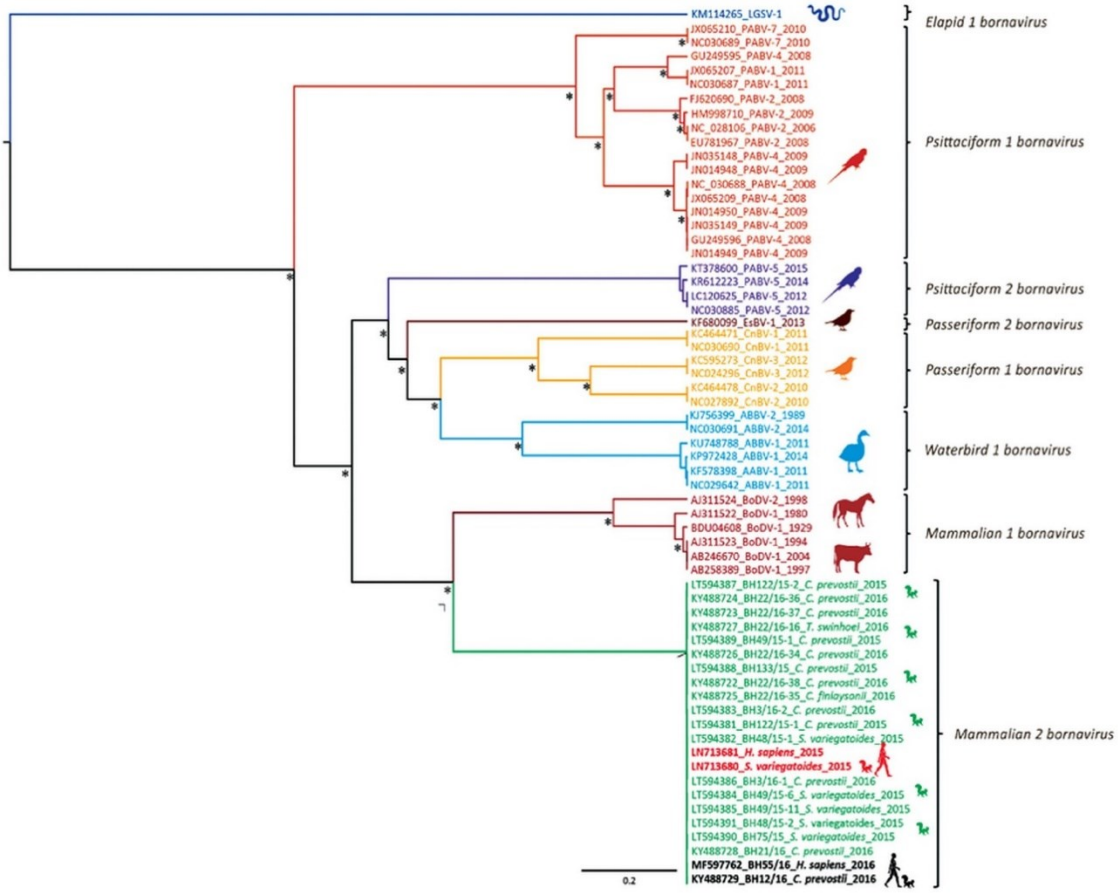


The genome is composed of a linear molecule of RNA with negative polarity, represented the genes that encode the nucleocapsid protein (N), viral phosphoprotein (P), overlapped with the regulatory X protein (X), matrix protein (M) overlapped with the type I surface glycoprotein (G), and the L-polymerase (L). Modified from Herden et al. 2013.

The bicoloured white-toothed shrew (*Crocidura leucodon*) is the only known natural reservoir of Borna disease virus 1 (BoDV-1, *Mammalian 1 orthobornavirus*) (Dürrwald et al., 2014; Nobach et al., 2015; Puorger et al., 2010; Weissenböck et al., 2017). Meanwhile, variegated squirrels (*Sciurus variegatoides*) and Prevost's squirrels (*Callosciurus prevostii*) naturally infected with variegated squirrel bornavirus 1 (VSBV-1, *Mammalian 2 orthobornavirus*) depicted a broad viral tissue tropism, similar to naturally BoDV-1-infected *C. leucodon* (Petzold et al., 2019). Natural Borna disease (BD) occurs mainly in horses and sheep, and sporadically in cattle, donkeys, goats, dogs, and alpacas, all infected by BoDV-1 (Bode et al., 1994; Jacobsen et al., 2010; Vahlenkamp et al., 2002; Weissenböck et al., 1998; Herden et al., 2013). Recently, cases of severe and fatal neurological disease in humans have been demonstrated to be caused by BoDV-1 and VSBV-1 (**Fig. 6**) (Hoffmann et al., 2015; Liesche et al., 2019; Niller et al., 2020). Additionally, experiments including rabbits, birds, and primates, potentialize the host range to all warm-blooded animals (Herden et al., 2013; Lipkin et al., 2011).

Clinical disease of horses is restricted to endemic areas in Germany, Austria, Liechtenstein and Switzerland, but there are few reports in other areas such as Great Britain which could be traced back to endemic infection (Dürrwald & Ludwig, 1997; Gosztonyi & Ludwig, 1984; Priestnall et al., 2011; Weissenböck et al., 1998; Weissenböck et al., 2017). However, BoDV-1 specific antibodies have been also detected in horses outside the endemic area, like in Finland (Kinnunen et al., 2007), Iceland (Björnsdóttir et al., 2013), Italy (Pisoni et al., 2007), Japan (Inoue et al., 2002; Taniyama et al., 2001), Turkey (Yeşilbağ et al., 2012; Yilmaz et al., 2001), Iran (Bahmani et al., 1996), China (Hagiwara et al., 2001; Zhang et al., 2014), and in the United States of America (USA) (Kao et al., 1993). Meanwhile in Brazil, there are no clinically diseased nor seropositive horses, and only parrots and macaws were demonstrated to be infected by the *Psittaciform 1 orthobornavirus* parrot bornavirus 4 (PaBV-4, **Fig. 6**) (Donatti et al., 2014; Encinas-Nagel et al., 2014; Philadelpho et al., 2014).

Fig. 6: Phylogenetic analysis of the Orthobornavirus genus



The phylogenetic tree was modified from Tappe et al. (2018) to show branches of bornaviruses species (in different colors) based on N protein gene datasets.

The route of transmission to horses is most likely to occur intranasally, through saliva, nasal and lacrimal secretions, and urine shed by reservoirs (Kupke et al., 2019; Nobach et al., 2015; Petzold et al., 2019). The incubation period in horses ranges from one to four weeks, until the onset of clinical signs and death, which occurs in 80-90% of cases (Bilzer et al., 1996; Herden et al., 2013). By the time of infection, the virus has access to the CNS by retrograde intra-axonal migration through the olfactory nerve and replicates locally in neurons (Kupke et al., 2019; MacLachlan & Dubovi, 2011; Sauder & Staeheli, 2003; Solbrig & Koob, 2003). The virus is then transported through the sensory tracts of the olfactory nerve into the limbic system, spreading to most areas of the grey matter, infecting astrocytes, neurons, occasionally ependymal cells and oligodendrocytes, and leading to prolonged activation microglia cells (Carbone et al., 2001; González et al., 2014; Gosztonyi & Ludwig, 1995; Herden et al., 2005, 2013; Werner-Keišs et al., 2008). BD of horses and sheep is not a direct result of virus replication, but is provoked by a virus-induced, delayed immunopathological reaction. Due to the T cell-mediated immunopathogenesis, the inflammation does not induce immune defence with virus elimination (Stitz et al., 2002).

Neurological clinical signs in horses are complex and variable, and the disease can run a peracute, acute to subacute, and in rare cases a chronic course (Grabner et al., 2002; Herden et al., 2013). Clinical signs consist of depression, apathy, somnolence, and stupor. Mostly, the signs observed consist with infection of the cerebrum and limbic system, including neuro-behavioural abnormalities and repetitive motor activity such as circling (Grabner & Fischer, 1991). Moderate fever, pharyngeal paralysis, lack of food intake, muscle tremor, defects in proprioception, and hyperesthesia have also been described (Bilzer et al., 1996; Grabner et al., 2002; Herden et al., 2013; Katz et al., 1998). In later stages, the horse may also display disturbances in movement coordination and balance, inadequate reactions to pain stimuli, and as sign of impaired proprioception, abnormal posture (Grabner et al., 2002). Non-neurologic signs like colic, emaciation, chronic lameness of unknown cause have also been described, but it is unclear whether they are related to BD (Herzog et al., 2008).

Gross lesions are usually unremarkable but histologically, BoDV-1 infection in horses usually appears as a severe non-suppurative poliomeningoencephalomyelitis with extensive perivascular and parenchymal infiltration. The perivascular cuffs might have thickness of more than seven-cell layers, and consist predominantly of macrophages, CD4+ and CD8+ T lymphocytes, and plasma cells later in the course of infection (Cantile & Youssef, 2016; Herden et al., 2013; Long et al., 2014; Richt et al., 2000). The highest viral concentration is found in the hippocampus and thalamus (Algermissen, 2010; Bilzer et al., 1996). Degeneration of neurons and neuronophagia are not prominent, however, loss of pyramidal cells of the hippocampus might be observed, and a reactive astrocytosis is usually accompanying all areas with inflammatory lesions. In less than 50% of the cases, intranuclear eosinophilic inclusion bodies (Joest-Degen bodies) can be found, particularly in the hippocampus, and are considered pathognomonic when detected (Herden et al., 2013; Richt et al., 2000). No significant lesions are apparent in the cerebellum, and lesions are inconsistent in the spinal cord.

In Germany, inapparent infection in horses has a prevalence of about 10% of antibodies against BoDV in serum, and therefore the presence of virus-specific antibodies is not related to disease unless there are clinical symptoms (Grabner et al., 2002; Stitz et al., 2002). For antemortem diagnosis, demonstration of specific antibodies is achieved in the serum and CSF using Western blot (WB), ELISA, or indirect immunofluorescence assay (IFA), which is advocated as the most reliable antigen-specific antibody detection (Richt et al., 2000). In the final stages of BD, when convulsions occur, there is a high concentration of lactate in the plasma (Grabner et al., 2002). For the post-mortem diagnosis, including FFPE brain specimens, histopathology, IHC, and alternatively nucleic acid detection via ISH (for well-preserved

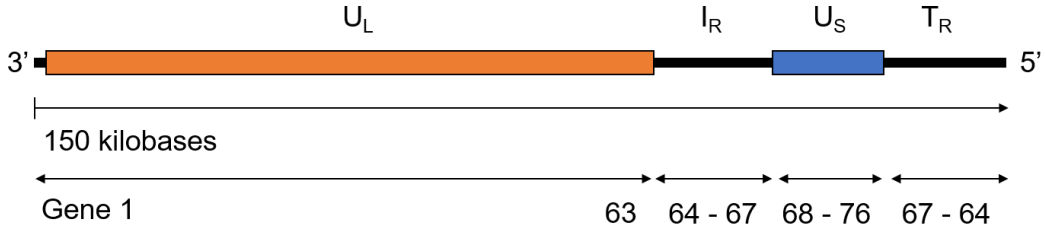
specimens), WB, or RT-PCR assays are confirmatory (Algermissen, 2010; Herden et al., 2000; Herden et al., 1999; Hoffmann et al., 2015; Tappe et al., 2018; Werner-Keišs et al., 2008). Monoclonal antibodies specific for the BoDV-1 nucleoprotein and phosphoprotein are frequently used to detect BoDV-1 in the nucleus and cytoplasm of neurons, neuronal processes, and glial cells (Bilzer et al., 1996; Herden et al., 1999).

There is no specific treatment for horses infected with BoDV-1 nor vaccines available. The main ways of preventing infections are in reducing the exposure of susceptible animal species to the reservoirs, in identifying clinically inapparent cases, and testing reservoirs that are in close contact to humans and horses, to avoid spillover infections (Tappe et al., 2018).

2.2.1.3 Equine herpesviral myeloencephalopathy

Equid herpesvirus type 1 and 4 are assigned under the order *Herpesvirales*, within the family *Herpesviridae*, subfamily *Alphaherpersvirinae*, genus *Varicellovirus* (Pellet & Roizman, 2013). While both viruses share high degree of genetic and antigenic similarities, they differ in host range and pathogenicity, being EHV-1 significantly more important in CNS infections (Patel & Heldens, 2005). They have the typical herpesvirion architecture of an enveloped, linear double-stranded DNA (dsDNA) genome of 150kb in length, encoding 76 ORFs, and replicating in the nucleus (**Fig. 7**). The genome contains the unique long region (U_L) which encodes genes 1 to 63, where ORFs largely used for EHV-1 detection (ORF33), characterization of neuropathogenic and non-neuropathogenic strains (ORF30), and latency factors (ORF63) are expressed (Baxi et al., 1995; Chesters et al., 1997), and the unique short region (U_S), which encodes genes 68 to 76. U_S is flanked by a repeat region creating identical, but inverted repeats, internal repeat (I_R) and terminal repeat (T_R), which encode genes 64 to 67, and 67 to 64, respectively (Pellet & Roizman, 2013). Herpesvirus genes have three general categories, 1) the ones encoding proteins with regulatory functions and virus replication, 2) encoding structural proteins, 3) heterologous set of genes not found in all herpesviruses and are not essential for replication in cultured cells (MacLachlan & Dubovi, 2011). The EHV-1 D752 genotype viruses contain a point-mutation within the DNA polymerase and are more commonly associated with equine herpesviral myeloencephalopathy (EHM), while the N752 genotype (wild-type) is responsible for approximately 15% to 26% of neurologic EHV-1 outbreaks (Pusterla & Hussey, 2014).

Fig. 7: Equine herpesvirus type 1 genome structure



Equine herpesvirus type 1genome is 150 kilobases long. I_R: internal repeat. T_R: terminal repeat. U_L: unique long region. U_S: unique short region. Modified from Pellet & Roizman, 2013.

Infection with EHV-1 is endemic in horse populations worldwide and it is estimated that up to 80% of all horses are latently infected (Gilkerson et al., 1999; Pusterla & Hussey, 2014). EHV-1 also causes disease in zebras (Blunden et al., 1998); is potentially pathogenic for llamas (*Lama glama*) (House et al., 1991) and was reported in other hosts such as a pregnant Indian rhinoceros, which aborted in mid-pregnancy, and that suffered from neurological disease (Abdelgawad et al., 2014). In Germany, zoo animals like four black bears (*Ursus americanus*), two Thomson's gazelles (*Eudorcas thomsonii*) and 18 guinea pigs (*Cavia porcellus*) suffered from neurological disorders all caused by EHV-1 (Wohlsein et al., 2011). In Brazil, the first record of EHV-1 isolation occurred in 1966 but the first case of EHM was described only in 2005, and a neuropathogenic strain described in 2009 (Lara et al., 2008; Mori et al., 2011). EHM outbreaks occur more commonly during winter and early spring, due to crowding, with older animals, pregnant or nursing mares considered at increased risk (Friday et al., 2000; Radostits et al., 2007). On the other hand EHV-1 infections are less likely to occur in horses younger than 3 years of age, and in a variety of robust small pony breeds, in Mangalarga, Brazilian Sport Horse, and Paint Horse (Goehring, 2015; Lara et al., 2008; Mori et al., 2011).

EHV-1 transmission to susceptible horses occurs by direct contact, through aerosolised droplets of respiratory secretions from acutely infected or reactivated virus-shedding horse, or from contact with aborted foals, foetal membranes and placenta, which are rich in infectious particles (Franco & Roehe, 2007; Rimstad & Evensen, 1993; Slater, 2014). Indirect transmission may occur via fomites and personnel (Lunn et al., 2009). By the time of transmission, EHV-1 enters and damage the respiratory epithelium and is from there transported to regional lymph nodes. It enters peripheral blood mononucleated cells, inducing T-cell and monocyte-associated viremia, and subsequently circulates in the blood stream (Cantile & Youssef, 2016; Goehring, 2015). The ability of EHV-1 to infect cells of the immune system enables it to cross the basal membrane and disseminate to other organs, including the pregnant uterus and the CNS (Gryspeerdt et al., 2010). The common final pathway for injury in each of these body systems is damage to vascular endothelium with subsequent necrosis, thrombosis,

and ischaemia. Impairment of blood flow will result in hypoxia and dysfunction or death of adjacent neural tissue resulting in the EHM (Radostits et al., 2007).

There are two cycles observed during the virus replication, the acute infection or productive (lytic cycle) and the latent infection. The lytic replication cycle occurs at the virus penetration sites at the host, (i.e. epithelium), and probably at neurons, before the establishment and during the reactivation of a latent infection. It is further characterized by viral nucleocapsid uncoating, expression of all viral genes, viral DNA replication (within host cell nucleus), and production of infectious viral progeny. It is also incompatible with the host cells survival (Franco & Roehe, 2007; Oladunni et al., 2019). The latent infection occurs in specific classes of neurons, especially in neurons of the sensorial ganglions and the autonomic ones, to where the nucleocapsids are transported by the axons or dendrites after the productive replication in the mucosae. This transport occurs by retrograde axoplasmic flow, through which the nucleocapsids reach the neural bodies. In some neurons, the expression of alpha genes is early suppressed. As the products of these genes are necessary to the next steps of the viral gene expression and to the genome replication, the cycle is interrupted. Thus, the viral genome persists at the nucleus of these neurons at the episomal form for the rest of the life (Franco & Roehe, 2007). Reactivation occurs when latently infected animals are stressed, like by malnutrition, super population, and transport of mares in advanced stage of pregnancy, and experimentally after corticosteroid administration (Edington et al., 1985; Franco & Roehe, 2007).

Clinical signs appear during or towards the end of the viremic phase of infection, e.g. in case of abortion. Neurologic signs are usually observed between six to 10 days post infection and peak in severity within two to three days (Pusterla & Hussey, 2014). There are invariably no premonitory clinical signs of respiratory disease, and pyrexia is likely to be the only warning clinical sign (Burgess et al., 2012; Goehring et al., 2010). The caudal segments of the spinal cord and sacral plexus are affected most often, although outbreaks have been described in which horses develop acute onset paralysis with cerebral signs, followed by rapid death. Neurologic dysfunction ranges from temporary ataxia and paresis to complete paralysis (McFadden et al., 2016). The hind limbs are usually the most severely affected due to sacral nerve involvement, resulting in notable hind limb weakness, bladder dysfunction with urine dribbling, cutaneous perineal sensory deficits, and decreased tail and anal tone with faecal retention (Johnson, 2011; McFadden et al., 2016). Less frequently there is cortical, brainstem, or vestibular disease characterized by depression, head tilt, ataxia, and cranial nerve dysfunction (Pusterla & Hussey,

2014; Slater, 2014). The prognosis for recumbent horses is poor, and requires euthanasia (Slater, 2014).

Gross lesions are not common, but small, random, multifocal haemorrhages may be visible throughout the brain and spinal cord (Cantile & Youssef, 2016; Summers et al., 1995). Multifocal necro-haemorrhagic or malacic areas can be present in severe cases, especially in the white matter of the spinal cord or the white or grey matter of the brain (Cantile & Youssef, 2016). Other relevant microscopic finding is vasculitis (viral cytotoxic effect or immune-mediated), that may end in thrombo-occlusive necrotizing vasculitis with secondary injury to the neuroparenchyma resulting in myeloencephalopathy (Cantile & Youssef, 2016). Usually, the affected venules are in the leptomeninges, brain, spinal cord, and spinal ganglia (Summers et al., 1995). Adjacent to the affected blood vessels, other lesions such as perivascular oedema, haemorrhage, focal areas of malacia, and infarction might be present. Intranuclear eosinophilic inclusion bodies within airway epithelial and hepatic cells from aborted foetuses are a typical histologic feature, but do not occur in the CNS (Slater, 2014).

Usually it is not possible to diagnose any of the diseases associated with infection by EHV with certainty clinically (Slater, 2014). Virus culture and isolation is considered the gold standard test for diagnosis, especially during epidemics of EHM, concurrently with PCR assays for the detection of viral DNA. Samples collected from nasal and nasopharyngeal swabs or buffy coat are strongly supportive in EHM cases of horses with compatible clinical signs (Lunn et al., 2009). Aborted foetus, placenta, brain and spinal cord, FFPE material, and infected cell cultures are also useful for PCR diagnostics and can be used also to discriminate between neuropathogenic and non-neuropathogenic strains in the CNS of horses (Leutenegger et al., 2008; Rimstad & Evensen, 1993). Besides, several PCR tests have been devised for the detection of viral DNA, capable of distinguishing between different EHVs (Carvalho et al., 2000; Daly & Doyle, 2003; Marshall & Field, 1997). Also, with real-time PCR (qPCR), it is possible to estimate the virus load in samples, as the virus copy number in latently infected leukocytes is between two and three orders of magnitude less than in lytically infected cells (Slater, 2014). Detection of viral antigen with direct immunofluorescence (DIF) in impression smears from nasopharyngeal swabs was demonstrated to be rapid and simple, with acceptable sensitivity and specificity and even faster than virus isolation (Oladunni et al., 2019; Slater, 2014). Histopathology is an essential method for confirming EHV infection in aborted foetuses through the identification of intranuclear inclusion bodies and samples collected from horses with necrotizing vasculitis due to EHM (Slater, 2014). IHC demonstrates viral antigen expression by infected epithelial and endothelial cells and is an additional technique to confirm

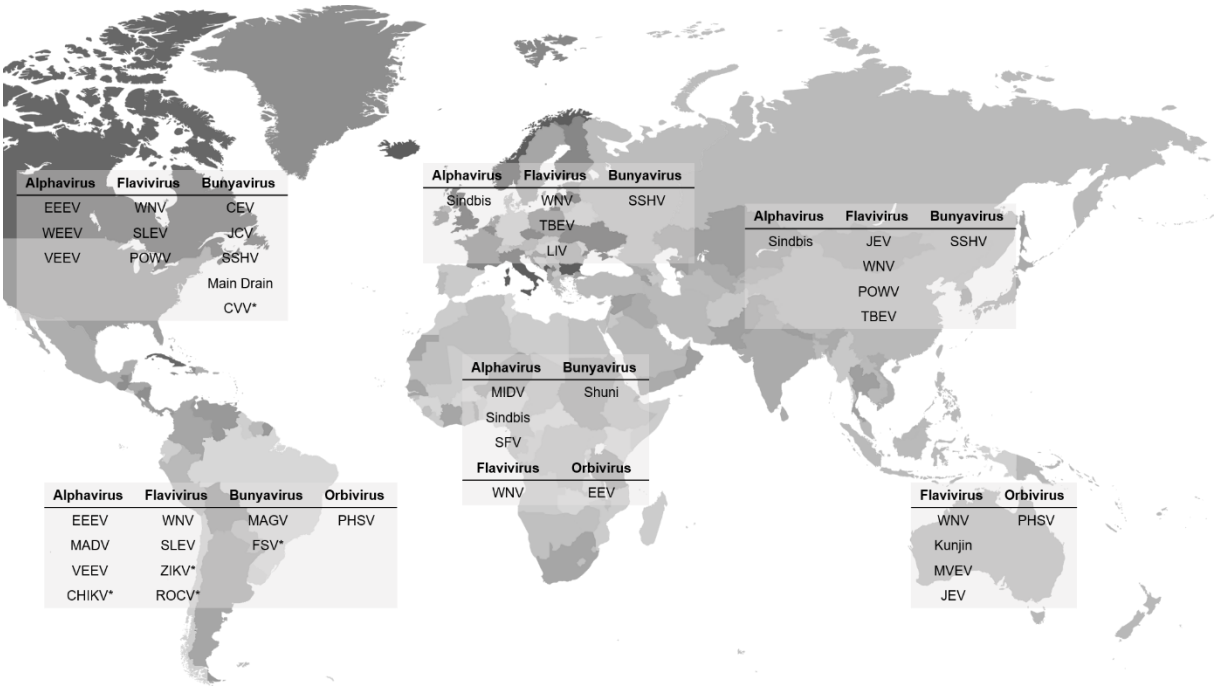
the infection (Schultheiss et al., 1997; Slater, 2014; Studdert et al., 2003). In the CNS, virus antigen is also present in neurons and astrocytes and has been linked to chorioretinitis in a foal (Schultheiss et al., 1997; Slater et al., 1992). EHV-1 can also be detected by ISH, providing additional sensitivity over IHC, since all virus-infected cells contain viral DNA, but only a proportion express viral proteins (Schmidt et al., 1994; Slater, 2014).

Because of the highly contagious nature of EHV-1 infections, horses with respiratory disease, abortion, or neurologic disease, especially if these occurs as an outbreak, should be isolated until the cause of the disease is identified (Radostits et al., 2007). Prevention of neurologic disease due to EHV-1 is difficult, thus it is important to minimize the risk of introduction of infection by periodic serologic monitoring and testing the new animals. If seropositive animals are detected, they should be kept separated from the others, avoid new animals without making quarantine. Also, minimizing stress risks could prevent the reactivation of latent infections. Vaccination with attenuated and inactivated vaccines have been used, in combination with management measures as a way of prevention (Franco & Roehe, 2007). There is no specific treatment for EHV infection, although the antiviral valacyclovir hydrochloride was used to treat horses in an outbreak of EHM (Goehring et al., 2010). Administration of corticosteroids has been discussed controversial, it could be contraindicated due to the presence of replicating virus (Radostits et al., 2007). The use of antibiotics with large spectre are recommended to prevent secondary infections (Riet-Correa et al., 2001).

2.2.1.4 Arboviruses of interest to equine CNS health

Arboviruses is a term to describe a group of hundreds of viruses that are transmitted by arthropods, mostly mosquitos and ticks, that infect a vertebrate host, where the virus replication usually also occurs (Fauci & Morens, 2016). More than 500 arboviruses are known, of which approximately 40 cause disease in domestic animals and many are zoonotic (MacLachlan & Dubovi, 2011). The arboviruses contain members of the families *Flaviviridae*, *Togaviridade*, *Peribunyaviridae*, *Rhabdoviridae*, *Reoviridae*, *Orthomyxoviridae*, and also of the single-stranded DNA (ssDNA) virus Asfarvirus, which causes the African swine fever disease (Hernandez et al., 2014). For horses and main focus of this section, the worldwide distributed *Flaviviridae*, *Togaviridae*, and *Peribunyaviridae* are most likely to cause encephalitis, followed by viruses in the family *Reoviridae*, genus *Orbivirus* (**Fig. 8**).

Fig. 8: Global distribution of equine encephalitic arboviruses

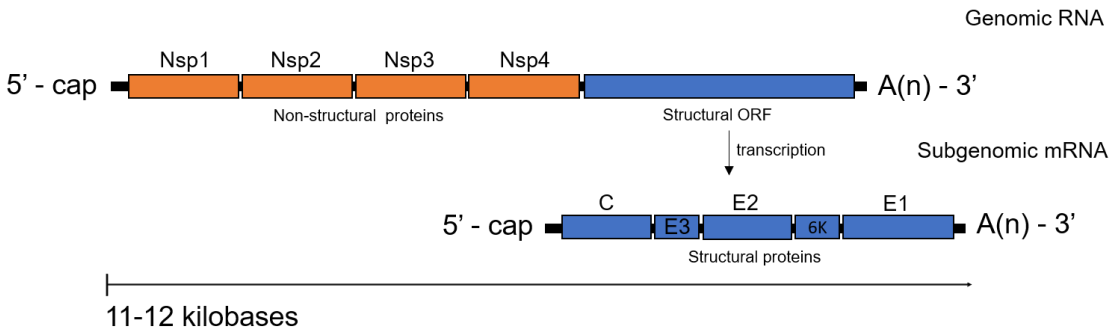


CEV: California encephalitis virus. CHIKV: Chikungunya virus. CVV: Cache Valley virus. EEV: Equine encephalosis virus. EEEV: Eastern equine encephalitis virus. FSV: Fort Sherman virus. JCV: Jamestown Canyon virus. JEV: Japanese encephalitis virus. LIV: Louping ill virus. MADV: Madariaga virus. MAGV: Maguari virus. MIDV: Middelburg virus. MVEV: Murray Valley encephalitis virus. PHSV: Peruvian horse sickness virus. POWV: Powassan virus. ROCV: Rocio virus. SLEV: Saint Louis virus. SFV: Semliki Forest virus. SSHV: Snowshoe hare virus. TBEV: Tick-borne encephalitis virus. VEEV: Venezuelan equine encephalitis virus. WEEV: Western equine encephalitis virus. WNV: West Nile virus. ZIKV: Zika virus. * Detected by serology in horses but cause neurological disease in humans. World map modified from http://pngimg.com/uploads/world_map/world_map_PNG30.png. Creative Commons license CC BY-NC available at https://creativecommons.org/licenses/by-nc/4.0/, unknown author.

2.2.1.4.1 Family *Togaviridae* (Arbovirus group A)

The family *Togaviridae* is composed of two genera, *Rubivirus* and *Alphavirus*, where only the later, distributed in more than 30 species, possess relevance for both human and animal health (**Appendix 1**) (Chen et al., 2018). Togaviruses are small, single-stranded, enveloped, unsegmented positive-sensed RNA viruses with a genome of 10-12kb (**Fig. 9**) (Chen et al., 2018). The non-structural proteins (Nsp) are produced by the cleavage of the precursor polyprotein and are responsible for the synthesis of RNA (-) capping (Nsp1), the helicase protease (Nsp2), RNA (+) (Nsp3), and polymerase/replicase (Nsp4). Meanwhile, the structural proteins are expressed by the translation of a subgenomic (26S) messenger RNA (mRNA) and they encode the capsid (C), and envelope glycoproteins (E3, E2, and E1), as well as the signal peptide (6K) and its frameshifting product (TF, not shown) (**Fig. 9**) (Chen et al., 2018). The capsid, E2 and E1, are the minimal proteins required for an infectious virion, but the signal peptide (6K) and the frameshifting product (TF) have been shown to have viroporin and virulence factor activities, respectively (Snyder et al., 2013).

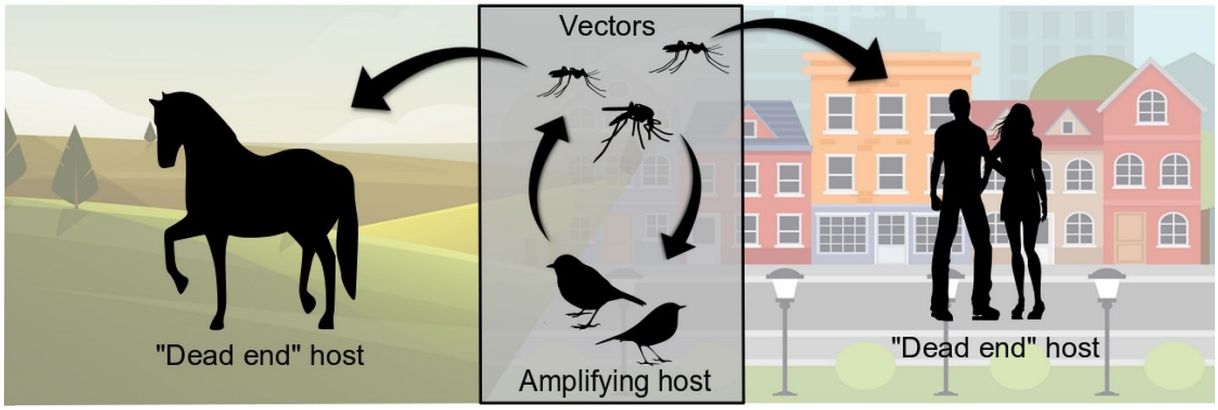
Fig. 9: Alphavirus genome structure



Sindbis virus, prototype for *Togaviridae*. The genome is linear and is represented by the continuous line, while the blocks represent the proteins encoded. 6k: signal peptide. C: capsid protein. E3, E2, E1: envelope glycoproteins. Nsp: non-structural proteins. ORF: open reading frame. Modified from Chen et al. (2018).

Most of the alphaviruses perpetuate in sylvatic cycles between asymptomatic avian hosts and mosquitos usually from *Culiseta* spp. (North America) and *Culex* spp. (Central and South America) (Fernandez et al., 2003; Molaei et al., 2006; Pisano et al., 2010). During epizootics, all species of mosquitos that feed on horses, including the culicid *Aedes*, *Psorophora* and *Deinocerites* spp. can help spreading the infection (Brault et al., 2002; Radostits et al., 2007). Passeriform birds species are the primary reservoir and amplifying hosts for the most significant alphaviruses causing encephalitis in horses, EEEV, Madariaga virus (MADV), Venezuelan equine encephalitis virus (VEEV), and Western equine encephalitis virus (WEEV) (**Fig. 10**, **Appendix 1**) (Molaei et al., 2016). Small rodents are also reservoirs for enzootic VEEV, like the genera *Sigmodon*, *Oryzomys*, *Zigodontomys*, *Heteromys*, *Peromyscus*, and *Proechimys,* and for EEEV during years of high virus transmission (Aguilar & Estrada-Franco, 2011; Carrara et al., 2007). Opossums, bats, and wild birds were also considered for the dispersal of enzootic VEEV (Scherer et al., 1972).

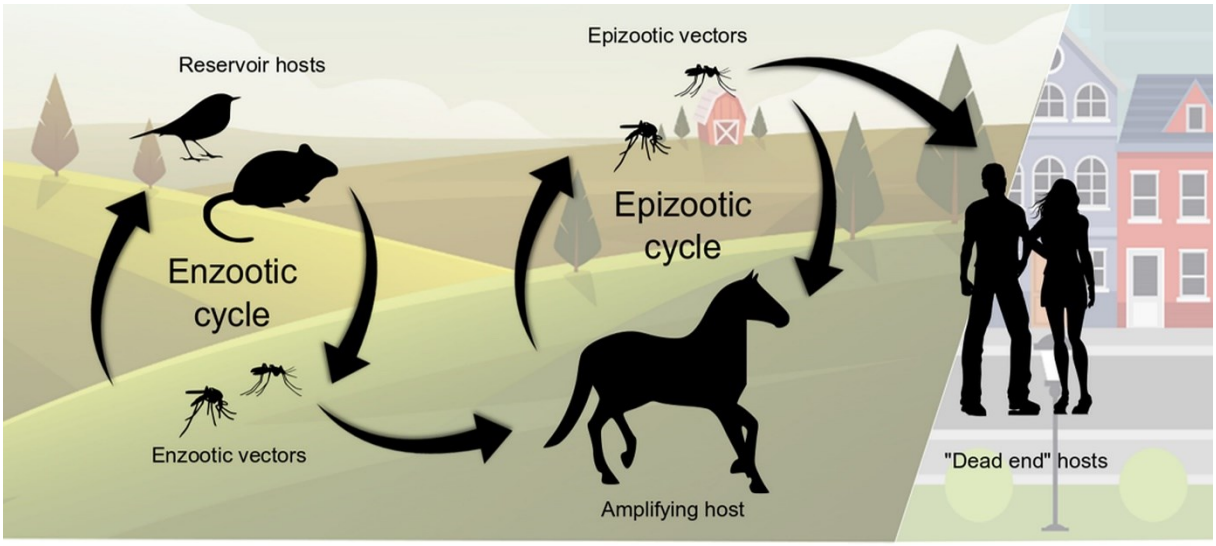
Fig. 10: Transmission cycle of Eastern and Western equine encephalitis virus and Madariaga virus



Horses and humans are considered dead end hosts, meaning that they keep viremic titers that are too low to induce infection of vectors. Illustration prepared on Inkscape© with VectorStock® royalty free icons.

In contrast, efficient amplification of VEEV by equids is the hallmark of its epizootic strains IAB and IC (**Fig. 11**) (Aguilar & Estrada-Franco, 2011).

Fig. 11: Venezuelan equine encephalitis virus transmission cycles



Differently from EEEV, MADV, and WEEV, horses are considered amplifying hosts within the VEE cycle, actively infecting susceptible mosquitos. Illustration prepared on Inkscape© with VectorStock® royalty free icons.

Besides the dead-end hosts horses and humans (and especially children for MADV – Luciani et al., 2015), other animal species are susceptible to infections with encephalitic alphaviruses, like alpacas and llamas (Nolen-Walston et al., 2007), cattle (McGee et al., 1992), swine (Elvinger et al., 1994), and dogs to EEEV (Farrar et al., 2005). Exotic birds are susceptible to EEEV and WEEV, like emus, the ring-necked pheasant, Pekin ducks, Chukar partridges, and whooping cranes (Ayers et al., 1994; Dein et al., 1986; Kissling et al., 1954). Domestic rabbits, small ruminants, and dogs develop potentially fatal clinical disease after VEEV infection (Sudia et al., 1975).

The alphaviruses have a worldwide distribution, but the viruses with the highest potential to cause encephalitis are so far geographically limited to the Americas, with overlaps in their distribution in the continent (Ferreira et al., 1994; Long, 2015a; Pauvolid-Corrêa et al., 2010; Radostits et al., 2007; Weaver et al., 1994). In South and Central America, CNS infections in horses and humans are predominantly caused by MADV, EEEV, and VEEV (Luciani et al., 2015; Pisano et al., 2013; Silva et al., 2015). Most infections by EEEV in North America occur east of the Mississippi river, while WEEV is generally detected west to the river and in Florida (Chénier et al., 2010; Del Piero et al., 2001). Alphaviruses linked to equine encephalitis in other regions are caused by infections with Middelburg virus (MIDV) and

Sindbis virus (SINV) in South Africa (van Niekerk et al., 2015), and Semliki Forest virus (SFV) in Senegal (Robin et al., 1974).

The transmission of alphaviruses to vertebrates occurs through the bite of an infected insect. Mosquitoes are infected by feeding on viremic birds or by vertical transmission, (Radostits et al., 2007). The pathogenesis of encephalitic alphaviruses is based on limited studies from human cases and animal models, usually with laboratory rodents and can be summarized in three main phases, 1) the initial peripheral phase of virus replication and spread, 2) neuroinvasion, and 3) terminal CNS phase in which virus spreads within the brain, infecting neurons primarily, which results in a neurodegenerative state that is often fatal (Steele & Twenhafel, 2010). Susceptibility to encephalitic alphaviruses varies between viruses and strains, age of animal infected, dose and route of virus inoculation (Steele & Twenhafel, 2010).

On the range of clinical signs, alphaviruses infections are indistinguishable, and only a fraction of diseased horses will develop signs of encephalitis. The incubation period for EEEV is 1-3 days, 2-9 days for WEEV and 1-6 days for VEEV (Radostits et al., 2007). The clinical course ranges from 24 hours to 15 days (Chénier et al., 2010; Silva et al., 2011). Initial neurologic signs are hyperexcitability, which then change to somnolence, depression, and recumbency. Circling, cortical blindness, propulsive walking, head pressing, and ataxia are commonly observed (Oberste et al., 1998; Silva et al., 2015; Silva et al., 2011; Sousa et al., 2015). True blindness and pharyngeal paralysis occur only in the late stages (Radostits et al., 2007). A more severe form of the disease is manifested by tachycardia, tachypnoea, depression, anorexia, occasional diarrhoea, and fever (Chénier et al., 2010; Radostits et al., 2007). The prognosis for horses with signs of encephalitis is unfavourable. Some affected horses that do not become recumbent may stabilize and survive. However, they may suffer permanently from cerebrocortical damage (Furr & Reed, 2008). Morbidity of infected animals may reach 60%, while fatal cases rate may reach almost 100% of infected animals (Silva et al., 2011). Death may occur after 5-14 days of rapid deterioration (Furr & Reed, 2008).

Usually there are no gross lesions in horses infected by alphaviruses (Cantile & Youssef, 2016). CNS infection is mostly observed in the grey matter of cerebral cortices, thalamus, and hypothalamus, with milder and less frequent lesions in the brainstem and spinal cord (Campos et al., 2013; Del Piero et al., 2001; Long & Gibbs, 2014; Sousa et al., 2015). The trigeminal ganglia are not affected (Cantile & Youssef, 2016). Histological characteristics are neuronophagia, early microglial reaction appearing as rod cells, and in severe cases malacia and necrosis of the neuropil (Furr & Reed, 2008). A neutrophilic infiltration is typical of EEEV in early infection, while VEEV is characterized by a more mixed lymphocytic-neutrophilic

infiltrate. Histology of WEEV infection is usually more lymphocytic/plasmacytic than suppurative (Cantile & Youssef, 2016; Furr & Reed, 2008). Venules with swollen endothelial cells are common, with cuffed neutrophils. Necrotizing vasculitis with thrombosis and cerebrocortical malacia can be additionally present in severe cases (Cantile & Youssef, 2016). Although uncommon in horses, extra neural lesions associated with presence of EEEV antigen can also occur in myocardium, stomach, intestine, urinary bladder, renal interstitial cells, and spleen (Del Piero et al., 2001; Poonacha et al., 1998). Extra neural lesions involved in a VEEV infection include necrotizing lesions in lymphoid organs, pancreas, liver, and heart (Kissling, 1967).

Antemortem diagnosis for alphaviral infections in horses are presumptive at best (Pennick et al., 2012). CSF may show a neutrophilic pleocytosis in EEEV cases, sometimes accompanied by an increase in protein concentration (Long, 2015a). Serology may detect silent activity/circulation of the virus and can be used to identify sub-clinical infections. PCR from serum and virus isolation, however, do not detect the virus in this case (Pauvolid-Corrêa et al., 2010). The OIE recommends specific tests for different purposes (**Table 1**). Their success and sensitivity varies with the material available and the work conditions (Lambert et al., 2003; Pennick et al., 2012).

Table 1: Methods available for the diagnosis of alphaviruses and their purpose using equine samples

Continued

Method	Purpose				
	Population free from infection	**Individual animal free from infection**	**Confirmation of clinical cases**	**Prevalence of infection/ surveillance**	**Immune status in animals or populations post-vacc.**
Agent identification					
Virus isolation (brain)	-	++	+++[a]	-	-
Molecular methods (brain, virus isolates)					
RT-PCR	-	++	+++	-	-
RT-qPCR	-	++	+++	-	-
Antigen detection					
IHC (brain)[b]	-	++	+++	-	-
IgM-capture ELISA (mosquitos)	n/a	n/a	n/a	++	n/a

Method	Purpose				
	Population free from infection	Individual animal free from infection	Confirmation of clinical cases	Prevalence of infection/ surveillance	Immune status in animals or populations post-vacc.
Detection of immune response					
Serological tests (serum) [c]					
IgM capture ELISA	-	+[d]	++	-	-
Hemagglutination inhibition	+	++	++	++	++
Complement fixation	-	+	++	-	-
Plaque reduction neutralization	+++	+	++	+++	+++

Modified from OIE (2019b). +++: recommended method. ++ suitable method. +: may be used in some situations, but cost, reliability, or other factors severely limits its application. -: not appropriate for this purpose. ELISA: enzyme-linked immunosorbent assay. IHC: immunohistochemistry. n/a: purpose not applicable. RT-(q)PCR: (real-time) reverse transcription polymerase chain reaction. Vacc.: vaccination.
[a] VEEV: ++. [b] : negative results with the technique do not rule out infection. [c]: Interpretation must be done with clinical signs onset, epizootic situation, and vaccination history. [d] VEEV: -.

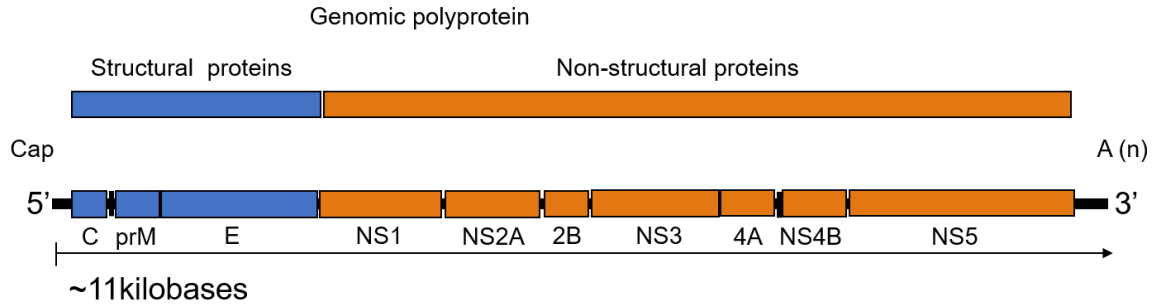
There is no definitive or specific treatment for horses infected with alphaviruses and only supportive therapy is foreseen (Radostits et al., 2007). Vaccination is considered the core component of immune-prophylaxis for horses, and although it confers immunity of variable duration, it is recommended to re-vaccinate the horses annually and before the vector season in the spring (Balasuriya et al., 2015). Prevention of alphaviral infections can be also achieved by housing horses indoors at night, especially in fly proofed stables, with the use of insect repellents, and to quarantine infected animals might restrain the spread of the virus (Radostits et al., 2007).

2.2.1.4.2 Family Flaviviridae (Arbovirus group B)

The family is composed by three genera: *Flavivirus*, *Hepacivirus*, and *Pestivirus*. The genus *Flavivirus* – composed of 53 species - poses the highest concern for human and animal health; its zoonotic pathogens cause asymptomatic up to severe infections, fatal haemorrhagic fever or neurological disease (Radostits et al., 2007; Simmonds et al., 2017). The 40-60 nm virions are enveloped, contain a positive-sense ssRNA genome that measures 9-13 kb (**Fig. 12**) (Simmonds et al., 2017). This genus is the only one in the *Flaviviridae* family that contains a capping (Cap) structure in the 5' end terminal. The structural proteins are essential for virion assembly through the capsid protein (C); after formed by its precursor membrane protein (prM), later the M protein assumes a role in viral replication, and envelope protein 1 (E1), which has

three structural domains, is responsible for structural rearrangements for fusion and binding of the virion. The non-structural proteins (Nsp) are cleaved after translation into Nsp1, Nsp2A, Nsp2B, Nsp3, Nsp4A, Nsp4B, and Nsp5 and are required for viral replication and assembly (Lindenbach et al., 2013; Long, 2015a). Flavivirus entry into cells is a crucial virulence factor, which is coordinated by E proteins on the surface of the virion, and consists on one of the major targets for anti-viral therapies (Pierson & Kielian, 2013).

Fig. 12: Flavivirus genome structure



Boxes below the genome indicate precursors and mature proteins generated by the proteolytic processing cascade. Cap: capping structure in the 5' end terminal. C: capsid protein. E: envelope glycoprotein. NS, 2B, and 4A: non-structural proteins. prM: precursor membrane protein. Modified from Lindenbach et al., 2013.

Mosquitos from *Culex* spp. and ticks from *Ixodes* spp. are the main vectors, and infections are maintained by the amplifying hosts. In North America, transmission cycle of mosquito-borne flaviviruses is maintained by birds, like the house sparrow (*Passer domesticus*), and in Latin America by Passeriformes, Columbiformes, and Galliformes birds (**Appendix 2**) (Diaz et al., 2008; Bosch et al., 2007; Dupuis et al., 2003; Melandri et al., 2012; Osorio et al., 2012; Radostits et al., 2007; Ulloa et al., 2003). In addition, free-ranging New World monkeys (*Alouatta caraya*, *Sapajus nigritus*, *S. cay*), armadillos, and marsupials are infrequently associated (Figueiredo, 2000; Svoboda et al., 2014). Pigs are the main amplifying hosts and targets for Japanese encephalitis virus (JEV), while for Murray Valley encephalitis virus (MVEV) rabbits and possibly grey kangaroos are accounted (Mackenzie et al., 2017; OIE, 2019d). For the tick-borne infections, lagomorphs, small rodents, skunk, dogs, sheep, and grouse are the best-known hosts (Fatmi et al., 2017; Hubálek et al., 2014; Kaiser, 2016).

Horses of all ages appear to be equally susceptible to infection (Radostits et al., 2007) and along with humans, they are dead-end hosts for encephalitic flaviviruses. For instance, neurologic disease due to WNV infection occurs in squirrels, chipmunks, bats, dogs, cats, reindeer, sheep, alpacas, alligators and a harbour seal (OIE, 2019h); the primary animals infected by tick-borne viruses are sheep (Louping ill virus), lambs, kids (tick-borne encephalitis virus, TBEV) and dogs (TBEV and Powassan virus) (**Appendix 2**).

While the *Flavivirus* genus presents worldwide distribution, the viral species are restricted to areas, usually related to the presence of the transmission vector (Ridpath & Flores, 2007). WNV has the widest geographic distribution. It is enzootic to Africa, it exploded in Europe (available in https://ec.europa.eu/food/animals/animal-diseases/not-system_en#) and Middle East (reviewed by May et al., 2011), and was introduced in 1999 in North America (Nash et al., 2001). WNV also spreads to Latin America, unmasked by several serological studies and by the first cases of clinical disease in humans and horses after 2015 (Silva et al., 2019; Vieira et al., 2015) (**Fig. 13**). SLEV circulates at least since 2005 in South America (Burgueño et al., 2013; Tauro et al., 2012), while

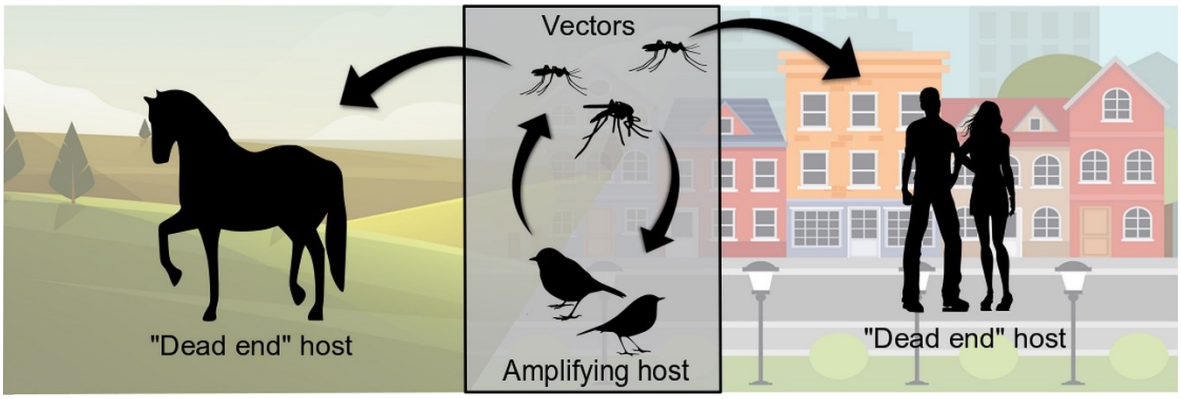
other encephalitic flaviviruses, like Ilheus virus are reported in Brazil since 1944 in Bahia state, and Rocio virus (ROCV) in the 1970's in São Paulo state (Laemmert & Hughes, 1947; Rosemberg, 1980). JEV outbreaks occur in Asia and northern Australia, TBEV and Louping ill in Europe, while Powassan virus is endemic in North America and sporadic in Russia (Fatmi et al., 2017; Hubálek et al., 2014; Kaiser, 2016; Krow-Lucal et al., 2018).

Fig. 13: Timeline of investigations carried out with flaviviruses posing risk of causing neurological disease, Brazil

ILHV: Ilheus virus. MG: Minas Gerais. MS: Mato Grosso do Sul. PB: Paraíba. PCR: Polymerase-chain reaction. RJ: Rio de Janeiro. RN: Rio Grande do Norte. RS: Rio Grande do Sul. SLEV: Saint Louis virus. SP: São Paulo. WNV: West Nile virus. Illustration prepared on Inkscape© with VectorStock® royalty free icons.

When mosquitos infected by viremic hosts feed on horses it initiates the transmission cycle (**Fig. 14**). Keratinocytes and skin dendritic cells (Langerhans cells) are the primary targets and can transport the virus to draining lymph nodes where the virus replicates and allow to disseminate into circulation and internal organs (Pierson & Kielian, 2013). Virus entry receptors involved in CNS invasion are virus species- and cell target-dependent, and occurs via C-type lectin dendritic cells specific intercellular-3 grabbing non integrin (DC-SIGN), toll-like receptor 3 and 7 (TRL3, TRL7), retinoic-acid-inducible gene 1 (Rig-1), Claudins-1, -6, -9, glycosaminoglycans (GAG), and heat shock proteins (hsp) (Neal, 2014). Flaviviruses prefer developing neurons and neuronal progenitors, and can also infect pericytes, astrocytes, and microglia (Neal, 2014).

Fig. 14: Mosquito-borne flaviviruses transmission cycle



Infected blood meal-seeking mosquito vectors feed on avian hosts, which will develop a viremic phase. When these birds are then bitten again, they will infect new mosquito vectors. These will sporadically feed on dead end hosts, such as horses and humans, who are susceptible to the virus. Illustration prepared on Inkscape© with VectorStock® royalty free icons.

The incubation period after natural infection is eight to 15 days (Radostits et al., 2007). Affected horses may present mild lymphopenia, and hyperbilirubinemia due to anorexia, and occasionally azotaemia (Porter et al., 2003). While some horses show signs of an uncomplicated infection by WNV, around 8% of exposed naïve horses will display neurological disease (Gardner et al., 2007). A mild increase in rectal temperature to around 38.3-38.5°C usually accompanies the infection before muscle fasciculation of the face and neck, weakness, ataxia and dysmetria (Angenvoort et al., 2013; Murgue et al., 2001; Porter et al., 2003). Drooping of eyelid, head tilt, tongue weakness, and muzzle deviation reveal cranial nerve deficit. Behavioural changes likely result from infection of thalamus, medulla, and pons, with limited viral load in the cerebrum, and rare direct lesions in the cerebellum (Delcambre & Long, 2014; Porter et al., 2003; Toplu et al., 2015). Complicated cases of JEV may additionally result in blindness, coma, and death (Gould et al., 1964). SLEV also causes incoordination, depression,

and flaccid paralysis of hind limbs (Rosa et al., 2013).

Gross lesions are infrequently seen, but might consist of multifocal congestion and haemorrhage within the medulla oblongata, midbrain, and spinal cord (Radostits et al., 2007). Histopathological changes include a non-suppurative meningoencephalomyelitis with multifocal glial nodules, which usually contain a few neutrophils amidst, and neuronophagia. In most cases, lesions are mild and confined to thin cuffs of lymphocytes, with fewer macrophages, mostly in the grey matter. The inflammatory changes and viral distribution are concentrated in the brainstem and thoracolumbar spinal cord; the cerebral cortex and cervical cord are less affected (Autorino et al., 2002; Cantile & Youssef, 2016; Porter et al., 2003; Radostits et al., 2007; Toplu et al., 2015). Areas of haemorrhage and malacia appear in severe cases, especially in brainstem and the ventral horn of the thoracic and lumbar spinal cord. Axonal swelling, spheroid formation, and vacuolization are also observed (Cantile & Youssef, 2016; Toplu et al., 2015). Extra neural lesions, like hepatitis and myocarditis, which occur in avian WNV infection, do not occur in equine WNV infection (Cantile & Youssef, 2016; Smedley et al., 2007).

Confirmatory diagnose is achieved by typical clinical history and clinical signs, to the residence in a risk area and the season of infection. A complete blood count usually reveals lymphopenia, while the CSF shows plasmacytic/lymphocytic pleocytosis combined with increased protein concentration (Wamsley et al., 2002). Most serological tests present cross reactivity among flaviviruses; plaque reduction virus neutralization is the most reliable using a 90% neutralization threshold (OIE, 2019d). IgM ELISA to detect antibodies against nsp1, which is induced only by infection and not by vaccination, has increased serological tests reliability (Beck et al., 2017; Konishi et al., 2004). Other diagnostic techniques are recommended by the OIE according to their methods and purposes (**Table 2**). For post-mortem diagnosis, histopathology is ideal and should be correlated with presence of antigen by IHC and viral RNA by RT-PCR (Delcambre & Long, 2014; Rosa et al., 2013). It is recommended to analyse spinal cord sections and multiple CNS sites to increase the chances of finding a virus-rich focus (Cantile & Youssef, 2016; Del Piero et al., 2001). This strategy also increases viral isolation outcome (Kleiboeker et al., 2004). As WNV infection in horses coincide with a short viremic phase of four to six days that is cleared by the onset of clinical signs, only animals with RT-PCR positive results are meaningful (Angenvoort et al., 2013; Patel et al., 2013; Scaramozzino et al., 2001). ISH to detect viral RNA in nerve fibres, axonal hillocks, spheroids, glial cells and their processes, endothelial cells of the brainstem and spinal cord has also proven

useful; viruses are detected to a lesser extent within cerebral hemispheres and cerebellum (Toplu et al., 2015).

Table 2: Methods available for the diagnosis of flaviviruses and their purpose using equine samples

Method	Purpose				
	Population free from infection	Individual animal free from infection	Confirmation of clinical cases	Prevalence of infection/ surveillance	Immune status in individual animals or populations post-vaccination
Agent identification					
Virus isolation (brain, spinal cord, blood)					
Isolation in tissue culture	-	++[a]	++(+)	-	-
Molecular methods (brain, spinal cord, blood)					
qRT-PCR	++[b]	++	++(+)	++[b]	-
Nested RT-PCR	-	++	+++	-	-
Antigen detection (brain, spinal cord)					
IHC	-	-	+	-	-
Detection of immune response (serum)					
IgM capture ELISA	++ [b]	++ [b]	++	++ [b]	++ [b]
Plaque reduction neutralization	++	++ [b]	++(+)	++	++
IFAT	+	+	+	+	+

Modified from OIE (2019d, 2019h). +++ recommended method. ++(+) recommended method but might have reduced sensitivity depending on the virus. ++ suitable method. +: may be used in some situations, but cost, reliability, or other factors severely limits its application. -: not appropriate for this purpose. ELISA: enzyme-linked immunosorbent assay. IFAT: indirect fluorescent antibody test. IgM: immunoglobulin M. IHC: immunohistochemistry. RT-PCR: reverse transcription polymerase chain reaction. qRT-PCR: real-time RT-PCR. [a] – for Japanese encephalitis virus. [b] – for WNF.

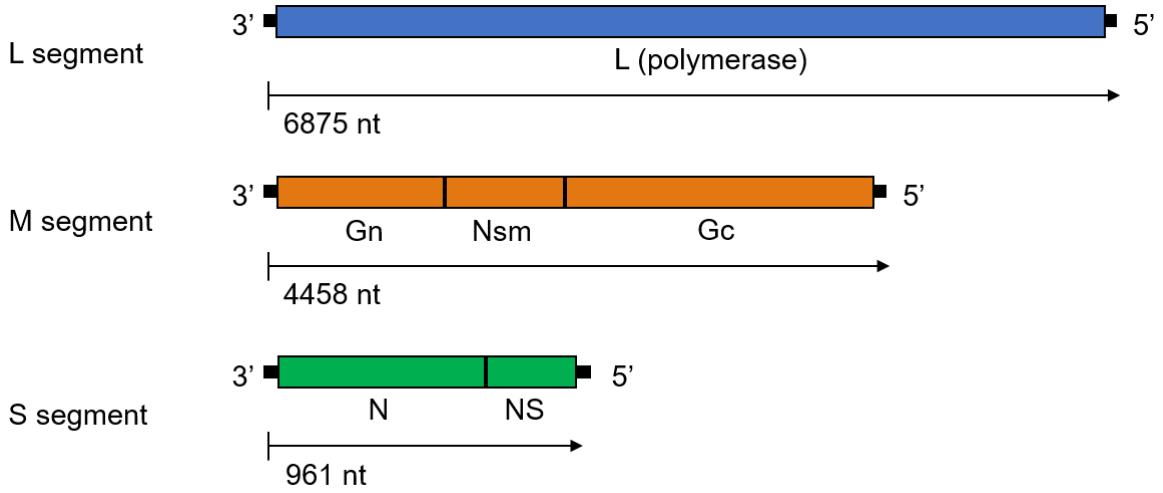
There is no specific treatment for flavivirus infection in horses and only supportive care is performed (Long, 2015a). Currently, there are four USDA-licensed WNV vaccines for horses that confer immunity for up to one year, and that should be applied in the spring, prior to the onset of the vector season (Balasuriya et al., 2015). Other effective preventive measures rely on vector control and the use of repellents (Long, 2015a).

2.2.1.4.3 Family *Peribunyaviridae* (Arbovirus group C)

This family is composed by four genera: *Orthobunyavirus*, *Herbevirus*, *Pacuvirus,* and *Shangavirus* (Hughes et al., 2020). Despite the tremendous impact that all bunyaviruses have on human and animal health, this review will focus on neurotropic orthobunyaviruses. The 80-

120 nm virions are enveloped and contain three distinct, single-stranded, negative-sense RNA segments comprising 11.2-12-5 kb in total (**Fig. 15**) (Hughes et al., 2020). The large segment (L) encodes the L protein, which has RNA-directed RNA polymerase and endonuclease functions, the medium segment (M) encodes two structural glycoproteins, Gn (amino-terminal coding) and Gc (carboxyl-terminal coding), and some viruses like the *Bunyamwera orthobunyavirus*, encode a non-structural protein (Nsm – movement protein). The small segment (S) encodes the nucleocapsid protein (N), abundant in infected cells, and the non-structural protein (NS) (Elliot & Schmaljohn, 2013; Hughes et al., 2020).

Fig. 15: Orthobunyavirus genome structure



Bunyamwera orthobunyavirus (BUNV) genome, segmented coding. Genomic RNAs are represented by the thick lines, above the gene products (colored blocks) are shown. The length is given in nucleotides (nt). Gc and Gn: envelope glycoproteins. N: ribonucleoprotein. NS: nonstructural protein s. Nsm: nonstructural protein. Modified from Elliot & Schmaljohn 2013.

More than 100 viruses compose the *Orthobunyavirus* family, all associated as mosquito-borne pathogens transmitted by *Aedes* spp., *Culicoides* spp., *Culiseta* spp., and *Culex* spp. (Cardoso et al., 2015; Sakkas et al., 2018). A variety of small mammals and birds serve as virus reservoirs for these worldwide-distributed viruses (**Appendix 3**). The most important CNS pathogens for horses are the Maguari virus (MAGV) (Tauro et al., 2015), Snowshoe hare virus (Heath et al., 1989), and Main Drain (Emmons et al., 1983), present in the Americas, and Shuni virus detected in Africa (van Eeden et al., 2012). Still, there are human febrile and neurotropic viruses like Fort Sherman virus (Oliveira Filho et al., 2020) and Cache Valley virus (Wilson et al., 2017), respectively detected in serological assays in horses, but their role in virus transmission and maintenance remain yet undetermined.

The infection cycle and the cytoplasmic replication undergone by bunyaviruses in equine infections is similar to the mosquito-borne flaviviruses (Elliot & Schmaljohn, 2013).

The virus entries host cells through clathrin-mediated endocytosis (Hughes et al., 2020), and the cytopathic effects vary widely between the virus species. Neurons are the primary target of CNS infection in mice models (Proenca-Modena et al., 2016).

Main infection signs are characterized as aborts and teratogenic effects, especially in ruminants, and CNS disease in horses suggestive of multifocal lesions – in cerebral and cerebellar cortices, thalamus, brainstem, cranial nerve nuclei, and possibly spinal cord (Heath et al., 1989; Hoffmann et al., 2013; Tauro et al., 2015). Clinical signs include incoordination, ataxia, stiffness of the neck, head-pressing, circling, inability to swallow, fever, tachycardia, and muscle spasms (Emmons et al., 1983; Heath et al., 1989). Histological lesions are predominantly found in the cerebral cortex, and characterized as histiocytic and lymphoplasmacytic meningoencephalitis, with thick perivascular cuffs, multifocal necrotizing panencephalitis, and neuronal necrosis (Cantile & Youssef, 2016; Heath et al., 1989). The CSF demonstrates a mild leucocytosis (primarily lymphocytic) and the hemagglutination inhibition seroconversion usually reveals more than one antigen simultaneously. Display of compatible symptoms associated to positive serum IgM detected with ELISA is considered diagnostic for acute infections in humans. Diagnosis can also be established by virus isolation and molecular detection of the viral RNA segments S or M (Romero-Alvarez & Escobar, 2018). Specific control measures are not determined so far (Goehring, 2008; Sakkas et al., 2018). However, deforestation and cultivation of land are risk factors - this potentially brings susceptible animals such as the horse (and people) in contact with the vector and the virus reservoir.

2.2.1.4.4 Family Reoviridae

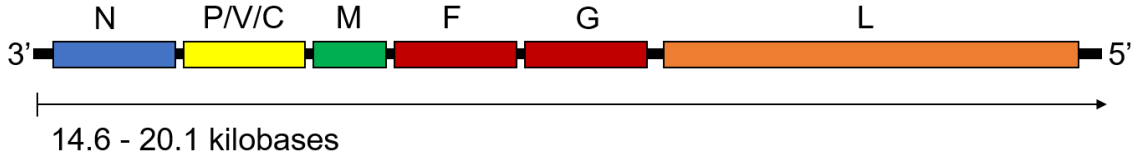
Two viruses from the *Orbivirus* genus cause sporadically fatal neurological disease in horses, the Equine encephalosis virus in Africa and the Peruvian horse sickness virus in Peru and Australia. They are transmitted to horses through biting midges from *Culicoides* spp. and mosquitos, respectively (Attoui & Mohd Jaafar, 2015).

2.2.1.5 Paramyxovirus encephalitis

Paramyxoviruses are enveloped pleomorphic virions with cytoplasmic replication. They contain a non-segmented negative single-stranded RNA (ssRNA) genome, which lengths between 14.6 to 20.1 kb and encodes six genes that produce six correspondent structural proteins N, P, M, F, G, L (**Fig. 16**) (Rima et al., 2019). The nucleocapsid protein (N), phosphoprotein (P), and large protein (L) genes provide RNA polymerase activity and are necessary for replication. The glycoprotein gene (G) encodes the respective protein for the viral attachment to the host cell surface and the fusion protein gene (F) for viral membrane fuse and

viral release into the host cell; it also induces syncytia formation, while the matrix protein gene (M) encodes the protein for virion budding and reduces host immune response to the virus (reviewed by Dawes & Freiberg, 2019). Two relatively new viruses that can cause encephalitis in horses within the *Paramyxoviridae* family, *Nipah henipavirus* (NiV) and *Hendra henipavirus* (HeV), belong to the *Henipavirus* genus, and although apparently geographically restricted, there are further evidences that similar viruses are circulating in non-pteropid fruit bats across the world (Mortlock et al., 2015; Muleya et al., 2014; Rima et al., 2019).

Fig. 16: Henipavirus genome organization



Genome structure not to scale. Each box represents separately the six encoded protein by its correspondent gene; slashes indicate where multiple distinct open reading frames are present within mRNA transcripts. Nucleocapsid-associated proteins: N - RNA-binding nucleocapsid protein, P (V,C) - polymerase-associated phosphoprotein, L - large protein. Membrane-associated proteins: M - matrix protein, envelope proteins – F - fusion protein and G - glycoprotein. Modified from Rima et al. (2019).

Nipah encephalitis, an emerging disease characterized by severe and rapidly progressive encephalitis and pneumonia, affects humans and several animals species; it has been detected in Malaysia, Cambodia, India, Bangladesh, and Philippines (Cantile & Youssef, 2016; Ching et al., 2015; Weatherman et al., 2018). The fruit bat flying fox (*Pteropus* spp.) is the natural reservoir host of NiV, and spreads the disease horizontally to pigs and, humans (Hinchcliff, 2014). Pigs are the most susceptible animal, but natural infection by direct contact with swine has also been reported in horses, cats, goats, and dogs. Morbidity rates in pigs are ~10%, with case mortality rates of <15%. The incubation period is estimated to be 1-2 weeks. In pigs, the virus targets the CNS and overcomes the blood-brain barrier through infection of brain endothelial cells and results in acute onset of nervous signs, like trembling, seizures, or tetanus-like spasms. Respiratory disease is also observed, characterized by signs of acute dyspnoea - laboured and harsh respiration, open-mouth breathing and severe cough. Additionally, abortion may occur to pregnant sows (Cantile & Youssef, 2016; Clayton, 2017; Hooper & Williamson, 2000). Neurological symptoms in horses presumably infected by NiV consist of head tilt, circling, and ataxia. However, no lesions or diagnostic tests were carried out, and infection is based on the report of human cases of encephalitis by NiV in the same villages in Philippines, few months later (Ching et al., 2015).

Hendra encephalitis is a rare emerging zoonosis affecting horses. It has been detected in Queensland and New South Wales in Australia, and like NiV, the reservoir hosts are flying foxes. However, transmission of HeV from bats to horses is rare. There is epidemiologic evidence of horse-to-horse transmission, particularly in stables or veterinary clinics, and through surface or equipment contaminated by infectious fluids (Savage et al., 2014).

There is little pathologic information available from post-mortem naturally infected animals. Lesions are similar to those observed with NiV, where the characteristic histologic lesion is vasculitis with fibrinoid degeneration and necrosis of the vascular wall. This lesion typically involves smaller vessels in lung, brain, meninges, spleen, lymph nodes, and kidney. Severe involvement of the lung is common, with diffuse areas of necrotizing alveolitis and marked fibrinous alveolar exudates accounting for the respiratory signs. Syncytial cells may also develop in vascular and lymphatic endothelium, and respiratory epithelial and lymphoid cells (Middleton, 2014; Savage et al., 2014). After an incubation time varying from 4 to 16 days, horses may develop severe, acute, febrile respiratory disease, sometimes accompanied by facial swelling, ataxia, and terminally, copious frothy nasal discharge as the result of pulmonary oedema. Recently, neurological signs have predominated in outbreaks, where the animals display signs of hypersensitivity, ataxia, disorientation, facial paralysis, head tilt, circling, head pressing, and strangury (Field et al., 2010).

NiV and HeV are biosafety level 4 (BSL4) agents and all diagnostic methods that involve viral cultures, blood samples, and fresh tissues must be performed with appropriate biosafety standards. Serological tests like virus neutralization and ELISA are available, they provide definitive antibody identification and screening, respectively. Differentiating infected from vaccinated animals (DIVA) assays might be also carried out. Identification of the agent is achieved with virus isolation and nucleic acid detection with conventional RT-PCR and qRT-PCR for N, M, P, and L genes (reviewed in OIE, 2019c; Tong et al., 2008). IHC to detect henipavirus nucleoprotein is a safe tool, but still not widely available; suitable tissues are lung, brain, lymph nodes, spleen, and kidney, and occasionally vascular walls (OIE, 2019e). There is no specific treatment for horses infected by henipaviruses and only a vaccine for HeV - EquiVac® HeV - is available (Middleton et al., 2014), so other preventive measures to avoid human and other animal species infection should aim to reduce contact with flying fox secretions and infected tissues/secretions (Savage et al., 2014).

2.2.1.6 Miscellaneous viral encephalitis

Equine infectious anaemia (immune-mediated encephalitis)

The lentivirus causing the equine infectious anaemia (EIA) is found almost worldwide and is also widespread in Brazil (Tigre et al., 2017). Although it rarely causes primary CNS disease, EIA is an immunosuppressive condition that allows co-infections like by *Trypanosoma evansi* to severely reduce athlete horses performance (reviewed by Parreira et al., 2016). EIA is mechanically transmitted by *Tabanidae* flies (the same vector for *T. evansi*), or iatrogenically (Mealey, 2014).

General clinical signs involve weight loss, cyclic anaemia and thrombocytopenia, fever, hypergammaglobulinemia, oedema, and haemorrhage (Summers et al., 1995; Oaks et al., 1998). Most horses that develop chronic disease will decreasingly present signs over about a year, and after that, they remain clinically normal (Sellon et al., 1994). CNS signs can occur as brain and/or spinal manifestations, such as blindness, seizures, ataxia, and sudden death (Oaks et al., 2004). Microscopic post-mortem lesions are characterized by a nonsuppurative granulomatous ependymitis, meningitis, and encephalomyelitis (Oaks et al., 2004). There are focal to locally extensive infiltrations of lymphocytes, plasma cells, macrophages, and occasional giant cells (Summers et al., 1995).

Clinical diagnosis can be confirmed by ELISA complemented by agar gel immunodiffusion test (AGID or Coggins test) for detection of p26 antigen with blood or spleen pulp samples (OIE, 2019c). Foals nursing infected dams might be tested positive temporarily, and recently infected horses may be tested negative. As with other retrovirus infections, a positive serological result can be confirmed by western immunoblot analysis and the presence of proviral DNA in peripheral blood leukocytes confirmed by virus-specific PCR assay (MacLachlan & Dubovi, 2011; Nagarajan & Simard, 2001; Oaks et al., 1998). Virus transmission in enzootic areas may be reduced by stabling horses in insect-secure facilities during those times of the year (summer) and that time of the day (dusk) when biting insects are most active. Careful hygiene can avoid iatrogenic transmission. EIAV infection now is controlled in many countries, including Brazil; seropositive horses are either euthanised or kept in quarantine for the rest of their lives (MacLachlan & Dubovi, 2011; Schneider et al., 2014).

Aujeszky's disease

Aujeszky's disease is also known as pseudorabies, mad itch, infectious bulbar paralysis, or porcine herpesvirus infection, and is caused by the *Suid herpesvirus 1* (SuHV-1) (Cantile & Youssef, 2016). The disease is worldwide endemic in domestic and feral swine, which are the primary reservoirs, who shed the virus in saliva, nasal secretions, and aborted material. Sick

rats may also contribute to farm-to-farm distribution. Other susceptible/accidental hosts include dogs, cats, horses, cattle, sheep, and goats, and the transmission may occur via ingestion, inhalation, or bites (reviewed by Long, 2015b). The disease is rare in horses but when infected, they become febrile after one week intranasal and conjunctive inoculation (Kimman et al., 1991). The primary behavioural change is usually frenzy, followed by severely ataxic animals, progressing to recumbency. Other signs observed are anorexia, depression, muscle tremors, hyperexcitability, chewing, hypersalivation, pruritus, and self-mutilation. Severe cortical signs are observed as head pressing, nystagmus, iridocyclitis, and blindness (Long, 2015b; van den Ingh et al., 1990).

For the diagnosis, a variety of methods of antigen detection in brain tissues can be used as well as viral isolation, IHC, PCR, ISH and serological tests as neutralization test and ELISA (van den Ingh et al., 1990). At necropsy of affected horses, no specific gross lesions are observed. Microscopically, the predominant lesions consist of severe non-suppurative meningoencephalitis characterized by marked perivascular cuffing of mononuclear cells, accompanied by focal gliosis, neuronal degeneration, neuronophagia and satellitosis (Cantile & Youssef, 2016; Long, 2015a). Acidophilic intranuclear inclusion bodies in neurons and astroglia can be observed in all susceptible species, including pigs, with some morphologic differences. In pigs, these inclusions are often solid and amphophilic, while in other species, they are characterized as multiple small-granular inclusions (Cantile & Youssef, 2016). There is no vaccine available for horses, therefore, prevention of infections is achieved with biosecurity, vaccination and identification of latently infected domestic swine, and implement measures to restrict the contact of horses with feral swine (reviewed by Long, 2015b).

2.2.2 Parasitic encephalitis

Parasitic infections of the CNS of equine are relatively uncommon. However, as parasites are widespread in the environment and several prophylactic therapies are available, infections by parasites might be good indicators of poor sanitary conditions and management failures in a herd (Dubey et al., 2015; Ehizibolo et al., 2012; Furr, 2015a; Laugier et al., 2012).

2.2.2.1 Protozoa

2.2.2.1.1 Equine protozoal myeloencephalitis (EPM)

EPM is mainly caused by the Apicomplexa *Sarcocystis neurona,* less commonly by *Neospora hughesi*, and was attributed to *Toxoplasma gondii* in the past (Cusick et al., 1974; Dubey et al., 2015; Furr & Howe, 2015). These protozoa parasites use two hosts during their life cycle (Lucius et al., 2018). For *S. neurona*, the opossum (*Didelphis* spp.) is the best

described definitive host, while for *T. gondii* are wild and domestic feline, and for *N. hughesi*, is still unknown (Mehlhorn, 2016; Witonsky et al., 2014). A wide range of warm-blooded vertebrates serve as intermediate/aberrant hosts, including horses and humans (Dubey et al., 2015). Due to its significant relevance compared to other protozoa, this revision will focus on *S. neurona* infection.

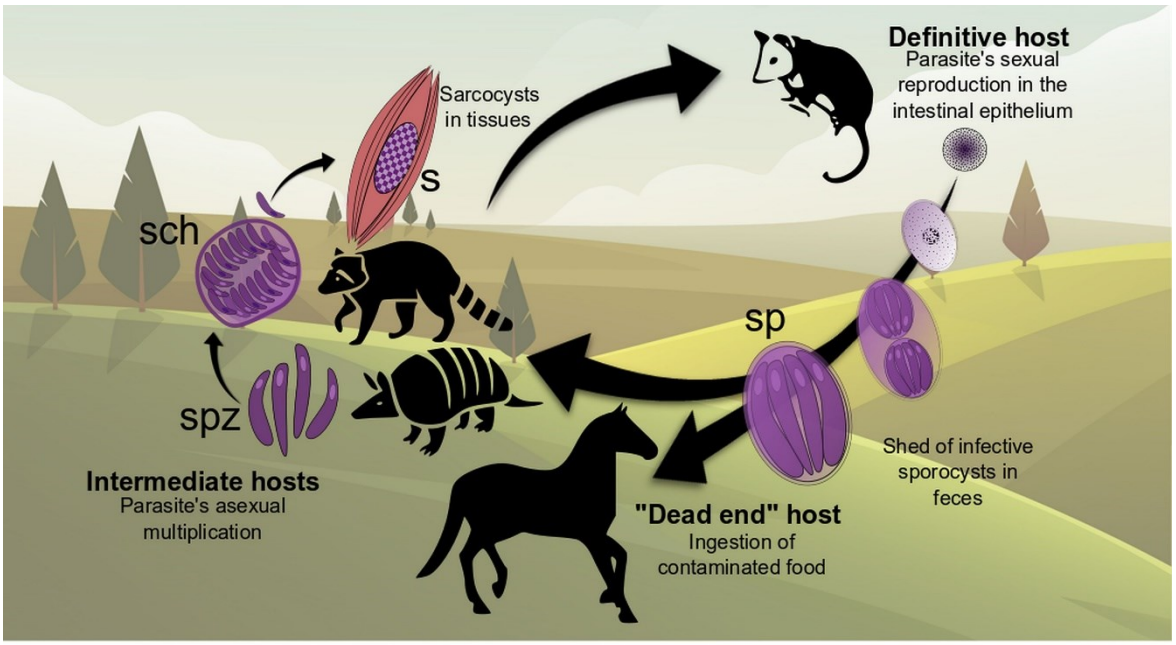
The risk factors associated with the infection of horses are the admission of sick animals in veterinary facilities during fall, hay storage not secure from wildlife, exposure of horses for races/shows , age between 1-5 years, previous diagnosis of EPM on the premises, and a recent adverse health event (Morley et al., 2008; Saville et al., 2000). Serological surveys have confirmed the widespread distribution of parasites in the Americas, where serum antibodies against *S. neurona* have been detected in up to 42% horses within a herd, *Neospora* spp. in 48%, and *Toxoplasma* spp. in 47% (Borges et al., 2017; Cazarotto et al., 2016; Dubey et al., 1999; Evers et al., 2013; Gennari et al., 2016; Magalhães et al., 2017; Masri et al., 1992; Portella et al., 2017; Ribeiro et al., 2016; Venturi et al., 2017).

The transmission cycle starts when the definitive host (e.g. opossum) sheds parasites with their faeces and the intermediate host (horse) feeds on contaminated (sporocysts) food or soil (**Fig. 17**). After hematogenous spread to the CNS, most likely within lymphocytes, *S. neurona* may localise in any area, from cerebrum to spinal cord, but is not found in peripheral nerves (Witonsky et al., 2014). Merozoites and schizonts may be observed in a variety of cell types in the CNS of horses with EPM, including neurons, mononuclear cells, and glial cells (Dubey, 1974).

The incubation period after experimental infection with *S. neurona* in young horses ranges from 28 to 42 days, but this is unknown for the natural disease (Fenger et al., 1997). Affected horses tend to be bright and alert with normal vital signs, while a wide range of clinical signs have been reported. Ataxia, limb weakness, and lameness are highly suggestive of EPM, especially if asymmetry of lesions is presumed (Howe et al., 2015; Radostits et al., 2007; Witonsky et al., 2014). Cerebral and brainstem involvement are characterized by depression, head tilt, facial paralysis, and difficulty in swallowing (Reed et al., 2016; Witonsky et al., 2014). Spinal cord ataxia is evident as hypo- or hypermetria, weakness, and defects in proprioception (Radostits et al., 2007). Multifocal spinal or cervical disease led to dysfunction of all four limbs, while lesions caudal to the cervical intumescence cause signs in the rear limbs only. Additionally, signs of cauda equine syndrome and asymmetric muscle atrophy are also associated to lesions on the spinal cord (Radostits et al., 2007). As the parasite may move along the CNS, clinical signs onset can expand to vestibular disease - unilateral facial nerve paralysis,

dysphagia, tongue paralysis, masseter atrophy, and hypoalgesia (Howe et al., 2015; Radostits et al., 2007; Reed et al., 2016).

Fig. 17: Sarcocystis neurona transmission cycle



The transmission cycle starts within the small intestine walls of the definitive host (opossum). There, the infective tissue cysts that contain bradyzoites and that were ingested by the opossum will transform into male and female gamonts without replication. Then the female is fertilised by the male, which produces oocyst, then oocyst sporulates in the lamina propria of the intestine and produces sporocysts (**sp**), each with four sporozoites – these are found in the opossum's faeces and are the infective form that will be ingested by the intermediate host and/or dead end host (horse). In the intermediate host, there will be sporozoite (**spz**) release and invasion of intermediate`s host intestinal epithelium. There, asexual multiplication (endopolygeny) occurs in many tissues, producing schizogonies, maturing to schizonts (**sch**). Merozoites are released by schizonts given rise to sarcocysts (**s**), the encysted stage. The sarcocysts are in a parasitophorous vacuole within the host cytoplasm. It is suggested that *S. neurona* multiplies in visceral tissues, lungs, and heart invades the CNS. Modified from Dubey et al. (2015), Fayer, Esposito & Dubey (2015), Witonsky et al. (2014), and Mehlhorn (2016). Illustration prepared on Inkscape© with VectorStock® royalty free icons.

In the horse, gross lesions are present only in severe cases and are restricted to the CNS. They range from a multifocal acute haemorrhage to the subacute and chronic discrete multifocal grey to dark yellow areas, and foci of malacia. Although the brainstem is more often involved, the lesions are more frequently seen in multiple segments of the spinal cord. Rarely, lesions may be present in both brain and the spinal cord (Cantile & Youssef, 2016; Dubey et al., 2015; Mayhew et al., 1978). Histological lesions are usually moderate to severe and characterized by multifocal areas of necrosis, malacia with presence of gitter cells, gliosis, and infiltration of large numbers of lymphocytes, histiocytes, plasma cells, and fewer eosinophils and neutrophils and additional severe involvement of the meninges. There might be multifocal to coalescing areas of haemorrhage, and the blood vessels have swollen activated endothelium. Particularly in spinal cord sections, there is axonal swelling or loss, besides the appearance of spheroids and

digestion chambers (Cantile & Youssef, 2016; Dubey et al., 2015; MacKay et al., 2000). Chronic lesions are seldom observed as most of the affected horses die or are euthanized during the acute stage, or may have been treated (Boy et al., 1990). When present, however, the inflammation can be predominantly histiocytic with occasional eosinophils and multinucleated giant cells (Cantile & Youssef, 2016; Dubey et al., 2015; MacKay et al., 2000).

The number of parasitic stages present are often few and finding *S. neurona* merozoites or schizonts can be a challenge. Developmental stages are more easily seen if organisms are present within neurons rather than in inflammatory cells (Dubey et al., 2015). Either way, parasitic organisms can be seen in association with lesions in fewer than 50% of the cases (10 to 36%) and serial sections must be examined in most cases (Boy et al., 1990; Hamir et al., 1993; Witonsky et al., 2014). Mature *S. neurona* schizonts are oval or irregularly round, have very thin walls (<0.5 µm), are up to 20 µm in diameter, and contain a few basophilic ovoid merozoites 5 µm x 1.5 µm, and are the only structure of *S. neurona* surely identified in horses. In early stages the uninucleate schizont sometimes resembles a macrophage or a degenerated host cell (Witonsky et al., 2014). Finding multiple nucleoli in a nucleus helps distinguish *S. neurona* from degenerating host cells (Witonsky et al., 2014). Schizonts and merozoites are periodic acid-Schiff (PAS) reaction negative, strongly basophilic, and agyrophobic (MacKay et al., 2000; Witonsky et al., 2014). These stages are observed in neurons, giant cells, neutrophils, and in macrophages (Dubey et al., 2015). The sarcocysts, incidental findings within cattle muscle fibres, are located within a parasitophorous vacuole in the host cell cytoplasm, lengthening approximately 700 µm with a cyst wall from 1 to 3 µm thick, filled with bradyzoites with 5 µm in length (Dubey, 1976; Dubey et al., 2015). The infective developmental stage, sporocysts, in the definitive host, can be found in tongue and other skeletal muscles. The *S. neurona* sporocyst is round (50-100 µm) or elongate (500 µm long and 40 µm wide), and contains a number of bradyzoites (Cantile & Youssef, 2016).

Definitive diagnosis in live horses is challenging. Detection of *S. neurona* or *N. hughesi* antibodies in serum and cerebrospinal fluid by immunoblot test is possible. Other serological tests available are direct agglutination, IFAT, and ELISA (Duarte et al., 2004; Ellison et al., 2003; Lindsay & Dubey, 2001). However, positive results only indicate exposure, and active disease is only considered if positive serology is accompanied by the onset of weakness and acute ataxia (Cantile & Youssef, 2016; Granstrom et al., 1993). Besides, the gold standard for diagnosis remains post-mortem identification of characteristic lesions and parasites in the CNS (Witonsky et al., 2014).

IHC can be carried out to detect schizonts within neuronal cytoplasm (Granstrom et al., 1991; Hamir et al., 1993; Masri et al., 1992), and banana-shaped merozoites within inflammatory cells, glial cells, vascular endothelium and extracellularly in the neuropil (Dubey & Hamir, 2000; Paixão et al., 2007). However, parasite reactivity is variable, as immature sarcocysts react brilliantly and mature sarcocysts are stained irregularly or not at all within the same histological section (Dubey et al., 2014; Thomas et al., 2007). Molecular detection of the small subunit ribosomal RNA gene (18S ss rRNA) by PCR has the advantage to detect several apicomplexa that may be infective for horses, while the internal transcribed spacer 1 (ITS1) detects the parasites in a restricted range, but at the species level (Fehlberg et al., 2017; Miller et al., 2009). However, if using CSF, positive results can only be detected if parasites are present (Marsh et al., 1996). Brain, heart, and skeletal muscle fragments collected during the necropsy offer a higher opportunity to detect parasitic stages as other parasitic life stages might be present (Dubey et al., 2015).

Horses suspected to be infected by EPM should be treated as quick as possible, since appropriate treatment results in the recovery of 70-75% horses. Approved anti-protozoal drugs are currently administered associated with anti-inflammatories along with adequate nutritional support (Dubey et al., 2015; Kennedy et al., 2001; Radostits et al., 2007). To effectively kill *S. neurona* sporocysts in feed or in the environment, Dubey et al. (2002) heated them at 55°C for 15 minutes or 60°C for one minute or more. The use of domestic sanitisers, on the other hand, is ineffective (Dubey et al., 2002). Thus, strategies to prevent infections should aim to minimise natural reservoirs access to feeds and pastures. Horse feed should be protected from wildlife access, and the same treatment should be given to domestic animal food (MacKay et al., 2000; Saville et al., 2000). Prompt treatment of illness and injury, monitor of pregnant mares, and management of stress associated to these conditions may also be beneficial (Witonsky et al., 2014).

2.2.2.1.2 Trypanosomiasis

The flagellate Euglenozoa parasites include some of the most important pathogens of tropical diseases, like Chagas in humans caused by *Trypanosoma cruzi* (Lucius et al., 2018). In horses, Dourine, caused by *T. equiperdum*, is primarily a venereal disease, transmitted through seminal fluid and mucous membranes of the genitalia of the infected animal during coitus (Brun et al., 1998). Therefore, the disease is almost exclusively present in stallions and breeding mares, although foals infected with the parasite may indicate an additional transmission route during parturition or through the milk (OIE, 2019a). Infections occur mainly in the Balkans, most of the African continent, Asia, and South America (Schlafer & Foster, 2016). The signs

of Dourine are divided in genital, cutaneous, nervous, and general manifestations, and might be observed separately or concurrently. The nervous manifestations develop late in disease and usually lead to death (Schlafer & Foster, 2016). Initially, there is hyperesthesia, followed by diminished sensitivity and/or anaesthesia, accompanied by paresis or paralysis of individual motor nerves, usually from the face (Summers et al., 1995; Schlafer & Foster, 2016). Evidence of a predominantly nonsuppurative meningoencephalitis, harbouring the parasite, has been found and led to changes in the CSF (Summers et al., 1995). In the spinal cord, degenerative lesions in white (axonal degeneration) and grey matter (neuronal degeneration) are reported (Yasine et al., 2019). There is neuropathy especially of large trunks in the lumbar and fifth and seventh cranial nerves, accompanied by mononuclear cells, fibrosis of the perineurium and oedema (Schlafer & Foster, 2016). Diagnosis depends on the onset of clinical signs combined with serological complement fixation tests. There is no vaccine available, and the most effective control measure is the slaughter of infected animals (OIE, 2019a).

Caused by *T. evansi*, Surra is transmitted by blood-sucking insects of the genera *Tabanus*, *Stomoxys*, *Atylotus*, and *Lyperosia* (Brun et al., 1998). In South and Central America, it might be also transmitted by vampire bats (*Desmodus rotundus*), which serve as both vector and reservoir host (Hoare, 1965). It can also be directly transmitted through milk or coitus (OIE, 2019g). Moreover, *T. evansi* occurs also in Africa and Asia (Brun et al., 1998). The clinical signs are characterized by fever and anaemia, emaciation, oedema, cachexia, and enlargement of the lymph nodes and spleen. Similarly to Dourine, neurological signs appear late in the disease, and are manifested as encephalic and medullar alterations, as hind limb paresis, wobbling, incoordination, and reluctance to move (Rodrigues et al., 2009; Rodrigues et al., 2005).

In the histology, *T. evansi* infections present a severe necrotizing non-suppurative panencephalitis or meningoencephalitis affecting both grey and white matter, with thick perivascular cuffs in which Mott cells are observed (Rodrigues et al., 2009; Seiler et al., 1981). IHC demonstrates parasites in the Virchow-Robin spaces and in the neuropil (Rodrigues et al., 2009). The parasite can be also identified in thick or thin blood films, and in fine needle aspirates from lymph nodes, or its DNA detected with PCR. For all these techniques, false negative results are frequent when parasitaemia is low, like in chronic infection. Treatment is performed with diminazene aceturate, although drug resistance has been reported. Surra is potentially zoonotic, therefore, suspicious, and/or infected samples should be handled with proper biosafety measures (OIE, 2019g).

2.2.2.2 Helminths

Although a substantial number of worms might cause neurologic disease in horses, their presence in the CNS is usually due to erratic migration or infestation of an aberrant host (Cantile & Youssef, 2016). Both conditions have sporadically been reported worldwide and are of considerable importance for equine and human health (Onyiche et al., 2018; Summers et al., 1995).

Infections can be caused by the nematodes *Strongylus vulgaris*, *Angiostrongylus cantonensis*, *Draschia megastoma* (*Habronema muscae*), *Halicephalobus gingivalis*, *Parelaphostrongylus tenuis*, *Setaria digitata* (cerebrospinal nematodiasis), and *Setaria labiato-papillosa* (Cantile & Youssef, 2016; Frauenfelder et al., 1980; Little et al., 1974; Mayhew et al., 1982). How they infect horses is unclear in several cases, but contaminated wounds in the mouth, skin, and eyes have been considered followed by hematogenous spread, along with eggs shed in faeces and urine, accidentally ingested (Onyiche et al., 2018; Radostits et al., 2007).

Affected horses may show variable symptoms, such as lethargy, behavioural abnormalities, circling, ataxia, subluxation of atlanto-occipital joint (C1-C5), head tilt, cranial nerve signs, and blindness (Furr, 2015a; Hermosilla et al., 2011; Radostits et al., 2007; Tanabe et al., 2007)

Gross lesions in the CNS vary from localized oedema (Tanabe et al., 2007) to malacic areas associated to parasite tracks (Vasconcelos et al., 2007). Like the infection in other organs, *P. tenuis* produces a granulomatous eosinophilic inflammation, accompanied by eggs, larvae, and adult parasites (Tanabe et al., 2007). Alternatively, in a case of acquired cervical scoliosis, *P. tenuis* led to extensive necrosis with proliferation of perivascular collagen in the spinal cord, spheroids, reactive astrocytes and debris-filled macrophages scattered within a mild inflammation composed mostly of lymphocytes with occasional eosinophils (van Biervliet et al., 2004). Horses affected with *H. gingivalis* produce predominant granulomatous inflammatory infiltration of brain and meninges, with variable amounts of eosinophils, numerous macrophages, and giant cells (Hermosilla et al., 2011; reviewed by Onyiche et al., 2018). Similar inflammatory lesions are observed in the kidneys, disseminated infections, and skin (Furr, 2015a; Ruggles et al., 1993). *Setaria* spp. are involved in ocular filariasis, while the lesions in the CNS consist of focal areas of malacia, loss of myelin, axonal swelling, degeneration and occurrence of gitter cells (Radostits et al., 2007). Larvae and adult parasites of *S. vulgaris* are frequently observed within lumen of mesenteric arteries, propitiating their hematogenous spread. In the CNS, especially in the cerebromedullar pathways, larvae migration produces haemorrhagic tracts of necrosis (Summers et al., 1995). As opposed to *P.*

tenuis parasites, which usually remain in the CNS, ascarids and strongyles are expected to be on the move. Therefore, to find these parasites might be difficult, even in the early stages of clinical signs onset (Cantile & Youssef, 2016). By the time the parasites reach the CNS, few treatment approaches are successful, and diagnosis is usually made during necropsy, based on parasite morphometry, tissue tropism, and geographic range (Furr, 2015a; Tanabe et al., 2007).

2.2.2.3 Arthropods

The only arthropod larvae known to migrate through the spinal canal of horses belong to the flies of *Hypoderma bovis* (Cantile & Youssef, 2016; Furr, 2015a). While these parasites have cattle as natural hosts, in horses the larvae do not complete their life cycle. *H. bovis* migrates in the epidural fat of the spinal cord canal and produces acute neurologic disease. Clinical signs are the sudden onset of muscular weakness or localised paralysis that proceeds to profound loss of motor control, convulsions, and death within a day to a week. This sudden onset should be differentiated from traumatic injury (Radostits et al., 2007). Hadlow et al. (1977) also described stumbling gait in circle, along with right side mouth drooping. Focal extensive haemorrhage are the main macroscopic and microscopic findings. There is moderate neutrophilic and eosinophilic infiltrate around vessels (Hadlow et al., 1977).

2.2.3 Bacterial encephalitis

Bacterial infections of the CNS of horses are uncommon, affecting up to 10% of animals surveyed (Laugier et al., 2009). At least half of the cases refer to horses less than 6 months old that have complications of septicaemia or secondary infections (Gomes et al., 2010; Morresey et al., 2011; Viu et al., 2012). Other possible routes of bacterial colonization occur after septic traumatic lesions in the head, and ingestion of contaminated food (Baker & Ellis, 1981; Kaplan & Moore, 1996; Smith et al., 2004). Immunosuppressed animals are also described as susceptible to bacterial infections (Pellegrini-Masini et al., 2005) Despite the rarity of primary bacterial infections in the CNS, *Listeria monocytogenes* and *Borrelia burgdorferi* sensu lato engage significantly as human and animal pathogens (Allerberger & Wagner, 2010).

L. monocytogenes is an aerobic, gram-positive rod found in nature that causes listeriosis more frequently in sheep, cattle, goats, and chickens (Radostits et al., 2007). Horses are rarely infected, but it happens usually due to spoiled silage fed incompletely fermented and with pH above 5.5, during winter and early spring (Cantile & Youssef, 2016). *L. monocytogenes* invades the apical side of intestinal epithelial cells and disseminates to neighbouring ones via plasma membrane protrusions, which guarantees the organism will avoid the host defence mechanisms. Damaged oral, nasal, or ocular mucosa are also viable entries to the CNS, where the bacteria

invade the trigeminal nerves and travel centripetally via axons to the brain (Cantile & Youssef, 2016). Besides neurological signs – depression, weakness, and seizures - listeriosis is related to septicaemia, kerato-conjunctivitis, abortion, and diarrhoea in horses (Revold et al., 2015; Rütten et al., 2006; Wilkins et al., 2000). Listeria has a remarkable affinity for the brainstem. Histologically, mononuclear perivascular cuffing, especially in the medulla and pons, and less severe in the thalamus and in the cervical parts of the spinal cord is present. The cuffs are composed mainly of lymphocytes and histiocytes with a few admixed neutrophils and eosinophils; granulocytes predominate in some cases (Cantile & Youssef, 2016; Seino & Long, 2014). Typically, this is accompanied by microabscess and occasionally spheroids. A diagnostic pathognomonic finding of listerial encephalitis is gram staining of the CNS that demonstrates gram-positive intra-monocytic or intra-neutrophilic bacilli in association with lesions (Cantile & Youssef, 2016). Imaging techniques, like computed tomography and magnetic resonance are used in equine clinics to provide precise location of *Streptococcus* spp., *Pseudomonas aeruginosa*, and *E. coli* suppurative infections and abscesses (Allen et al., 1987; Audigié et al., 2004; Cornelisse et al., 2001) and can beneficiate in listeriosis diagnosis as in human medicine (Hatipoglu et al., 2007). IHC to demonstrate antigen of *L. monocytogenes* serotype 1/2a has already successfully been used in FFPE material since the 1980's (Domingo et al., 1986). Listeriosis can be treated with most antibiotics available for equine, as long as carried out through an adequate (usually long) period, and preventive measures consist of adequate feed management (Radostits et al., 2007; Revold et al., 2015).

B. burgdorferi is a gram-negative spirochete transmitted mostly by *Ixodes* ticks and causes Lyme neuroborreliosis (Divers et al., 2018). Pathogenicity of *B. burgdorferi* relies on the spirochete attachment to a receptor with the outer-surface protein (OspA) expressed in the midgut of infected ticks, which allows the pathogen to remain in the tick. Also, immunosuppression and migration through connective tissues are key factors that help the organism to protect from humoral antibodies (Divers et al., 2018; Johnstone et al., 2016).

Neuroborreliosis is described with signs of muscle atrophy and weight loss, cranial nerve deficit, ataxia by general proprioceptive deficits in conjunction with limb paresis, respiratory distress, uveitis, fever, joint effusion, and cardiac arrhythmias (Johnstone et al., 2016). A lymphohistiocytic infiltration of the leptomeninges and the vessel walls might also be present during infection with *B. burgdorferi* in horses, accompanied by reactive astrogliosis and Wallerian degeneration (James et al., 2010; Johnstone et al., 2016). Sclerosing vasculitis, cranial and peripheral ganglionitis, radiculoneuritis, and neuritis are also reported, with fewer lesions affecting the parenchyma of the brain and spinal cord (Johnstone et al., 2016).

To detect infections by *B. burgdorferi*, serological tests are the gold standard if combined in a two-step protocol (i.e. ELISA or IFAT confirmed by Western blot) (Basile et al., 2017). Histology is a reliable test, also in combination with spirochete detection (i.e. Warthin-Starry or Steiner staining), PCR, and IHC (Imai et al., 2011; James et al., 2010; Johnstone et al., 2016). Treatment is achieved with antibiotics, extrapolated from human guidelines, while prevention is better achieved by avoiding tick infestations (Divers et al., 2018).

2.2.4 Mycotic encephalitis

Mycotic encephalitis is rare in horses and most often associated with complications of guttural pouch mycosis or fungal sinusitis (McLaughlin & O'brien, 1986). These conditions are usually related to *Aspergillus* spp. infections, presenting non-specific clinical signs, usually with brainstem and cerebral involvement - dysphagia, head shaking, ataxia, blindness, and seizures. Histological lesions consist of infiltration with neutrophils and mononuclear cells, along with thrombosis of vessels, and fungal elements (Furr, 2015c). Infections with *Cryptococcus neoformans* led to chronic granulomatous inflammation, usually in the respiratory tract of immunocompromised animals. The same lesions can be found in cases of meningitis and encephalitis (Barclay & DeLahunta, 1979; Cho et al., 1986; Hart et al., 2008). Direct mycotic transfer from a pre-existing lesion, like in sinusitis, might produce single, large foci of cerebral malacia, different from a hematogenous spread from the guttural pouch (Hunter & Nation, 2011).

2.3 Differential diagnosis for infectious non-suppurative equine encephalitis

Differential diagnosis of a primary infectious CNS disease is a challenge. Pathogens involved in these cases appear predominantly in multifocal regions of the brain and spinal cord, and there is predominance of non-suppurative lesions (**Table 3**). There are variable degrees in severity of lesions observed throughout the CNS and in some cases the affinity of some pathogens to determined areas might help in the diagnosis. Also, formation of inclusion bodies, ganglial lesions, and infection in other organs can hint towards a pathogen or a group of pathogens (**Table 3**, revision in this study).

Table 3: Key histological lesions caused by infectious agents in the CNS of horses

Continued

Disease	Main histological features	Frequency of histological lesion location										
		Forebrain			Midbrain			Cereb.	Sp. cord		Ganglia	Others
		Cer. cortices	Hippoc.	Thalamus	Mesen.	Pons	Medulla oblongata		Gm	Wm		
Rabies	+ to ++ non-suppurative encephalomyelitis, ICB (neuron)											
Borna Disease	Non-suppurative encephalomyelitis, INB (neuron, astrocyte)											
Equine herpesviral myeloencephalopathy	Thrombo-occlusive necrotizing vasculitis, myeloencephalopathy, INB (airway epithelial, hepatic)											A, LI, LU
Aujeszky's disease	+++ non-suppurative meningoencephalitis, INB (neuron, astrocyte)											H
Alphavirus encephalitis	+ to +++ non-suppurative meningoencephalitis, necrotizing vasculitis, neutrophils beginning of infection											B, H, K, LI, S
Flavivirus encephalitis	+ to ++ non-suppurative encephalomyelitis											
Bunyavirus encephalitis	++ to +++ non-suppurative meningoencephalitis, thick cuffs, multifocal necrotizing encephalitis											A
Hendra encephalitis	+, ++, +++ focal non-suppurative meningoencephalitis, necrotizing lymphadenitis and alveolitis, syncytial cells (vascular/lymphoid endothelium)											K, LU, LN, NS, SP
Equine protozoal myeloencephalitis	++ to +++ mixed (meningo-) encephalomyelitis, segmented lesions spinal cord, schizonts/ merozoites in lesions											M
Dourine	Non-suppurative meningoencephalitis											G, SK

Disease	Main histological features	Frequency of histological lesion location										
		Forebrain			Midbrain			Cereb.	Sp. cord		Ganglia	Others
		Cer. cortices	Hippoc.	Thalamus	Mesen.	Pons	Medulla oblongata		Gm	Wm		
Surra	+++ necrotizing non-suppurative (meningo)encephalitis, +++ white matter lesions, AIIa	■	■	■	■				▪	▪		M
Arthropod aberrant migration	Focal extensive haemorrhage, neutrophilic and eosinophilic perivascular infiltrate								■	■		
Helminth aberrant migration	++(+) granulomatous eosinophilic (meningo)encephalitis	■	▫	▫	▫	▫	■	▫	▫	▫		E, K, MA
Listeriosis	Microabscesses; ++(+) non-suppurative encephalitis			▫	■	■	■		▫		▪	A, E, I, S
Neuroborreliosis	Non-suppurative meningoencephalitis/ myelitis	▫	▫	▫	▫	▫	▫		▪	▪	▪	M
Mycotic encephalitis	Chronic granulomatous encephalitis/ meningitis	▪	▪	▪	▫	▫	▫	▪	▪	▪		LU, NS

Severity of lesions: +++ severe, ++(+) moderate and severe, ++ moderate, + mild, - no lesions reported. Frequency of lesions observed in each location: ■ lesions are predominantly observed in this region, ▪ lesions are frequently observed in this region, ▫ lesions can also be observed in this region. Empty space indicates that lesions have not yet been described in this region. AIIa: Alzheimer type II astrocytes. Cer. cortices: cerebral cortices, including frontal, parietal, temporal, and occipital, as well as olfactory bulb. Cereb.: cerebellum. Gm: grey matter. Hippoc.: hippocampus. ICB: intracytoplasmic bodies. INB: intranuclear bodies. Mesen.: mesencephalon. NS – nasal cavity and/or superior respiratory tract. Others: organs or structures that might also present lesions, A – abortion; B – urinary bladder; E – eye; G – genital; H – heart; I – intestine; K – kidney; LI – liver; LU – lung; LN – lymph node; MA – mesenteric artery; M – muscle (atrophy); S – septicaemia; SK – skin; SP – spleen. Sp. cord: spinal cord. Wm: white matter.

The differential diagnoses of meningitis and encephalitis in horses include viral, parasitic, bacterial, and fungal aetiologies (Seino & Long, 2014). Also, poorly known neuroinvasive pathogens of animals herded with horses, like cattle infected with astrovirus, could spill over to a new host (Schlottau et al., 2016).

Infectious encephalitis may also be difficult to distinguish from an encephalopathy with metabolic and toxic causes (Divers, 2011; Radostits et al., 2007). Hepatic encephalopathy in horses is associated to hyperammonaemia, gut-derived neurotoxins, endogenous false neurotransmitters, increases in aromatic amino acids, hypoglycaemia, and blood-brain barrier disruption (reviewed by Divers, 2011), while uremic encephalopathy (UE) indicates chronic renal failure (Frye et al., 2001). Both produce nearly identical symptoms such as progressive anorexia, altered mentation, lethargy, head pressing, and seizures; UE leads additionally to azotaemia. Histological lesions consist of marked status spongiosus and diffuse reactive astrocytosis (UE) with Alzheimer type II astrocytes mainly in hepatic encephalopathy (Bouchard et al., 1994; Frye et al., 2001).

Although rare, *toxicoinfections* (due to toxins released by infectious agents) and plant intoxications should be included in the differential diagnosis. These conditions tend to occur due to poor management, when animals are fed or graze in contaminated/infested food/pasture, more commonly in regions where horses can freely grass, like in the Americas. *Clostridium botulinum* and *C. tetani* are good examples of environmental contaminants; botulism is usually associated to signs of progressive muscular weakness, dysphagia, paddling, gastrointestinal signs, usually affecting more than one horse in the herd (Ostrowski et al., 2012; Schoenbaum et al., 2000), while tetanus is reported in cases of limb spasticity, stiffness, trismus and/or jaw locking, dysphagia is seldomly observed, and history of wound is common (Gračner et al., 2015; Ribeiro et al., 2018). Mycotoxins released by *Aspergillus* spp. and *Fusarium* spp. tend to induce signs of depression, ataxia, and are involved in cases of sudden death (Giannitti et al., 2011; Headley et al., 2014; Tunev et al., 1999; Vendruscolo et al., 2016). Among the neurotoxic plants, bracken fern (*Pteridium aquilinum*) is widely described, although lesions are not observed. Other plant species are also reported in Brazil (**Table 4**), and the main clinical signs consist of ataxia, depression, and cranial nerves deficits (Bandarra et al., 2010; Lorenzett et al., 2018; reviewed by Riet-Correa et al., 2017).

Table 4: Neurotoxins originated from bacteria, fungi, and plants of risk for horses

Agent (toxin)	CNS histological lesions	Predominant lesion location
Bacterial		
Clostridium botulinum (A, B, C_1, C_2, D, E, F, G)	Usually no lesions, but perivascular haemorrhages, gelatinous oedema of head and neck can occur	Not specific
Clostridium tetani (tetanospasmin)	Not observed	Not observed
Mycotic		
Aspergillus spp. (unknown)	Intralesional hyphae, coagulative necrosis (grey matter), haemorrhage, vasculitis, thrombosis, gitter cells	Cerebrum
Fusarium spp. (fumonisin B_1)	Leucoencephalomalacia, diffuse brain congestion, oedema, severe micro- and astrogliosis, reactive endothelium, haemorrhage, mononuclear perivascular cuffs	Thalamus, brainstem
Plant toxin		
Bambusa vulgaris (unknown)	Oedema, axonal degeneration	Medulla oblongata
Pteridium aquilinum (thiaminase)	Not observed	Not observed
Sida carpinifolia (swainsonine)	Vacuolation of perikaria in neurons	Cerebellum, ganglia
Trema micrantha (unknown)	Hepatic encephalopathy with AIIa, severe fibrinoid vasculitis, thrombosis, haemorrhage	Cerebral cortex, cerebellum

AIIa: Alzheimer type II astrocytes. Not specific lesion: if found, it may be observed anywhere in the CNS.

Other conditions to consider in the differential diagnosis are disorders like equine degenerative myeloencephalopathy and equine motor neuron disease that might mimic signs of EPM, but low serum/plasma vitamin E concentration and symmetry of lesions are supportive for the correct diagnosis (MacKay, 2015). Fractures of cranial cervical vertebrae are fairly common in horses (Tyler et al., 1993) and they can destruct the primary sensory neurons in the dorsal roots and ganglia. This leads to orthograde fibre degeneration in the spinal dorsal funiculus (Summers et al., 1995) and be misinterpreted as parasitic tracts, similar to what is found in acquired scoliosis associated to *P. tenuis* migration (Van Biervliet et al., 2004). An undocumented systemic hypertension in older animals can lead to signs of depression and weakness similar to rabies i.e. (Radostits et al., 2007). Cholesterol granulomas are usually incidental necropsy findings from older and over-weight horses but can mimic signs of cerebrocortical disease (Radostits et al., 2007). Neoplasia parallels signs of increased intracranial pressure and local tissue destruction - opisthotonos, convulsions, nystagmus, head-

pressing, hyperexcitability or dullness – and localizing signs of circling, head tilt, and disturbance of balance (Radostits et al., 2007).

2.4 Zoonotic potential and (re)emergence of equine encephalitis

In the last two decades a remarkable increase in emergent, re-emergent, and neglected diseases have been noted. Several pathogens involved in these conditions are zoonotic, potentially, or knowingly neuro-invasive for humans and horses and represent serious sanitary and economic threats worldwide. This situation is aggravated due to the inevitable strict contact between humans and horses, especially in places where these animals ensure the survival of poor communities (Kumar et al., 2018; Lecollinet et al., 2019; Powell, 2000).

Although a high proportion of zoonosis circulate in humans and animals from tropical and subtropical regions, history suggests that climate and socioeconomic changes, added to high adaptability of pathogens, allow the spread of diseases to new, temperate regions (Weaver & Reisen, 2010). Economically developed regions, like Europe, experience an increase in the number of cases of vector-borne zoonosis, both imported and indigenous (Calzolari, 2016). Adversely, invasion of natural areas for pasture and disorganized urbanization as well as intense human and animal movement across the world have practically extinguished disease boundaries (McNerney, 2015; Morens & Fauci, 2013; Mota et al., 2016). Also, increase in bat-borne breakouts are allegedly consequences of man-made environment changes, due to bat-housing invasion (reviewed by Mildenstein et al., 2016). Other impact by the decline of ecosystems health due to human activity is the dilution effect: a less diversified environment heightens the probability of spreading diseases to naïve populations – humans and domestic animals (Khalil et al., 2016; Wood et al., 2014; Young et al., 2017).

Although few diseases are transmitted directly from horses to people, equine are in close contact to humans and present susceptibility to several similar agents as humans so that they are suitable sentinels for respective risk assessments (Bender & Tsukayama, 2004; Gossner et al., 2017; Timoney, 2000). In other circumstances, equine infections maintain epizootics, like VEEV outbreaks, where human disease has never been demonstrated in the absence of equine encephalitis (Kissling et al., 1954; Miller et al., 1973), or by handling horses infected with HeV, which spreads the disease among horses and humans (Field et al., 2010).

For other diseases, horses can also serve as carriers. With the increase in the human immunocompromised population – cancer survivors, HIV patients, transplanted, and ageing – there is a higher risk to be infected. Although rare, transmission of pathogens such as *Cryptosporidium* spp., *Salmonella* spp., *Campylobacter* spp., *Rhodococcus equi*, and *L. monocytogenes* have been associated from horses to HIV-infected persons (reviewed by Bender

& Tsukayama, 2004). Horses are also carriers and susceptible to *B. burgdorferi*, which causes neuroborreliosis, and that in humans the acute phase characterizes by expanding erythematous skin lesions, and late manifestations include neurological signs (Basile et al., 2017). More recently, it was demonstrated that strains of the bacteria *Elizabethkingia anophelis* isolated from horses in Oklahoma, USA, were similar to isolates from human clinical samples, suggesting a potential zoonotic spread involving horses as carriers (Johnson et al., 2018). Horses can also carry *Babesia* spp. known to infect humans and bovine cattle (Gaffar et al., 2003); these protozoa are transmitted to susceptible hosts through hard ticks, like *Ixodes* spp., *Rhipicephalus* spp., that also feed on horses (Lucius et al., 2018).

2.5 Strategies for pathogen detection

With the increase in cases of emergent, re-emergent zoonotic and neglected diseases not only in developing regions, but also in economically stable countries, there is the need of detecting pathogens with methodologies accessible to all levels of infrastructure (Nel et al., 2017; Powell, 2000; Timoney, 2000). This strategy can prevent that outbreaks get out of control and burden already fragile public health systems (McNerney, 2015). In this scenario a *One Health* approach is the most appropriate tool for implementing appropriate surveillance, managing outbreaks, and preventing infections as it explores the relation of human, animal, and environmental health in a multidisciplinary effort (Nel et al., 2017). On the other hand, scarcity of resources and the lack of assistance in geographically remote areas are especially detrimental for appropriate assessments and interventions. Thus, the choice of material to conduct investigations should strategically match the reality of the cohort studied (McNerney, 2015).

2.5.1 Formalin-fixed paraffin-embedded tissue as diagnostic tool for equine encephalitis

Routine formalin fixation from biopsies or from necropsies are still the most common type of sample archive in pathology. The use of FFPE material is essential for the diagnostics of infectious diseases, which combined to other techniques can improve the diagnose of unresolved cases (Rech & Barros, 2015). In some regions, like in Brazil, necropsy of large animals is performed in the field. Under the circumstances of varied climatic demands, degrees of body conservation, and sample transport from isolated areas, preserving material in formalin may be the only alternative of acquiring reliable material (Rech & Barros, 2015). Besides, histotechnical procedures alleviate the risk of infection to laboratory staff for pathogens like the novel coronavirus (SARS CoV-2) and Ebola virus (Henwood, 2018, 2020). Moreover, FFPE archives are largely employed in pilot and retrospective studies and are wide sources of material from natural disease that require fewer transport regulations (Abed & Dark, 2016).

There are though some limitations in the use of FFPE material (**Table 5**). Techniques like IHC, molecular and genomic assays might have reduced performance with samples that are long-term stored/fixed, contaminated (chemical and environmental), or have low pathogen density (Bhudevi & Weinstock, 2003; Frickmann et al., 2013; Layton et al., 2019; Ramos-Vara, 2005; Ramos-Vara et al., 2008). This happens because the epitope and the nucleic acid required are masked by protein cross-linked with formalin in bounds that are almost insoluble over time (Bhudevi & Weinstock, 2003; Layton et al., 2019; Masuda et al., 1999; Nam et al., 2014). Therefore, proper adaptation into stringent methods are on demand (**Table 5**).

Table 5: Factors to consider when using formalin-fixed paraffin-embedded tissues in research and diagnostics

Technique	Problem	Solution
Immunohistoche-mistry	Antigen detection in different species using the same antibody	Standards for different species
	Reduced antigen detection due to prolonged storage/fixation	Stringent antigen retrieval
Molecular and genomic assays	Nucleic acid contamination	Improve sampling/storage conditions
	Low yield/quality of nucleic acid; low pathogen density	Improve nucleic acid extraction protocols
		Adapt PCR to amplify shorter amplicons
		Adapt PCR to amplify shorter amplicons, smaller contigs cut-off
		Pan-PCRs followed by Sanger sequencing
		Target enrichment

Moreover, the potential of FFPE archives can be unravelled by a combination of hypothesis-driven techniques (IHC and PCR for pathogen detection) with hypothesis-free techniques (IHC to characterize host response to injury and metagenomic WGS). For that, the establishment of standards is required, while considering methods applicable also in areas of limited resources, which are historically burdened by the majority of deadly pathogens (Frickmann et al., 2019; Gu et al., 2019; McNerney, 2015; Ramos-Vara et al., 2008).

3. Materials and methods

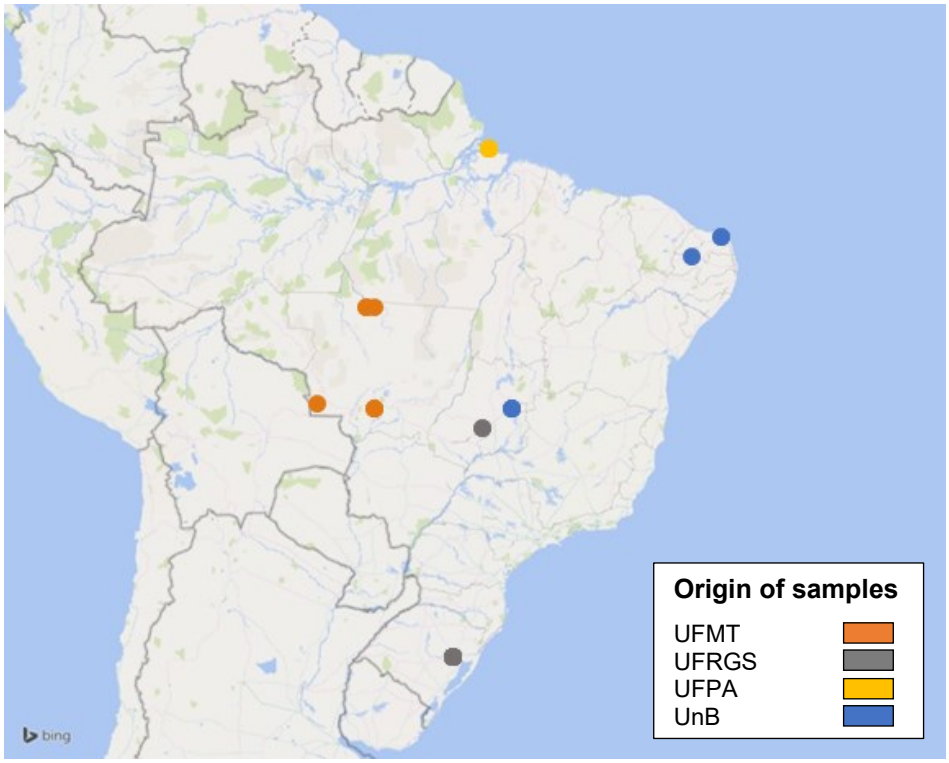
3.1 Sample selection

Thirty-five samples consisting of archived formalin-fixed paraffin-embedded (FFPE) tissues of the central nervous system (CNS) of horses from Brazil were selected. Selection criteria consisted of necropsy cases that remained with unclear etiologic diagnosis from animals that presented neurological clinical signs associated to a) nonsuppurative inflammatory lesions; b) degenerative and/or c) reactive lesions that do not rule out an infectious cause.

3.2 Origin of the samples used in the study

Sample material were kindly provided by Prof. Dr. Edson Moleta Colodel, Prof. Dr. David Driemeier, Prof. Dr. José Diomedes Barbosa, and by Prof Dr. Márcio Botelho Castro. Paraffin blocks were identified and packed according to the World Organization for Animal Health (OIE) instructions (**Fig. 18**).

Fig. 18: Origin of samples from the CNS of horses from Brazil (N=35)



UFMT: Universidade Federal do Mato Grosso, Prof. Dr. Edson Moleta Colodel. UFRGS: Universidade Federal do Rio Grande do Sul, Prof. Dr. David Driemeier. UFPA: Universidade Federal do Pará, Prof. Dr. José Diomedes Barbosa. UnB: Universidade de Brasília, Prof. Dr. Márcio Botelho Castro.

3.3 Epidemiological data assessment

Data from each horse were gathered, through the clinical history and suspected etiology sent with the tissues (**Appendix 4**). If available, information regarding sex, age, breed, clinical

signs, CNS fragments available for investigation, season, year and nature of death, and relevant gross lesions were noted. Upon availability in 24/35 cases, the tests performed *in vivo* or after the necropsy in Brazil were added (**Table 6**).

Table 6: Diagnostics tests performed *in vivo* or after necropsy in 24/35 cases of equine neurological disease in Brazil

Suggested disease/lesion	Diagnostic technique	Result	Horse ID
EEE	PCR (fresh CNS) IHC	Positive Positive	26, 27, 31 29, 30, 31, 32, 33
EHV-1	IHC	Negative	9
Rabies	IHC IF IF Clinical	Negative Negative Positive Inconclusive	9, 21, 24, 25, 26, 27 1, 16, 19 2 5, 17
EPM	Clinical	Inconclusive	20
Hematozoan	Not specified	Negative	22
Bacteria	Bacteriology (spinal cord swab)	Negative	15
Botulism	Clinical	Inconclusive	17
Tetanus	Clinical	Inconclusive	14
Trauma	Clinical	Inconclusive	8, 18, 21

Clinical: investigation based on clinical signs. CNS: central nervous system. EEE: Eastern equine encephalomyelitis. EHV-1: equine herpesvirus 1. EPM: equine protozoal myeloencephalitis. ID: horse identification. IF: immunofluorescence. IHC: immunohistochemistry. PCR: polymerase chain reaction.

Neurological clinical signs were classified according to the presumptive neuroanatomical localization of the lesions (**Table 7**). Additionally, the categories *multifocal with*, and *multifocal without cerebral signs* were assigned to animals presenting signs that implied involvement of more than one region of the CNS (Furr & Reed, 2015; Radostits et al., 2007).

Table 7: Presumptive neuroanatomic location of lesions according to the clinical signs

Anatomic region	Predominant clinical signs
Cerebral cortex	Postural deficits, seizures, altered mentation, blindness
Brainstem	Mild to moderate ataxia, weakness and dysmetria; dysphagia, anisocoria, or dilated pupils possible
Vestibular system	Ataxia, head tilt, and pronounced postural deficits
Cerebellum	Ataxia and intention tremors
Spinal cord/ UMN	Mild to moderate paresis, ataxia, and dysmetria, all present; spasticity is prominent
Peripheral nerve/ LMN	Weakness predominates; mild postural deficits and ataxia

UMN: upper motor neuron. LMN: lower motor neuron. Modified from Furr and Reed (2015).

3.4 Pathological studies

3.4.1 Tissue preparation and histochemical stains

Tissue preparation for histochemical stains were carried out according to Suvarna et al. (2019) specifications. The paraffin-blocks were archived at room temperature and protected from light. From each block, sections with 4µm-thickness were obtained and placed at glass slides SuperFrost® Plus Objektträger (R. Langenbrinck, Emmendingen, Germany). Afterwards the slides were dried at 60ºC for 15 minutes in the heat incubator and stored in plastic slide-boxes at room temperature. All slides were mechanically cover slipped by the end of every staining with Miles TissueTek® Film® (Sakura®, Alphen aan den Rijn, The Netherlands).

Hematoxylin-eosin staining

Deparaffinization and rehydration of tissue sections followed by staining with hematoxylin-eosin (HE) were carried out in the automated Leica slide stainer (Leica Biosystems Nussloch GmbH, Nussloch, Germany).

Periodic acid Schiff staining

Periodic acid Schiff (PAS) staining was used for demonstration of potential infectious agents. Deparaffinization and rehydration were carried out with immersion of slides in three rows of xylol, followed by two immersions in isopropanol, once in 96% ethanol, and once in 80% ethanol, for 3 min each. Afterwards, slides were incubated in 0.5% periodic acid for 10 min and rinsed three times for two minutes with distilled water. An incubation with filtered ready-to-use Schiff-Reagent was carried out for 30 min at 60°C. Samples were then rinsed three times for 2 min each with fresh sulphur water, and with distilled water, respectively. For nuclear staining, a 3 min immersion in Papanicolaou was made, followed by 5 min washing in tap water. In the end, the slides were dehydrated in increasing alcohol series, isopropanol, and xylol.

Congo red staining

Congo red was used for demonstration of potential amyloid deposit. Deparaffinization and rehydration were carried out as described above. Slides were incubated in Congo red solution for 25 min, washed in distilled water, then in running tap water for 5 min. Counterstain was carried out with Harris' hematoxylin for 1 min. Slides were then dehydrated, cleared, and mounted as described above.

Giemsa staining

Giemsa staining was carried out for demonstration of potential blood parasite infections. Deparaffinization and rehydration were carried out as described above. Sections were incubated in a dilution of 1:50 Giemsa (Merck, Darmstadt, Germany) for 2h, and rinsed once with distilled

water, which contained light acidity of glacial acetic acid. When the sections gained a reddish colour, which indicates staining of connective tissue, 96% ethanol was rinsed, until no cloudy staining was seen. Afterwards, two immersions of 30 seconds in isopropanol were carried out, followed by three in pure xylol.

Luxol fast blue-cresyl violet staining

Luxol-fast-blue was used to demonstrate potential demyelination. Slides were deparaffinized and rehydrated using ethanol 95%. After an overnight-incubation in Luxol fast-blue-solution at 57°C, slides were let to cool, and the excess stain washed out with 95% ethanol and distilled water. Rapid immersion in 0.05% lithium carbonate and then in 70% ethanol ensured adequate staining until the colours blue and white could be differentiated. Afterwards, slides were rinsed with distilled water and incubated for 8 min in 0.1% cresyl-violet-solution at 60°C added to 10% glacial acetic acid (5 drops/30ml). To finalize, slides were washed with 95% ethanol, and immersed in xylol.

Masson Goldner trichrome staining

Masson Goldner trichrome staining was carried out to demonstrate connective tissue deposits and/or alterations. Slides were deparaffinized and rehydrated according to the HE-staining protocol. Afterwards, slides were placed in Weigert's iron hematoxylin for 1-2 min (40 ml Solution A, 30 ml Solution B) for nuclear staining. Sections were then washed for 10 min in running tap water and immersed for 5 min in ponceau-acid-fuchsin (Solution I). They were then briefly rinsed with 1% acetic acid and differentiated with mordent solution (Solution II) for 30 min, followed by another rinse in 1% acetic acid. Counter staining was performed with 0.1% light green solution for 5 min, finalized by washing in 1% acetic acid for 5 min, 3 immersions in absolute ethanol, and once in xylol.

Gram staining

For identification of possible bacterial infections, Gram staining was carried out with deparaffinized and rehydrated sections. For nuclear staining, incubation with Harris-hematoxylin for 5 min was followed by a brief rinse with 1% ammonium hydroxide solution and washing for 10 min under running tap water. 1% gentian violet and 5% sodium bicarbonate solutions were mixed, applied to the slides, and incubated for 2 min, until being washed with distilled water. Fixation and differentiation were carried out with incubation for 1 min in Lugol solution, followed by washing under running tap water and ether-acetone solution. Counter staining was performed with 0.01% fuchsin for 5 min and washing under running tap water.

The last differentiation was performed with brief immersions in acetone, picric-acid-acetone solution, a second time in acetone, and in acetone-histological clearing agent for 2 min.

3.4.2 Histological evaluation

The terminology and description of histological alterations were classified according to Cantile and Youssef (2016), and Kaufmann et al. (2012).

3.4.2.1 Degree of inflammatory cell infiltration

To establish a scoring system for the *degree of inflammatory cell infiltration* (DICI), available CNS fragments were sorted into four subcategories, similar to descriptions used by the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (Kaufmann et al., 2012): 1) forebrain (paired cerebral hemispheres and the diencephalon); 2) midbrain (mesencephalon, ventral metencephalon, medulla oblongata); 3) cerebellum, and 4) spinal cord. Severity of lesions was determined by calculating the mean of inflammatory cells observed within five microscopic fields, under a 200x magnification field from:

A) The perivascular inflammatory cuffs
B) The number of inflammatory cell layers of the cuffs
C) Inflammatory cells in the parenchyma

Sample analysis was carried out with the Nikon Eclipse 80i microscope (Nikon, Düsseldorf, Germany). Acquired images and counting of inflammatory cells were obtained with the high-resolution microscope-camera Nikon DS-Fi1 (Nikon, Düsseldorf, Germany), and visualized with NIS-Elements Basic Research 3.2 64bit software (Nikon, Düsseldorf, Germany).

3.4.2.2 Inflammatory cell morphology

The percentage of each cell type constituting the inflammatory infiltrates - lymphocytes, macrophages, plasma cells, and eosinophils – was determined within five 400x magnification fields and graded according to Algermissen (2010):

- No inflammatory cells
\+ Small number of inflammatory cells (between 1 – 33%)
++ Moderate number of inflammatory cells (between 34 – 66%)
+++ Accentuated number of inflammatory cells (> 67%)

If transitions were observed, they were classified as <(+), (+), +(+) or ++(+).

3.4.2.3 Meningeal inflammatory infiltrate

Likewise, meningeal infiltrates were categorized according to Schwab et al. (2007):

- \- No inflammatory infiltrate
- \+ Mild (one or no more than a few infiltrates)
- ++ Moderate (one moderate infiltrate or several mild infiltrates)
- +++ Severe (several moderate infiltrates or confluent infiltrates)

If transitions were observed, they were classified as <(+), (+), +(+) or ++(+).

3.4.2.4 Additional lesions

Further tissue alterations were categorized as neuronal necrosis, satellitosis/neuronophagia, vascular alterations (subcategories vasculitis, haemorrhage, reactive endothelium). Vacuolation and malacia were considered if they were colocalized with other alterations. Morphologic alterations observed in astrocytes and microglia cells were described under the IHC section (3.5.10, 3.5.11).

3.4.2.5 Statistical analysis

The statistical evaluation of the histopathological investigation was carried out by the Biomathematics and Data Processing working group of the Department of Veterinary Medicine of the Justus-Liebig-University Giessen (JLU Giessen). To assess the DICI based on number and morphology of cells involved in the lesions, data processing and statistical tests were conducted with the BMDP Statistical Software.

Quantitative score for inflammatory cells infiltration

The first step for the establishment of DICI, weights were given to each histological characteristic roughly composing an inflammatory infiltrate:

A) mean of inflammatory cells in perivascular infiltrates (weight 3),

B) mean of layers of these infiltrates (weight 1), and

C) mean of inflammatory cells in the parenchyma (weight 2)

For each CNS region, “severe” infiltration was defined as highest numbers of cells observed among the samples. The definition of severe was used as scale to construct the following scheme applied similarly to other brain areas and spinal cord:

$$Horse\ Y_{forebrain\ A} = \frac{mean\ of\ cells\ in\ the\ forebrain\ for\ parameter\ A}{max.value\ of\ parameter\ A\ found\ in\ the\ forebrain\ of\ all\ horses}$$

$$Horse\ Y_{forebrain\ B} = \frac{mean\ of\ layers\ in\ the\ forebrain\ for\ parameter\ B}{max.value\ of\ parameter\ B\ found\ in\ the\ forebrain\ of\ all\ horses}$$

$$Horse\ Y_{forebrain\ C} = \frac{mean\ of\ cells\ in\ the\ forebrain\ for\ parameter\ C}{max.value\ of\ parameter\ C\ found\ in\ the\ parenchyma\ of\ the\ forebrain\ of\ all\ horses}$$

This was followed by the final equation that established a score ranging from mild to severe, or 0 to 6, respectively:

$$Score\ forebrain\ Horse\ Y = 3 \times Horse\ Y_{forebrain\ A} + Horse\ Y_{forebrain\ B} + 2 \times Horse\ Y_{forebrain\ C}$$

This scoring system provided mid-scale values, where CNS fragments presenting score from 0 to 2 are considered to have mild DICI, from >2 to 4 considered moderate DICI, and above 4 considered severe DICI. A general final score was defined for each CNS region. To describe the data obtained, quantitative, approximately normally distributed characteristics were shown through descriptive statistics, with values for mean, standard deviation, standard error of mean, minima, and maxima values. For statistically significant results ($p \leq 0.05$), the data description was carried out by specifying the medians ($\tilde{x}$), the quartiles (Q1 and Q3) and the smallest and largest observations with presentation in box and whisker plot.

Qualitative analysis of lesions

Wilcoxon-Mann-Whitney-test (WMWT) assessed whether there was relation between the DICI and the occurrence of other alterations that were usually observed with CNS lesion (3.4.2.4). If this relation showed significant difference ($p \leq 0.05$), the alterations were assessed with Spearman's Correlation test. Data description was carried out by specifying the medians ($\tilde{x}$), the quartiles (Q1 and Q3) and the smallest and largest observations with presentation in box and whisker plot.

3.5 Immunohistochemical studies

Immunohistochemistry (IHC) was carried out with all 35 brain samples, and as a rule, every CNS tissue available was tested. Repetitions were performed when inconclusive staining was observed.

3.5.1 Deparaffinization and rehydration

The slides were placed in glass cuvettes and deparaffinized in a three-row immersion in xylol for 3 min each, followed by 2 rounds of 3 min in isopropanol. To rehydrate the tissues, the cuvettes were incubated for 3 min in 96% ethanol and, 3 min in 80% ethanol.

3.5.2 Endogenous peroxidase blocking

For each cuvette containing 20 slides, 177 ml methanol added to 3 ml of 30% H_20_2 was incubated for 30 min at room temperature. Afterwards the slides were rinsed with tris-buffered saline (TBS) for 5 min.

3.5.3 Antigenic retrieval

Enzymatic digestion

For epitope retrieval of double-stranded RNA (dsRNA), and antigens of *Toxoplasma gondii* and *Neospora caninum*, slides were first incubated with buffer NaCL-PBS pH7.4 for 5 min at 37°C. Right after, followed an incubation with 5 µg proteinase K (Sigma Life Science, Mannheim, Germany) to each 10 ml NaCL-PBS buffer, pH 7.4 for 5 min at 37°C. Slides were washed twice for 5 min with ice-cold TBS and placed on Sequenza™ Slide Rack with Coverplates™ (Thermo Fischer Scientific, Carlsbad, USA).

Heat-induced epitope retrieval

Retrieval of alphavirus, flavivirus, and *Sarcocystis neurona* antigens was carried out with the incubation of the slides in citrate buffer pH 4.0 at 95°C in water bath for 20 min. After cooling down in the buffer, the slides were transferred to Sequenza™ Slide Racks with Coverplates™ (Thermo Fischer Scientific, Carlsbad, USA) and rinsed once with TBS for 5 min.

3.5.4 Positive controls

For each IHC, positive controls were used to assess the correct functioning of protocols (**Table 8**). Unless signalized, controls originated from cases with confirmed infection of the respective agent at the Institute of Veterinary Pathology, JLU Giessen. For negative controls see 3.5.5.

Table 8: Positive controls used for immunohistochemistry

Antibody against antigen of	Animal sp. tissue	Organ/cells
Rabies virus	Mouse	Cerebellum
Borna-disease-virus nucleoprotein	Horse	Hippocampus
Borna-disease-virus phosphoprotein	Horse	Hippocampus
Equine herpesvirus type 1	Horse	Liver, brain
Glial fibrillary acid protein	Mouse	Spinal cord
Iba1	Horse	Hippocampus
Double-stranded RNA	Pelican (WNV)[a] Mouse (rabies virus) Swine (SuHV-1) Horse (EHV-1)	Hippocampus Cerebellum Brain Brain, lymph node
Alphavirus	Horse	Mesencephalon
Flavivirus envelope	Pelican [a]	Brain
Listeria sp.	Ovine	Spinal cord, obex
Toxoplasma gondii	Kangaroo	Liver
Neospora caninum	Dog	Cerebellum
Sarcocystis neurona	Cattle [b]	Cardiac muscle

EHV-1: equine herpesvirus-1. Iba1: Ionized calcium-binding adapter molecule 1. SuHV-1: Suid herpesvirus 1. WNV: West Nile virus.
[a] provided by Prof. Dr. Arno Wünschmann, Veterinary Diagnostic Laboratory, University of Minnesota, USA. [b] provided by Dr. Fabiana Marques Boabaid, Laboratory of Veterinary Pathology, University of Cuiabá, Brazil.

3.5.5 Antibodies and sera

Primary antibodies for the detection of viral, bacterial, and protozoal agents are listed in Table 4 including further information on origin and respective use. Additionally, glial cells reaction pattern was investigated using the glial fibrillary acid protein (GFAP, Dako A/S, Glostrup, Denmark) and the ionized calcium-binding adapter molecule 1 (Iba1, Wako Chemicals GmbH, Neuss, Germany) (**Table 9**). Also, the monoclonal double-stranded RNA (dsRNA, Scicons English and Scientific Consulting, Szirák, Hungary) was used for detection of positive-strand RNA viruses and DNA viruses (Richardson et al., 2010; Weber et al., 2006). After antibody application, slides were incubated in the Sequenza™ Slide Rack with Coverplates™ (Thermo Fischer Scientific, Carlsbad, USA) at 4°C, overnight. On the next day, slides were rinsed three times for 5 min each with TBS buffer before secondary antibody incubation.

For each primary monoclonal antibody, a negative control isotype-matched originated from chicken T-lymphocytes (Hirschberger, 1987) was applied alongside to positive controls. For the polyclonal antibodies, rabbit-control serum (Dako A/S, Glostrup, Denmark) was used instead.

Table 9: Primary antibodies tested for immunohistochemistry in the CNS of horses Continued

Antigen (target) Clone Specificity	Source	Dilution Buffer Negative control	Blocking-serum	2ry antibody (IgG) Dilution, incubation Buffer	Detection system
Rabies virus Polyclonal, rabbit Not stated	Dr. Ulrich Eskens	1:2000 20% swine serum/TBS Rabbit-control serum	20% swine serum/TBS, 15’	Biot. goat anti-rabbit 9µl/ml, 30’ - RT 20% swine serum/TBS	ABC
Borna disease virus Monoclonal, mouse Nucleoprotein (Bo18)	Dr. Herzog	1:500 1% BSA/TBS Chicken T-lymphocytes	20% swine serum/TBS, 30’	Biot. horse anti-mouse 9µl/ml, 30’ - RT 1% BSA/TBS	ABC
Borna disease virus Polyclonal, rabbit Phosphoprotein (p24)	Institute of Virology, Marburg	1:2000 20% swine serum/TBS Rabbit-control serum	20% swine serum/TBS, 15’	Biot. goat anti-rabbit 9µl/ml, 30’ - RT 1% BSA/TBS	ABC
EHV-1 Polyclonal, rabbit Not stated	Dr. Michael Hardt	1:500 20% swine serum/TBS Rabbit-control serum	20% swine serum/TBS, 10’	Swine anti-rabbit 1:100, 30’ - RT 20% swine serum/TBS	PAP
GFAP (Z0334) Polyclonal, rabbit Full length, all isoforms	Dako A/S	1:500 20% swine serum/TBS Rabbit-control serum	20% swine serum/TBS, 15’	Swine anti-rabbit 1:100, 30’- RT 20% swine serum/TBS	PAP
Iba1 Polyclonal, rabbit C-terminus of Iba1	Wako Chemicals GmbH	1:500 1.5% goat serum/TBS Rabbit-control serum	1.5% goat serum/TBS, 2hs	Biot. goat anti-rabbit 1:200, 1h - RT, 1.5% goat serum 1% BSA/TBS	ABC
dsRNA (J2) Monoclonal, mouse Large dsRNA from unspecified agent	Scicons English and Scientific Consulting	1:200 3% BSA/PBS Chicken T-lymphocytes	3% BSA/PBS, 30’	Biot. horse anti-mouse 1:500, 30’ - 37°C 3% BSA/TBS	ABC
Alphavirus (MBS832145) Monoclonal, mouse Whole virus lysate	My Bio Source	1:50 1% BSA/TBS Chicken T-lymphocytes	No blocking	Biot. goat-anti mouse 1:500, 30’ - RT 1% BSA/TBS	ABC

Antigen (target) Clone Specificity	Source	Dilution Buffer Negative control	Blocking-serum	2ry antibody (IgG) Dilution, incubation Buffer	Detection system
Flavivirus (3571) Monoclonal, mouse Envelope protein	Santa Cruz Biotechnology	1:50 1% BSA/TBS Chicken T-lymphocytes	No blocking	Biot. goat-anti mouse 1:500, 30' - RT 1% BSA/TBS	ABC
Listeria sp. Polyclonal, rabbit Not stated	Dr. Domingo	1:100 20% swine serum/TBS Rabbit-control serum	20% swine serum/TBS, 10'	Swine anti-rabbit 1:100, 30' - RT 20% swine serum/TBS	PAP
Toxoplasma gondii Polyclonal, rabbit Not stated	Dako A/S	1:800 20% swine serum/TBS Rabbit-control serum	20% swine serum/TBS, 15'	Swine anti-rabbit 1:100, 30' - RT 20% swine serum/TBS	PAP
Neospora caninum Monoclonal, mouse Not stated	Dr. Schares	Concentrate - Chicken T-lymphocytes	100% horse serum	Biot. horse anti-mouse 9µl/ml, 30' - RT 1% BSA/TBS	ABC
Sarcocystis neurona Polyclonal, rabbit Unspecified proteins from cultured merozoites	Dr. Jitender Dubey	1:100 1.5% goat serum/TBS Rabbit-control serum	1.5% goat serum/TBS, 10'	Biot. goat anti-rabbit 1:500, 30' - RT 1.5% goat serum/TBS	ABC

ABC: Avidin-biotin complex. Biot.: biotinylated. Bo18: Borna disease virus-nucleoprotein 18. BDV-p24: Borna disease virus-phosphoprotein 24. BSA: bovine serum albumin. dsRNA: double-strand RNA. EHV-1: equine herpesvirus-1. GFAP: glial fibrillary acid protein. Iba1: ionized calcium-binding adapter molecule 1. PAP: peroxidase-anti-peroxidase. RT: room temperature. TBS: tris-buffered saline. T1: negative control originated from T-lymphocytes.

3.5.6 Detection systems

A commercial horseradish peroxidase-linked (HRP) conjugate detection kit and an HRP peroxidase-anti-peroxidase (PAP) antibody were applied according to the manufacturer's instructions (**Table 10**). Afterwards, slides were rinsed three times with TBS for 5 min each.

Table 10: Enzymatic detection systems

Detection system	Source	Dilution, incubation
Avidin-biotin complex (Vectastain® ABC Kit peroxidase standard)	Vector Laboratories	4.5µl Avidin (A) + 4.5µl Biotin (B) in 1000µl 1% BSA/TBS, 30'[a] - RT
Mouse peroxidase anti-peroxidase (PAP)	Jackson ImmunoResearch	1:600 in 1% BSA/TBS, 30' - RT

BSA: bovine serum albumin. RT: room temperature. TBS: tris-buffered saline.
[a]: Iba1 incubated for one hour.

3.5.7 Staining, counter staining, and mounting

After incubation with the detection system, the slides were transferred from the racks to glass cuvettes. The slides were then incubated in an immersion of fresh diluted and filtered 3,3' –diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma-Aldrich®, St. Louis, USA)/Imidazol solution (Carl Roth GmbH, Karlsruhe, Germany) and 70µl 30% hydrogen peroxide (H_2O_2, Carl Roth GmbH, Karlsruhe, Germany) for 2 min at room temperature. Exceptionally, for detection of Iba1, alphavirus, and flavivirus slides were stained for 10 min, and *S. neurona* for 5 min. Subsequently, the slides were rinsed for 5 min three times in TBS buffer immersion, followed by one immersion in distilled water. Then, the slides were immersed for 5 min in Kardasewitch solution and washed in distilled water twice. Counter staining was carried out with Papanicolaou's-hematoxylin (Merck KGaA, Darmstadt, Germany) for approximately 30s. Afterwards, two final washings were made during 5 min each, with tap water and distilled water, respectively.

Dehydration of the slides was carried out in an ascending series of alcohol (50%, 80%, and 96%), twice in isopropanol and then cleared three times in xylol Roti-Histol® (Carl Roth GmbH, Karlsruhe, Germany), for 3 min each time. To conclude, slides were mechanically coverslipped with Miles TissueTek® Film® (Sakura®, Alphen aan den Rijn, The Netherlands) machine.

3.5.8 Antigen distribution

Sections from all available brain locations were examined. Except for immunostaining of GFAP and Iba1, the IHC reactivity was scored according to Stein et al. (2010):

- Specific staining is absent
+ Weak, mean of 1 focus of positive cell per high-power field
++ Moderate, mean of 2 to 5 foci of positive cells per high power field
+++ Strong, mean of more than 5 foci of positive cells per high power field

Cellular staining pattern for each agent was compared to previous reports (**Table 11**).

Table 11: Immunohistological cellular staining pattern for each agent

Detection of	Cellular staining pattern	Animal spp. description	Reference
Rabies virus	Fine to large intracytoplasmic granules (neurons, glia cells, oligodendrocytes)	Mammals	Pedroso et al. (2008), Wilkins & Del Piero (2014)
Borna disease virus	Nucleus and cytoplasm of neurons, neuronal processes, glial cells	Horse	Herden et al. (1999), Algermissen (2010), Long et al. (2014)
EHV-1	Nuclei, cytoplasm and axonal processes of neurons and glial cells	Horse, llama, black bear	House et al. (1991), Wohlsein et al. (2011), Slater (2014)
dsRNA	Cytoplasm (RNA viruses), nucleus (DNA viruses)	Eukaryotic cells	Weber et al. (2006)
Alphavirus	Neuronal cytoplasm, dendritic processes, glial cells, lymphocytes	Horse	Pennick et al. (2012), Sousa et al. (2015)
Flavivirus	Neuronal cytoplasm, axon, cytoplasm of glial cells, macrophages, neutrophils, and endothelial cells	Horse, raptor, mice	Ellis et al. (2007), Cantile et al. (2001), de Barros et al. (2011)
Listeria sp.	Intra and extra-cellular bacteria within inflammatory cells	Horse	Rütten et al. (2006)
Toxoplasma gondii	CNS (cysts with bradyzoites amidst parenchyma)	Sheep	Silva et al. (2013)
Neospora caninum	Tachyzoites and bradyzoites within neural tissue	Mouse, cattle	Lindsay & Dubey (1989), Uzêda et al. (2013)
Sarcocystis neurona	Schizonts within neuronal cytoplasm, merozoites within inflammatory and glial cells, vascular endothelium, and neuropil	Horse, mink, racoon, cat, skunk	Granstrom et al. (1991), Masri et al. (1992), Hamir et al. (1993), Dubey & Hamir (2000), Paixão, Rêgo & Santos (2007)

dsRNA: double stranded RNA. EHV-1: equine herpesvirus type 1.

3.5.9 Antigen cross-reactivity test

After antibody standardization, the protocols were also tested with samples from animals infected with morphologically similar agents when concerning protozoa and bacterial

agents, according to Ramos-Vara et al. (2008). Cross-reactivity tests were also carried out with the viral antigens assessed in the study.

3.5.10 Immunostaining of GFAP

Available CNS fragments were categorized under four main regions, 1) forebrain, 2) midbrain, 3) cerebellum, or 4) spinal cord. Each region was sub-categorized in lesioned and non-lesioned areas, added to specification whether lesions were predominantly inflammatory or reactive/degenerative. Four non-altered CNS of horses necropsied at the Institute of Veterinary-Pathology, JLU Giessen were used as controls to characterize the normal equine astrocytic morphology and GFAP staining pattern. Samples were examined using a Nikon Eclipse 80i microscope, and images were taken with the high-resolution microscope camera Nikon DS-Fi1. Immunostained cells were counted with the NIS-Elements Basic Research 3.2 64bit software. For quantitative and qualitative analysis, 200x magnification field was used and the following methodology was performed:

Quantitative assessment of astrocytes

The mean of GFAP positive astrocytes was calculated within five microscopic fields in lesioned areas and in five fields of non-lesioned areas.

Astrocytosis grading

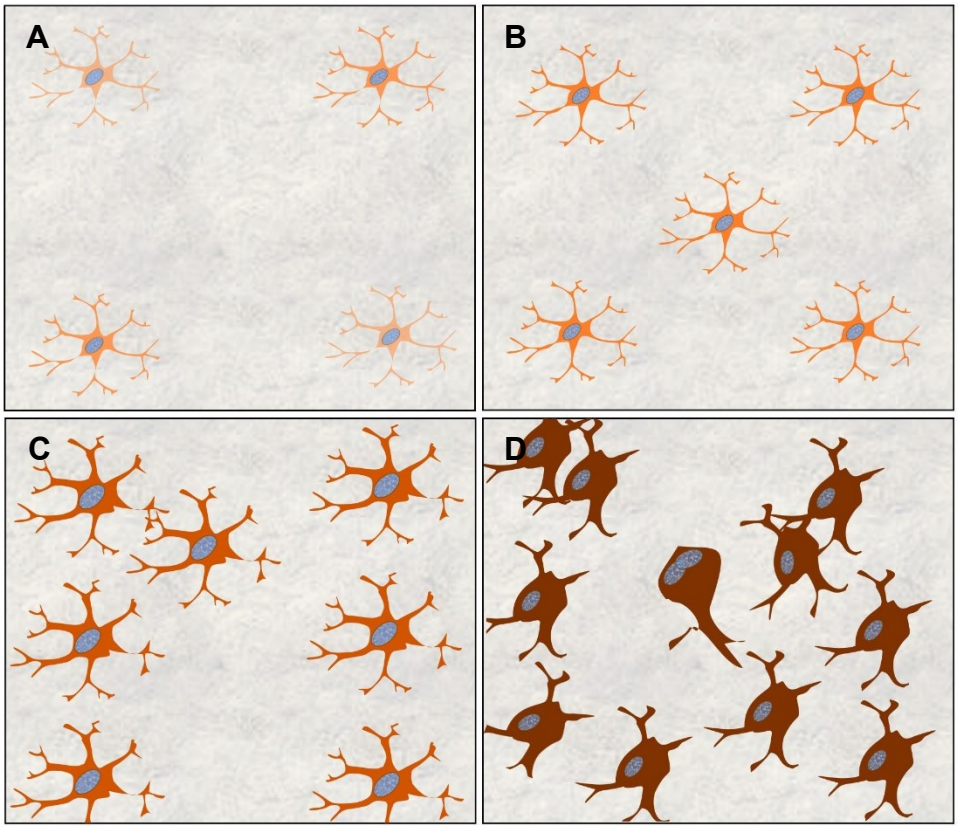
A scale from grade 0 to 3 was established for astrocytic alterations observed (**Table 12**, **Fig. 19**). To grade these alterations, four morphologic characteristics were taken into account, a) apparent cellular proliferation, b) nuclear alterations, c) cytoplasmic staining, and d) alterations of cellular processes (Sofroniew & Vinters, 2010; Sofroniew, 2009).

Table 12: Morphologic criteria to grade astrocytic injury in the CNS of horses

Morphologic structure	Non-altered	Definition according to severity of lesions		
		Mild	Moderate	Severe
Cellular proliferation	No	No or discrete	Moderate	Yes
Nucleus	No alterations	Mild increase in volume	Moderate increase in volume	Severe increase in volume
Cytoplasm	Few mildly stained cells	Mildly stained	Moderately stained	Accentuated stain
Processes[a]	Long, thin, well ramified	Long, thin, well ramified	Long, moderately thickened	Thickened, trespassing other cell processes, gemistocytes
Grade	**0 (Fig. 19A)**	**1 (Fig. 19B)**	**2 (Fig. 19C)**	**3 (Fig. 19D)**

[a] Protoplasmic (in the grey matter, with numerous, shorter, and branched processes) and fibrous (in the white matter, with relatively few, long processes) astrocytes taken into consideration according to Sofroniew and Vinters (2010).

Fig. 19: Schematic representation of the different astrogliosis grading



Statistical analysis

The data were evaluated by the Biomathematics and Data Processing Working Group of the Department of Veterinary Medicine of the JLU Giessen. The statistical tests were conducted with the statistical program BMDP / Dynamic, Release 8.1 (Statistical Solutions Ltd., Cork, Ireland) (Dixon, 1993) and data were sorted according to the IHC quantitative and morphologic analyses carried out with each of the four CNS regions available.

T-test for dependent variables was conducted to determine if the number of immunostained astrocytes occurred differently between lesioned and non-lesioned areas of each CNS region. Astrocytosis grade in lesioned and non-lesioned areas from the CNS was investigated with Wilcoxon Signed Ranks Test. Moreover, a rough classification to indicate a range of astrocytes stained within each grade was carried out with Spearman's Rank Correlation Coefficient. Statistical significance was assigned as $p \leq 0.05$. Regarding the lesion type conferred to each CNS region, Wilcoxon-Mann-Whitney test (WMWT) and WMWT with exact inference determined if the mean of astrocytes and the astrocytosis grade in inflammatory lesions and in reactive/degenerative lesions occurs differently, respectively. To describe approximal normally distributed quantitative data, arithmetic mean values ($\bar{x}$), standard deviation (sd), minima (min), maxima (max), and sample sizes (n) were calculated and tabulated. In the case of semiquantitative variables, the data description was carried out by

specifying the medians ($\tilde{x}$), the quartiles (Q1 and Q3) and the smallest and largest observations with presentation in box and whisker plot.

Range of GFAP-positive astrocytes related to the alteration grade

After the morphological and statistical assessment, the range of astrocytes stained in each grade of astrocytic alteration was determined as shown in **Table 13**.

Table 13: Range of GFAP-positive astrocytes for each astrocytic grade in different regions of the CNS of horses

CNS region	Astrogliosis			
	Grade 0	Grade 1	Grade 2	Grade 3
Forebrain	0 to 20 cells	20 to 56 cells	43 to 86 cells	>86 cells
Midbrain	0 to 20 cells	21 to 60 cells	58 to 96 cells	>115 cells
Cerebellum	0 to 30 cells	30 to 58 cells	40 to 100 cells	>90 cells
Spinal cord	0 to 20 cells	16 to 43 cells	43 to 77 cells	>77 cells

3.5.11 Immunostaining of Iba1

Available CNS fragments were categorized under four main regions as described above (3.5.10). Four non-altered CNS of horses necropsied at the Institute of Veterinary-Pathology, JLU Giessen were used as controls to characterize the morphology and Iba1 staining of equine resting microglia. Samples were examined as described above (3.5.10).

Iba1 staining quantitative assessment

The mean of Iba1-stained microglial cells was calculated within five microscopic fields in lesioned areas and in five fields of non-lesioned areas.

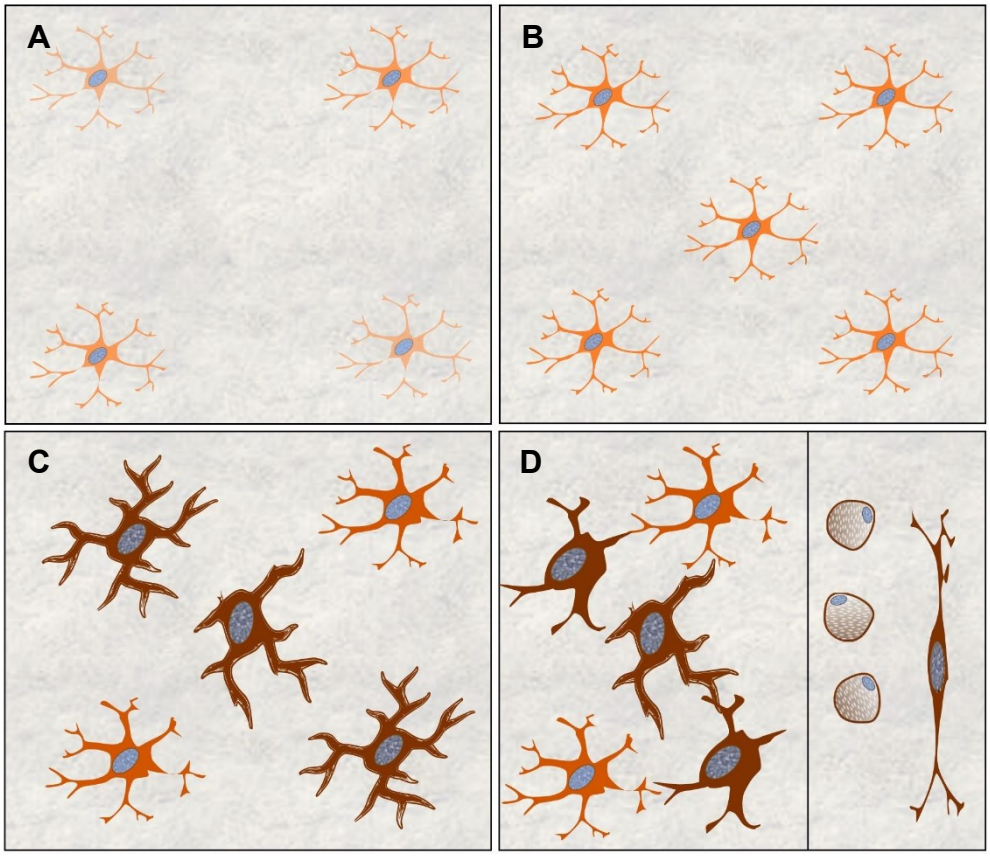
Microgliosis grading

A scale from grade 0 to 3 was established for the microglial alterations (**Table 14**, **Fig. 20**). To grade the alterations observed, four morphologic characteristics were taken into account if applicable: a) apparent cellular proliferation, b) nuclear alterations, c) cytoplasmic staining and/or cell body, and d) alterations of cellular processes (Crews & Vetreno, 2016; Kreutzberg, 1996; Lemstra et al., 2007; Taylor et al., 2014).

Table 14: Morphologic criteria to grade microglial activation in the CNS of horses

Morphologic criteria	Resting microglia	Definition according to severity of lesions		
		Mild	Moderate	Severe
Cellular proliferation	No	No or mild	Moderate	Yes
Nucleus	No alterations	Mild increase in volume	Moderate increase in volume	Severe increase in volume, vacuolated appearance
Cytoplasm/cell body	Small cell body	Mild increase in cell body	Enlarged cell bodies, bushy cells	Rounded macrophage-like, amoeboid shape (Gitter cells)
Processes	Long, thin well ramified	Long, thin, hyper-ramified	Long, moderately thickened	Thickened, shortened, few, or no processes, or bipolar (rod cells)
Grade	**0 (Fig. 20A)**	**1 (Fig. 20B)**	**2 (Fig. 20C)**	**3 (Fig. 20D)**

Fig. 20: Schematic grading for microglial activation



Statistical evaluation

The data were evaluated by the Biomathematics and Data Processing Working Group of the Department of Veterinary Medicine of the JLU Giessen. The statistical tests were conducted with the statistical program BMDP / Dynamic, Release 8.1 (Statistical Solutions Ltd., Cork, Ireland) (Dixon, 1993) and data was sorted according to the IHC quantitative and morphologic analysis carried out with each of the four CNS regions available.

T-test for dependent variables was conducted to determine if the number of immunostained microglial cells occurred differently between lesioned and non-lesioned areas of each CNS region. Microgliosis grade in lesioned and non-lesioned areas from the CNS was investigated with Wilcoxon Signed Ranks Test. Moreover, a rough classification to indicate a range of microglial cells stained within each grade was carried out with Spearman's Rank Correlation Coefficient. Statistical significance was assigned as $p \leq 0.05$. Regarding the lesion type conferred to each CNS region, WMWT and WMWT with exact inference determined if the mean of microglial cells and the microgliosis grade in inflammatory lesions and in reactive/degenerative lesions occurred differently, respectively. To describe approximal normally distributed quantitative data, arithmetic mean values ($\bar{x}$), standard deviation (sd), minima (min), maxima (max), and sample sizes (n) were calculated and tabulated. In the case of semiquantitative variables, the data description was carried out by specifying the medians ($\tilde{x}$), the quartiles (Q1 and Q3) and the smallest and largest observations with presentation in box and whisker plot.

Range of Iba1-positive microglial cells related to the alteration grade

After the morphological and statistical assessment, the range of microglial cells stained in each grade of alteration was estimated when possible as shown in **Table 15**.

Table 15: Range of Iba1-positive cells for each grade in different regions of the CNS of horses

CNS region	Microgliosis			
	Grade 0	Grade 1	Grade 2	Grade 3 [a]
Forebrain	0 to 15 cells	6 to 30 cells	20 to 60 cells	> 50 cells
Midbrain	0 to 10 cells	10 to 30 cells	30 to 60 cells	> 60 cells
Cerebellum	0 to 13 cells	10 to 17 cells	> 15 cells[b]	[c]
Spinal cord	0 to 11 cells	10 to 25 cells	17 to 37 cells	> 37 cells

[a] All cases had cells compatible with Gitter cells. [b] horse 24 only. [c] there were no microglial alterations compatible with grade 3 in the cohort studied.

3.6 Cell culture and transfection

3.6.1 Vero cell culture

The Vero cell line B4 was kindly provided by Prof. Dr. Friedemann Weber, Institute of Virology, JLU Giessen. The adherent cells were cultured in 25 cm^2 cell culture flasks (Sarstedt, Nümbrecht, Germany) at 5% CO_2 atmosphere and incubated at 37° C in an incubator. Once a week a medium change was performed. The culture medium used for the seeding and the four first passages was the minimum essential medium (MEM) enriched with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin. For the subsequent passages, Dulbecco's Modified

Eagle Medium (DMEM, PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FCS (PAA Laboratories GmbH, Pasching, Austria) and 1% penicillin / streptomycin (PAA Laboratories GmbH, Pasching, Austria) was used. Once a week, the cells were additionally passaged, and the confluence was initially monitored microscopically. Following this, first the old medium was aspirated, 2 ml trypsin-EDTA solution (PAA Laboratories GmbH, Pasching, Austria) was added and the cells were kept for 5 min in an incubator at 37°C and 5% CO_2 atmosphere. After addition of 5 ml of the DMEM medium, the cell suspension was transferred to a conical centrifuge tube and centrifuged for six minutes at 200×g, at 15°C (48 C Rotina, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). After that, the supernatant was aspirated, the cell pellet was resuspended in 3 ml DMEM medium and 50 µl of the cell suspension transferred and 5 ml DMEM medium in a new 25 cm^2 tissue culture flask was given (Sarstedt, Nümbrecht, Germany). Cells were kept incubated at 37°C and 5% CO_2 atmosphere.

3.6.2 Vero cell infection with La Crosse virus

Infection of Vero cells was carried out with a concentration of 2.05 x 10^7 pfu/ml and MOI 1 of La Crosse virus (LACV). The infection protocol of Cardoso et al. (2015) was modified, adapted to infection in 25 cm² cell culture flasks. After an incubation of 2 h at 37°C and 5% CO_2, the inoculum was removed, and the cells were washed with high glucose DMEM containing 1% penicillin/streptomycin. The cell medium was replaced, and cells were further incubated for 72 h. Harvesting of cells for further molecular investigation was carried out with Trizol® Reagent (Thermo Fisher Scientific Inc., Carlsbad, USA) and total RNA extracted with RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), performed as described by Chomczynski & Sacchi (1987).

3.7 Molecular analyses

3.7.1 Optimization of RNA isolation from FFPE material

The protocol used for RNA isolation was adapted from the one provided with the RNeasy FFPE-kit (Qiagen GmbH, Hilden, Germany) and depicted in detail in Boos et al. (2019). In summary, two deparaffinization methods and three lysis approaches where tested to establish the maximum yield and purity of RNA for FFPE material. Tests were firstly conducted with 18 samples from the CNS of horses from Brazil, and after statistical analysis with the program BMDP / Dynamic Release 8.1, the most appropriate protocol was used for the 35 samples. The RNA obtained was eluted in 24 µl RNase-free water, measurements of RNA yield, purity in relation to proteins (A260/280 nm) and in relation to contaminants (A260/230 nm)

were obtained with NanoDrop 2000 (Thermo Scientific, Spectrophotometer and Software) and kept frozen at -80°C for future analysis.

3.7.2 RNA isolation from inactivated-infected cells

The fast version of the RNA isolation protocol of the RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) was used for the samples of Nipah virus (NiV)-infected Vero76 cells. The first steps of RNA isolation were performed under biosafety S4 laboratory of the Institute for Virology, Philipps University Marburg by Dr. Laura Behner (AG Prof. Maisner). These consisted of treatment with 350 µl RLT buffer added to 1:100 β-mercaptoethanol, incubated at room temperature for 10 min and addition of 250 µl 100% ethanol. Further steps were carried out at the Institute of Veterinary Pathology, JLU Giessen following the manufacturer's instructions. RNA was eluted in 40 µl RNase-free water, measured with NanoDrop 2000 (Thermo Scientific, Spectrophotometer and Software) and was kept frozen at -80°C for future analysis.

3.7.3 Genomic DNA isolation from FFPE material

Genomic DNA (gDNA) isolation from FFPE material was carried out with the QIAamp® DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany), following the instructions of the manufacturer. Starting material consisted of seven to eight sections obtained from FFPE blocks, with 8 µm thickness. Genomic DNA was eluted in 30 µl buffer ATE and measured with NanoDrop 2000 (Thermo Scientific, Spectrophotometer and Software) after a water bath was set at 63°C for five minutes while the program was blanked using buffer ATE. Extracted DNA was kept frozen at -20°C.

3.7.4 Genomic DNA isolation from fresh tissues

Genomic DNA from fresh tissues was extracted with the QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Fresh-frozen CNS fragments from a horse previously diagnosed with EHV-1 was used as positive control and gDNA from a healthy horse, provided by the genetic bank of the Institute of Veterinary Pathology, JLU Giessen served as negative control for EHV-1 investigations and as positive control for the housekeeping gene equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.7.5 Reference material

To establish optimal cycling conditions and assess the proper working conditions for the RT-PCR or PCR assays, reference material consisting of knowingly infected cells/organs

were used (**Table 16**). Alongside each test, a negative control consisting of nucleic acid extracts from the CNS of a horse with no neurological signs/lesions was used as well and no-template controls (NTC), consisting of RNase-free water. The RNA extracted from reference material originated from FFPE material was obtained with the optimized RNA extraction protocol (Boos et al., 2019a).

Table 16: Reference material used for the optimization of RT-PCR or PCR assays

Target	Cell line/ organ	Nucleic acid ng/µl	Origin
RNA			
Rabies virus	BHK cells	5.8	Dr. Eickmann[a]
Rabies virus	CNS (FFPE)	*	IVP Giessen[b]
Borna disease virus	CNS (fresh)	79.2	Dr. Manon Bourg[b]
Borna disease virus	CNS (FFPE)	*	Dr. Manon Bourg[b]
Alphavirus (CHIK)	BHK cells	15.8	Dr. Eickmann[a]
Alphavirus (Horse 31)	CNS (FFPE)	*	Prof. Castro[c]
Flavivirus (YFV)	Vero cells	3.4	Dr. Eickmann[a]
Flavivirus (WNV)	CNS (FFPE)	*	Dr. Wünschmann[d]
Bunyavirus (LACV)	Vero cells	100	Prof. Friedemann Weber[e]
Paramyxovirinae (NiV)	Vero cells	5.1	Prof. Maisner and Dr. Behner[a]
Paramyxovirinae (CDV)	CNS (FFPE)	*	IVP Giessen[b]
DNA			
EHV-1	CNS (fresh)	747.1	IVP Giessen[b]
EHV-1	CNS (FFPE)	73.6	IVP Giessen[b]
Coccidia (*Eimeria arloingi*)	Jejunum (FFPE)	268.2	Prof. Hermosilla and Dr. Silva[f]
Horse 1 (GAPDH)	CNS (FFPE)	109.9	IVP Giessen[b]

BHK: baby hamster kidney cells. CDV: Canine distemper virus. CHIK: Chikungunya virus. CNS: central nervous system. GAPDH: glyceraldehyde phosphate dehydrogenase. LACV: LaCrosse virus. NiV: Nipah virus. WNV: Nest Nile virus. YFV: Yellow fever virus. * determined after RNA extraction optimization (see Results 4.5.1.1). [a] Institute of Virology, Philipps-University Marburg, Germany. [b] Institute of Veterinary Pathology, Justus-Liebig-University, Giessen, Germany. [c] Laboratory of Veterinary Pathology, Universidade de Brasília, Brasília, Brazil. [d] Veterinary Diagnostic Laboratory, University of Minnesota, USA. [e] Institute of Virology, Justus-Liebig-University, Giessen, Germany. [f] Institute of Parasitology, Justus-Liebig-University, Giessen, Germany.

3.7.6 Polymerase chain reaction (PCR)

To optimize PCR assays adequate for FFPE material, additional sequences of the targeted pathogens were obtained with the Basic Local Alignment Search Tool (BLAST®, National Center for Biotechnology Information, Bethesda, USA) and alignments were conducted with the alignment editor BioEdit v7.2.5 (Tom Hall, Ibis Biosciences, Carlsbad, USA). Afterwards, the primers set were purchased at biomers.net (biomers.net GmbH, Ulm, Germany). Temperature gradients were carried out to establish appropriate cycling conditions for primers designed for new targets. Tests for optimal dilution of reference materials were carried out from a total RNA or gDNA concentration of 1000 ng and 30 ng, respectively, and

afterwards diluted in 1:10, 1:100, 1:1,000, and 1:10,000 aliquots. Unless stated otherwise, cycling was carried out with the thermo-cycler Multicycler® PTC 200 (Biozym Scientific GmbH, Hessisch Oldendorf, Germany).

3.7.6.1 Reverse transcription

Reverse transcription was performed ahead all of protocols involving RNA viruses. The manufacturer's instructions for the use of the QuantiTect® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) were followed to obtain a master mix of 20 µl (**Table 17**). For the elimination of gDNA, each template RNA was diluted in RNase-free water in a final concentration of 1000 ng. The NTC consisted solely of gDNA wipeout buffer and 12 µl RNase-free water. An incubation at 42°C for 2 min was then carried out in the thermo-cycler. Afterwards, 6 µl of the RT-master mix was added to the 14 µl gDNA-free samples and incubated for 15 min at 42°C followed by 3 min at 95°C. The cDNA obtained was used for the following PCR protocols.

Table 17: Reverse-transcription reaction components

Reagent	Concentration of stock solution	Volume	Concentration/amount per reaction
1. Elimination of genomic DNA (gDNA)			
gDNA Wipeout buffer	7x	2µl	1x
RNase-free water		Variable	
Template RNA	Variable	Max. 12µl	Max. 1000ng
Partial volume		**14µl**	
2. Reverse-transcription master mix			
Quantiscript reverse transcriptase		1µl	
Quantiscript RT buffer	5x	4µl	
Polymerase		1µl	
Total volume		**20µl**	

3.7.6.2 Equid glyceraldehyde-3-phosphate dehydrogenase

To investigate RNA accessibility obtained with each RNA isolation protocols, six equid glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sets were tested in ascendant length (**Table 18**). For this first approach, 18/35 samples were selected showing inflammatory

lesions (Boos et al., 2019a). Equid GAPDH PCR was then carried out with all 35 samples by using RNA from the optimized extraction protocol (3.7.1).

Table 18: Sequences of equid glyceraldehyde-3-phosphate dehydrogenase primers

Target gene	Sequence 5'-3'	Position[a]	A.t.	Product
GAPDH eq 421F GAPDH eq 447R	TCT GCT GAT GCC CCA ATG TT ATT TCT CGT GGT TCA CGC CC	421-440 466-447	55°C	46bp
GAPDH eq 214F GAPDH eq 277R	TTC CAT GGC ACA GTC AAG GC AGA TGG TGA TGG CCT TTC CG	214-233 277-258	60°C	64bp
GAPDH eq 300F GAPDH eq 402R	CAT CAA ATG GGG CGA TGC TG CCT TTT GGC TCC ACC CTT CA	300-319 402-383	60°C	103bp
GAPDHeq170 F[b] GAPDHeq170 R[b]	ATC CCT GCT TCT ACT GGT TTC ACC ACC TTC TTG ATC TC	667-685 875-855	55°C	170bp
GAPDHeq298 F[b] GAPDHeq298 R[b]	TCT TCC AGG AGC GAG ATC ATG AGT CCC TCC ACG ATG	275-293 609-591	55°C	298bp
GAPDHeq517 F[b] GAPDHeq517 R[b]	AAA GGC CAT CAC CAT CTT C CAC GAC TGA CAC GTT AGG	262-280 812-794	55°C	517bp

A.t.: annealing temperature. bp: base pairs. eq: equine. F: forward. R: reverse.
[a] Reference gene NM001163856.1 for *Equus caballus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA. [b] Kristine Kehr (data not published), Institute of Veterinary Pathology, JLU Giessen.

GAPDH master mix of QIAGEN® Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany), was prepared according to **Table 19**. 5 µl of cDNA from each sample was added, and the solution cycled. A horse brain cDNA obtained from a fresh FFPE block was used as positive control, while RNase-free water as NTC. Cat and squirrel RNA extracted from FFPE blocks from the archives of the Institut of Veterinary Pathology, JLU Giessen, were used as negative controls for amplification products up to 130 bp and from 170-517 bp, respectively.

Table 19: Overview of reaction approaches for equid-GAPDH PCR

Reagent	Volume for 1 reaction	Stock concentration
QIAGEN Multiplex PCR Master Mix	7.8µl	2x
RNase-free water	1.3µl	
cDNA	5µl	
Forward primer	0.78µl	10µM
Reverse primer	0.78µl	10µM
Total volume	**15µl**	

Amplification was carried out for 15min at 95°C, followed by 35 cycles of denaturation for 30s at 94°C, annealing for 60s at temperatures ranging from 55°C to 60°C (see Table 16 for each primer pair), elongation for 90s at 72°C, and final elongation for 10 min at 72°C.

3.7.6.3 Pan-lyssavirus RT-PCR

Pan-lyssavirus semi-nested PCR for detection of the large polymerase (L) protein gene

A pan-lyssavirus semi-nested PCR was optimized to detect all viruses within the *Lyssavirus* genus. Primers sequences for the first PCR round were obtained from Fischer et al. (2014) to amplify a sequence fragment of the large polymerase (L) protein, while a new forward primer was designed to produce shorter amplicons for the semi-nested PCR using the Pasteur virus (Accession M13215) nucleotide sequence as reference (**Table 20**).

Table 20: Sequences of pan-lyssavirus for semi-nested RT-PCR

Primer	Sequence (5'-3')	Position	Product	Reference
Pan-Lyssa-7531F	TTC TTC GCT YTR ATG TCW TGG AA	7074-7096	218bp	Fischer et al. (2014)
Pan-Lyssa-7749R	ATG RTT GTT CCA CTT YTC ATA RTC	7269-7292		Fischer et al. (2014)
Rabies semi-nested F	ATG ACW GAC AAT TTG AAC AAG G	7170-7191	~120bp	This study

F: forward. R: reverse.

For each reaction, a master mix with 10 µl was obtained with BioLine MyTaq™ HS Mix kit (Bioline GmbH, Luckenwalde, Germany) added to 5 µl cDNA (**Table 21**).

Table 21: Reaction reagents for pan-lyssavirus semi-nested RT-PCR

Reagent	Volume for 1 reaction	Stock concentration
Two-step PCR		
BioLine MyTaq™ HS Mix	7.8µl	2x
RNase-free water	1.3µl	
Pan-Lyssa-7531R	0.7µl	10µM
Pan-Lyssa-7749R	0.7µl	10µM
cDNA	5 µl	
Total	**15µl**	
Semi-nested PCR		
BioLine MyTaq™ HS Mix	10µl	2x
Rabies semi-nested F	0.4µl	10µM
Pan-Lyssa-7749R	0.4µl	10µM
RNase-free water	7.2µl	
Two-step PCR product	2µl	
Total volume	**20µl**	

Amplification conditions consisted of 15 min at 95°C, followed by 40 cycles of denaturation for 30s at 94°C, annealing for 60s at 55°C, elongation for 90 s at 72°C, and final elongation for 10 min at 72°C. Another round of amplification with the same conditions was carried out, this time containing 2 µl of the first PCR's product.

3.7.6.4 Pan-Borna disease virus RT-PCR

Pan-Borna virus One Step Real-time RT-PCR for detection of X/P proteins

For screening of orthobornaviruses, the primer set designed by Nobach & Herden (2020) was used to amplify a 200 bp fragment of the X/P-open reading frame (ORF). These primers were designed to detect viruses of seven species of the genus *Orthobornavirus* (*Mammalian 1 orthobornavirus*, *Mammalian 2 orthobornavirus*, *Passeriform 1 orthobornavirus*, *Passeriform 2 orthobornavirus*, *Psittaciform 1 orthobornavirus*, *Psittaciform 2 orthobornavirus*, and *Waterbird 1 orthobornavirus*). Additionally, Bourg et al. (2016) demonstrated that other known pathogenic viruses, like BoDV-1, VSBV-1, PaBV-2, and PaBV-4 can also be detected by this assay (**Table 22**).

Table 22: Sequences of pan-Borna disease virus primers for Real-time RT-PCR

Primer	Sequence 5'-3'	Product	Reference
OrthoBorna Forward	CGC GAC CMT CGA GYC TRG T	200bp	Nobach & Herden (2020)
OrthoBorna Reverse	GAC ARC TGY TCC CTT CCK GT		
Probe Borna-1471-FAM	AAG AAC CCH TCC ATG ATC TCM GAC CMA GA		

Reactions were carried out with the OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany). Each reaction had a total volume of 25 µl including 5 µl of RNA template (**Table 23**). Amplification was obtained using the QIAGEN Rotor Gene-Q cycler and analyses were carried out with the Rotor-Gene Q Series software. Cycling conditions consisted of 30 min at 50°C, 15 min at 95°C, followed by 45 cycles of 15s at 95°C, 30s single at 57°C and, 30s at 72°C. Samples were considered positive when the threshold cycle (Ct) values produced were comparable to the positive controls.

Table 23: Reaction reagents for pan-Borna disease virus Real-time RT-PCR

Reagent	Volume for 1 reaction	Stock concentration
5x One Step Buffer	5µl	5x
dNTP Mix	1µl	10mM
BSA	1µl	1 mg/ml
Borna-1319-F	0,8µl	10uM
Borna-1529-R	0,8µl	10uM
Borna-1471-FAM	0,1µl	50uM
DEPC water	10,3µl	
Enzyme Mix	1µl	
Template RNA	5µl	
Total volume	**25µl**	

Pan-Borna disease virus two-step RT-PCR for detection of the X/P proteins gene

The two-step RT-PCR was carried out to amplify a 60 bp fragment of the genus *Orthobunyavirus* X/P ORF. It was used for samples that showed late amplification curves/reaction (Ct above 27) with the pan-Borna disease virus qRT-PCR, described above. In this case, primers set was provided by Daniel Nobach, Institute of Veterinary-Pathology, JLU Giessen (data not published) (**Table 24**).

Table 24: Sequences of pan-Borna disease virus primers for pan-Borna virus two-step PCR

Primer	Sequence 5'-3'	Product	Reference
Borna1424-F	GAA GAA CCC HTC CAT GAT CTC	60bp	Nobach (not published)
Borna1484-R	ATT CGA TAG TTG RTC CCT TCC		

F: forward. R: reverse.

After reverse transcription, the two-step PCR was carried out with BioLine MyTaq™ HS Mix. Each reaction had a total volume of 20 µl including 5 µl of cDNA (**Table 25**).

Table 25: Reaction reagents for pan-Bornavirus two-step RT-PCR

Reagent	Volume for 1 reaction	Stock concentration
BioLine MyTaq™ HS Mix	10.5µl	2x
RNase-free water	3.4µl	
Borna1424-F	1.1µl	10µM
Borna1484-R	1.1µl	10µM
cDNA	5µl	
Total	**20µl**	

Cycling consisted of a single step of 15 min at 95°C, followed by 35 cycles of denaturation for 30s at 94°C, annealing for 60s at 50°C, elongation for 90s at 72°C, and final elongation for 10 min at 72°C.

3.7.6.5 Equine herpesvirus-1 PCR

Equine herpesvirus-1 PCR for detection of the viral glycoprotein B (ORF33) and DNA polymerase (ORF33)

To detect EHV-1 DNA, primers and cycling conditions were adapted from Leutenegger et al. (2008). The assay was designed to amplify a 90 bp fragment of the viral glycoprotein B (ORF33) and/or a 53 bp fragment of the DNA polymerase (ORF30) (**Table 26**). Infection with a neuropathogenic variant was considered when there was amplification of the ORF30, and further demonstration of the single nucleotide polymorphism (snp) from A(2254) to G(2254), associated with clinical EHM.

Table 26: Sequences of Equine herpesvirus-1 primers

Target gene	Sequence (5'-3')	Primer	Product	Reference
Glycoprotein B (ORF33)	TAT ACT CGC TGA GGA TGG AGA CTT T	EHV1-387F	90bp	Leutenegger et al. (2008)
	TTG GGG CAA GTT CTA GGT GGT T	EHV1-476R		
DNA polymerase (ORF30)	ATC TGG CCG GGC TTC AAC	EHV1-29F	53bp	Leutenegger et al. (2008)
	GGT CAC CCA CCT CGA ACG T	EHV1-82R		

F: forward. ORF: Open reading frame. R: reverse.

The extracted gDNA was diluted in sterile water to achieve the final concentration of 30 ng. For the PCR, the BioLine MyTaq™ HS Mix kit (Bioline GmbH, Luckenwalde, Germany) was used in 15 µl reactions (**Table 27**).

Table 27: Reaction reagents for Equine herpesvirus-1 PCR

Reagent	Volume for 1 reaction	Stock concentration
BioLine MyTaq™ HS Mix	7.8µl	2x
RNase-free water	1.3µl	
Forward primer	0.7µl	10µM
Reverse primer	0.7µl	10µM
gDNA	5 µl	
Total	**15µl**	

Amplification conditions consisted of 15 min at 95°C, denaturation for 30s at 94°C, annealing for 60s at 55°C, elongation for 90s at 72°C repeated through 40 cycles, and final elongation for 10 min at 72°C.

3.7.6.6 Pan-alphavirus RT-PCR

Pan-Alphavirus One Step nested RT-PCR for detection of the non-structural protein 4 (nsP4) gene

Due to its high conservation, the non-structural protein 4 (nsP4) coding region of the alphavirus genus RNA was chosen as described by Grywna et al. (2010). The assay was designed to recognize the virus species Barmah Forest virus (BFV), CHIKV, Mayaro virus (MAYV), O'nyong-nyong virus, Ross River virus (RRV), Semliki Forest virus (SFV), Sindbis virus, Eastern, Western, and Venezuelan equine encephalitis viruses (EEEV, WEEV, and VEEV, respectively). Based on the available alphavirus sequences, it was necessary to use two antisense primers differing in one nucleotide for the nested step (**Table 28**, Grywna et al., 2010).

Table 28: Sequences of primers for pan-alphavirus nested RT-PCR

Primer	Sequence 5'-3'	Product	Reference
panAlpha 1 F	TTT AAG TTT GGT GCG ATG ATG AAG TC	~400bp	Grywna et al. (2010)
panAlpha 1 R	GTT GCT TCA ATG GTT CAR GGN GAY AA		
panAlpha 2 F	GGT GCG ATG ATG AAG TCT GGG ATG T	210bp	
panAlpha 2R1	CTA TGA TAT TGA CTT CCA TGT TCA **T**CC A		
panAlpha 2R2	CTA TGA TAT TGA CTT CCA TGT TCA **G**CC A		

F: forward. R: reverse.

For the first step of the nested RT-PCR, Qiagen OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany) was carried out in a total volume of 13 µl for each reaction and 12 µl template RNA, or RNase-free water for the NTC (**Table 29**). The cycling conditions consisted of 15 min at 45°C followed by 3 min at 95°. A touchdown step was included consisting of 10 cycles of 20s at 95°C, 20s starting at 65°C decreasing 1°C each cycle, and 20s at 72°C. To finalize, another 30 cycles of 20s at 95°C, 20s at 55°, and 20s at 72°C were done.

Table 29: Reaction reagents for pan-alphavirus nested RT-PCR

Reagent	Volume for 1 reaction	Stock concentration
One Step RT PCR		
5x One Step Buffer	5µl	5x
dNTP Mix	1µl	10mM
BSA	1µl	1 mg/ml
panAlpha 1 for	1.25µl	10uM
panAlpha 1 rev	1.25µl	10uM
DEPC water	2.5µl	
Enzyme Mix	1µl	
Template RNA	12µl	
Total	**25µl**	
Nested PCR		
2x Multiplex Master Mix	10µl	2x
panAlpha 2 for	0.4µl	10uM
panAlpha 2rev1	0.2µl	10uM
panAlpha 2rev2	0.2µl	10uM
DEPC water	7.2µl	
One Step RT PCR product	2µl	
Total	**20µl**	

In the nested phase, QIAGEN® Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany) was prepared in a total volume reaction of 18 µl, added to 2 µl from the product of the first step,

using the primers described for the nested phase. The cycling conditions were 10 min at 95° followed by 40 cycles of 20s at 95°C, 20s at 55°C and, 20s at 72°C.

Pan-alphavirus semi-nested RT-PCR for detection of the non-structural protein 4 (nsP4) gene

To improve the PCR conditions applicable for FFPE material, a primer sequence complimentary to the inner primer pair of Grywna et al. (2010) was selected for a semi-nested reaction to amplify a 90 bp fragment from the nsP4 (**Table 30**). Strains of Chikungunya virus were the reference gene for this new primer (Sidonia 2F).

Table 30: Primers used for the pan-alphavirus semi-nested PCR

Primer	Sequence 5'-3'	Product	Reference
panAlpha 2 F	GGT GCG ATG ATG AAG TCT GGG ATG T	210bp	Grywna et al. (2010)
panAlpha 2R1	CTA TGA TAT TGA CTT CCA TGT TCA **T**CC A		
panAlpha 2R2	CTA TGA TAT TGA CTT CCA TGT TCA **G**CC A		
Sidonia 2F	TGC GCA GCM TTC ATC GGC GAC GAC	90bp	This study

F: forward. R: reverse.

For the first step of the semi-nested PCR, each reaction consisted of 13 µl master mix added to 5 µl RNA template. The master mix reagents and the cycling conditions were the same as the ones previously described on the pan-alphavirus One Step nested RT-PCR (**Table 31**). While in the semi-nested step, the cycling was carried out initially for 15 min at 95°C, followed by 40 cycles of 30s at 94°C, 60s at 58°C, 90s at 72°C, and final elongation of 10 min at 72°C.

Table 31: Reaction reagents for pan-alphavirus semi-nested RT-PCR Continued

Reagent	Volume for 1 reaction	Stock concentration
One Step RT PCR		
5x One Step Buffer	5µl	5x
dNTP Mix	1µl	10mM
BSA	1µl	1 mg/ml
panAlpha 2 for	1.25µl	10µM
panAlpha 2rev1	0.63µl	10µM
panAlpha 2rev2	0.63µl	10µM
DEPC water	2.5µl	
Enzyme Mix	1µl	
Template RNA	5µl (adjusted to 1000ng)	
Total	**18µl**	

Reagent	Volume for 1 reaction	Stock concentration
Semi-nested PCR		
2x BioLine My Taq HS Mix	10µl	2x
Sidonia 2F	0.8µl	10µM
panAlpha 2rev1	0.8µl	10µM
panAlpha 2rev2	0.8µl	10µM
DEPC water	1.3µl	
One Step RT PCR product	2µl	
Total	**15µl**	

3.7.6.7 Pan-flavivirus RT-PCR

A broad-range-flavivirus Real-time RT-PCR was used according to Kuno (1998) and optimized by Scaramozzino et al. (2001). The assay was designed to amplify the non-structural protein 5 (NS5) genes from the main flavivirus complexes Dengue virus (DENV), Japanese encephalitis virus (JEV), WNV, Yellow fever virus (YFV), Tick-borne encephalitis virus (TBEV), and Rio Bravo virus. In this study, flaviviral screening was carried out in two approaches; RT-qPCR assay obtaining a final product of 250 bp, and a semi-nested RT-PCR, using the same primers as outer primers added to a new, complimentary inner primer, designed to amplify a product lengthening 130 bp (**Table 32**). To design this new primer (Louis R), sequence of the Saint Louis encephalitis virus (SLEV) strain MSI-7 NS5 gene was added (Accession EU074012.1).

Table 32: Sequences of primers for Real-time RT-PCR and semi-nested PCR for flaviviruses

Primer	Sequence 5'-3'	Position	Product	Reference
FU1 (F)	TAC AAC ATG ATG GGA AAG AGA GAG AA	8993-9018[a]	250bp	Kuno (1998)
cFD2 (R)	GTG TCC CAG CCG GCG GTG TCA TCA GC	9232-9258[a]		Kuno (1998)
Probe 788 (6FAM)	AAR GGH AGY MCD GCH ATH TGG T	9044-9065		Scaramozzino et al. (2001)
Louis R	CCA GTG GTC TTC ATT SAG GAA	9151-9172[a]	133bp[b]	This study

F: forward. R: reverse.
[a] Reference gene: Yellow fever virus complete genome, 17D vaccine strain (Accession X03700). [b] inner primer combined with primer FU1 (F).

Pan-flavivirus One Step Real-time RT-PCR for detection of the non-structural protein 5 (NS5) gene

Reactions were carried out with the OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany) consisting of 20 µl of the master mix, added to 5 µl of RNA template (**Table 33**).

Table 33: Reaction reagents for the pan-flavivirus Real-time RT-PCR

Reagent	Volume for 1 reaction	Stock concentration
One Step buffer	5µl	5x
dNTP mix	1µl	10mM
BSA	1µl	1mg/mL
DEPC water	11.3µl	
Enzyme mix	1µl	
FU1 (F)	0.3µl	100µM
cFD2 (R)	0.3µl	100µM
Probe 788 (6FAM)	0.1 µl	
Template RNA	5µl	
Total volume	**25µl**	

The reaction time and temperature in the Rotor Gene-Q cycler set were 30 min at 50°C, 15 min at 95°C, followed by 45 cycles of 15s at 95°C, 30s single at 52°C and, 30s at 72°C. Later, samples were analysed with the Rotor-Gene Q Series software and considered positive when the threshold cycle (Ct) values produced were comparable to the positive controls.

Pan-flavivirus semi-nested RT-PCR for detection of non-structural protein 5 (NS5) gene

To increase the sensitivity of the primer pair used for the pan-flavivirus One Step Real-time RT-PCR described on the section above, the reverse inner primer *Louis R* was applied to a second round of PCR reactions. The first round of the semi-nested PCR was carried out with the OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany), each reaction consisting of a 20 µL master mix, added to 5 µL of RNA template (**Table 34**). Cycling conditions consisted of 30 min at 50°C and 15 min at 95°C for the RT phase, followed by 40 cycles of 15s at 95°C, 30s at 52°C, and 30s at 72°C. The semi-nested phase was carried out with the BioLine MyTaq™ HS Mix, each reaction occurring in a total volume of 18 µL, including 2 µl of the two-step PCR product. The cycling conditions consisted of amplification for 15 min at 95°C, followed by 35 cycles of denaturation for 30s at 94°C, annealing for 60s at 55°C, elongation for 90s at 72°C, and final elongation for 10 min at 72°C.

Table 34: Reaction reagents used for the pan-flavivirus semi-nested PCR

Reagent	Volume for 1 reaction	Stock concentration
One Step PCR		
One Step buffer	5µl	5x
dNTP mix	1µl	10mM
BSA	1µl	1mg/mL
DEPC water	11.4µl	
Enzyme mix	1µl	
FU1 (F)	0.3µl	10µM
cFD2 (R)	0.3µl	10µM
Template RNA	5µl	
Total volume	**25µl**	
Semi-nested PCR		
2x BioLine MyTaq™ HS Mix	10µL	2x
FU1 (F)	0.4µL	100µM
Louis R	0.4µL	100µM
RNase-free water	5.3µL	
One Step PCR product	2µL	
Total volume	**18µl**	

3.7.6.8 Pan-bunyavirus RT-PCR

Pan-bunyavirus semi-nested RT-PCR for detection of the small viral RNA segment (S-segment)

For the screening of viruses in the *Orthobunyavirus* genus, a primer set designed by Kuno et al. (1996) was used to amplify a 210 bp fragment of the small RNA segment. This was demonstrated to amplify sequences of at least 44 viruses, including Main Drain virus, California encephalitis virus, and LACV. Moreover, for the semi-nested phase, new primers were designed to amplify a 115 bp fragment, including sequences of Maguari virus, Shuni virus, and Oropouche virus (**Table 35**).

Table 35: Sequences of pan-bunyavirus primers for semi-nested RT-PCR

Primer	Sequence 5'-3'	Position	Product	Reference
New-BCS82 F	TTY HAT GAT GTC GCA TC	97-114	210bp	Modified from Kuno et al. (1996)
New-BCS82 F2	TTY HAT GAT GC**T G**C	97-114		
New-BCS308 R	TGT TCC KGT TKC CAG GAA AAT	308-328		
SN-BCS197 R1[a]	TTR AGG AAG AAG ATC CTA A	197-216	115bp	This study
SN-BCS197 R2[a]	TTA ATG TAG AAG ATT CGW A	197-216		This study

F: forward. R: reverse.
[a] Additional reference genes: Maguari virus strain BeAr7272 (Accession KX100103.1), Shuni virus strain SAE1809 (Accession KC510272.1), Oropouche virus strain TRVL9375 (Accession KP026181.1).

The first step of the semi-nested PCR was carried out with BioLine MyTaq ™ HS Mix kit (Bioline GmbH, Luckenwalde, Germany), each reaction consisting of a volume of 16 µl, including 5 µl cDNA, or RNase-free water for the NTC (**Table 36**). Amplification was carried out for 15 min at 95°C, followed by 35 cycles of denaturation for 30s at 94°C, annealing for 60s at 51°C, elongation for 90s at 72°C, and final elongation for 10 min at 72°C. The semi-nested RT-PCR was also carried out with the BioLine MyTaq™ HS Mix, each reaction occurring in a total volume of 20 µl, including 2 µl of the two-step PCR product. The cycling conditions were the same as the first cycle, except amplification which was carried out at 53°C.

Table 36: Reaction reagents used for the pan-bunyavirus semi-nested RT-PCR

Reagent	Volume for 1 reaction	Stock concentration
Two-step PCR		
2x BioLine MyTaq™ HS Mix	7.1µl	2x
New-BCS82 F	0.8µl	10µM
New-BCS82 F2	0.8µl	10µM
New-BCS332 R	0.8µl	10µM
RNase-free water	1.5µl	
cDNA	5µl	
Total volume	**16µl**	
Semi-nested PCR		
2x BioLine MyTaq™ HS Mix	10µl	2x
New-BCS82 F	0.4µl	10µM
New-BCS82 F2	0.4µl	10µM
SN-BCS197 R1	0.4µl	10µM
SN-BCS197 R2	0.4µl	10µM
RNase-free water	6.4µl	
Two-step PCR product	2µl	
Total volume	**20µl**	

3.7.6.9 Pan-paramyxovirinae RT-PCR

Pan-paramyxovirinae semi-nested RT-PCR for detection of the large protein (L)

For the screening of viruses in the *Paramyxovirinae* subfamily, primers sets designed by Tong et al. (2008) were modified to amplify a 149 bp sequence fragment of the large protein (L). For the semi-nested phase, new primers were designed to amplify a 74 bp sequence fragment, including sequences of NiV, Hendra virus (HeV), and canine distemper disease (CDV) (**Table 37**).

Table 37: Sequences of pan-paramyxovirinae primers for semi-nested RT-PCR

Primer	Sequence (5'-3')	Position [a]	Product	Reference
Paramyxo F Nipah-based	GTC WGT TAT HTA TGT HGC AGA	13806-13826[a]	149bp	Modified from Tong et al. (2008)
Paramyxo F CDV-based	GTC GAT CCT ATA CGT AAG TGA	13806-13826[b]		This study
Paramyxo R1 Nipah-based	AT WGT TGC KAT MGT CCA TGT	13936-13955[a]		This study
Paramyxo R1 CDV-based	AT GGT TGC AAT AGT CCA TAA	13936-13955[b]		This study
SN-Paramyxo R2 Nipah-based	K AAT GRA TRA ATA TAT CAT CTT	13880-13901[a]	74bp	This study
SN-Paramyxo R2 CDV-based	T AAT TGA TGA ATA TTT GAG AGT	13880-13901[b]		This study

F: forward. R: reverse.
[a] Reference genes Nipah virus (Accession NC 002728.1) and Hendra virus (Accession NC 001906.3). [b] Reference gene Canine distemper virus (Accession KJ147057.1).

The first amplification round was carried out with BioLine MyTaq ™ HS Mix kit (Bioline GmbH, Luckenwalde, Germany), each reaction of 16 µl, including 5 µl cDNA, or RNase-free water for the NTC (**Table 38**). Cycling conditions consisted of 15 min at 95°C, followed by 35 cycles of 30s at 94°C, 60s at 51°C and 90s at 72°C, and final elongation for 10 min at 72°C. The semi-nested phase was carried out in 20 µl reaction including 2 µl of the first PCR product. Cycling conditions were the same as for the first round, with annealing at 55°C.

Table 38: Reaction reagents used for the pan-paramyxovirinae semi-nested RT-PCR

Reagent	Volume for 1 reaction	Stock concentration
Two-step PCR		
2x BioLine MyTaq™ HS Mix	7.8µl	2x
Paramyxo F Nipah-based	0.4µl	10µM
Paramyxo F CDV-based	0.4µl	10µM
Paramyxo R1 Nipah-based	0.4µl	10µM
Paramyxo R1 CDV-based	0.4µl	10µM
RNase-free water	1.6µl	
cDNA	5µl	
Total volume	**16µl**	
Semi-nested PCR		
2x BioLine MyTaq™ HS Mix	10µl	2x
Paramyxo F Nipah-based	0.4µl	10µM
Paramyxo F CDV-based	0.4µl	10µM
SN-Paramyxo R2 Nipah-based	0.4µl	10µM
SN-Paramyxo R2 CDV-based	0.4µl	10µM
RNase-free water	6.4µl	
Two-step PCR product	2µl	
Total volume	**20µl**	

3.7.6.10 Pan-coccidia PCR

Pan-Coccidia nested PCR

Two universal Coccidia PCRs, a semi-nested 18S and nested internal transcribed spacer 1 (ITS1), were carried out with the gDNA from 6/35 samples (Horses 1, 9, 18, 19, 20, 22) comprising the animals that presented lesions suggesting a protozoan infection. The PCR was conducted at the Institute of Parasitology, JLU Giessen by Dr. Joerg Hirzmann. While the semi-nested 18S PCR screened the presence of *T. gondii*, *S. neurona*, and/or *N. caninum*, the nested ITS1 PCR differentiated each coccidia by the amplicon produced (**Table 39**).

Table 39: Sequences of pan-coccidia primers

Target	Primer	Sequence (5'-3')	Product	Reference
18S	Cocc18S-F	GCA AGG AAG TTT GAG GCA AT	382bp	Miller et al. (2009)
	Cocc18S-R	TGC AGG TTC ACC TAC GGA AA		
	Cocc18S-iR	TCC TTC CTC TAA GTG TTA AGG TTC A		
ITS1	ApiITS1-F	TTA CGT CCC TGC CCT TTG TA	500-1000kb[a]	Gibson et al. (2011)
	ApiITS1-R	TGC GTT CTT CAT CGT TGC GC		
	ApiITS1nF (internal)	GTG AAC CTT AAC ACT TAG AGG		
	ApiITS1nR (internal)	GAG CCA AGA CAT CCA TTG CT		

F: forward. ITS1: internal transcribed spacer 1. Kb: kilo base. R: reverse.
[a]: 500kb – *Neospora caninum*, 1000kb – *Sarcocystis neurona*.

Pan-Coccidia semi-nested PCR for detection of 18S small subunit rRNA (18S ss rRNA)

For the screening of coccidia in a semi-nested assay, primers sequences optimized by Fehlberg et al. (2017) were modified and complemented by sequences of *Sarcocystidae*, *Eimeridae*, and *Cryptosporidae* families. The first round of PCR was designed to amplify a 341 bp product of the 18S small subunit rRNA, while the semi-nested phase, was to amplify a 146 bp (**Table 40**).

Table 40: Sequences of pan-coccidia primers used for semi-nested PCR investigations

Primer	Sequence (5'-3')	Product	Reference
Pan-Coccidia F	GTT GTT GCA GTT AAA AAG CTC GT	341bp	Modified from Fehlberg et al. (2017)
Pan-Coccidia R	ACT GTC AGA GGT GAA ATT CTT AGA T		Fehlberg et al. (2017)
SN-Pan-Coccidia F	TAG AGT GTT TCA AGC AGG CTT GT	146bp	This study

F: forward. R: reverse. Underline indicates nucleotide substitution.

Each reaction consisted of a 20 µl mix carried out with BioLine MyTaq™ HS Mix kit (Bioline GmbH, Hessisch Oldendorf, Germany) including 5 µl gDNA (**Table 41**). Cycling conditions for the first PCR consisted of 15 min at 95°C, followed by 35 cycles of 30s at 94°C, 60s at 50°C and 90s at 72°C, and final elongation for 10 min at 72°C. The semi-nested phase was carried out as a 20 µl master mix including 2 µl of the first PCR product. Cycling conditions were the same as for the first round, except the annealing temperature, carried out at 53°C.

Table 41: Reagents used for the pan-coccidia semi-nested PCR

Reagent	Volume for 1 reaction	Stock concentration
1st PCR		
2x BioLine MyTaq™ HS Mix	7.8µl	2x
Pan-Coccidia F	0.4µl	10µM
Pan-Coccidia R	0.4µl	10µM
RNase-free water	6.4µl	
gDNA	5µl (adjusted at 30ng)	
Total volume	**20µl**	
Semi-nested PCR		
2x BioLine MyTaq™ HS Mix	10µl	2x
SN-Pan-Coccidia F	0.4µl	10µM
Pan-Coccidia R	0.4µl	10µM
RNase-free water	7.2µl	
1st PCR's product	2µl	
Total volume	**20µl**	

3.7.6.11 Pan-mamastrovirus RT-PCR

Pan-mamastrovirus Real Time RT-PCR

To screen the samples for *Mamastrovirus* genera, Dr. Bernd Hoffmann, Friedrich-Loeffler-Institut, Greifswald – Island Riems, Germany kindly performed a RT-qPCR. The assay was designed to amplify a 160 bp product.

3.7.6.12 Electrophoresis gel preparation and separation of amplified products

To separate the DNA fragments amplified during the PCR protocols, horizontal gel systems were carried out in different gel concentrations, which varied with chambers size with pattern voltage of 7V/cm (**Table 42**).

Table 42: Electrophoresis gel preparation

Reagents	Chamber size			
	Mini (10 wells)		Middle (20 wells)	
Gel concentration	2%	4%	2%	4%
Agarose type	UltraPure™ Agarose, Invitrogen	Biozym Phor Agarose, Biozym Scientific	UltraPure™ Agarose, Invitrogen	Biozym Phor Agarose, Biozym Scientific
Agarose weight (g)	0.6	1.2	2.2	4.4
0.5x TBE buffer (ml)	30	30	110	110
Ethidium bromide 1:10 (µl)	1	1	3.6	3.6
Separation product (bp)	>300	<300	>300	<300

Alongside the samples tested, DNA ladders pUC DNA/Mspl (HpaII) (Thermo Fisher Scientific™, Carlsbad, USA) and GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific™, Carlsbad, USA) were used. For each 15 µl PCR product, 3 µl of Orange DNA loading dye (Thermo Fisher Scientific™, Carlsbad, USA) was added.

3.7.7 Specificity of sequences obtained with the PCRs

Assay specificity was verified by examining the size-specific PCR products from positive controls and afterwards, from positive samples. Size-specific amplification products, assessed through electrophoresis were then processed with ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific, Carlsbad, USA) for the cleanup of amplified PCR products. This method applies two hydrolytic enzymes, simultaneously used to purify the DNA. For each positive PCR reaction, 20 µl PCR-product was treated with ExoSAP-IT-mix, consisting of 4 µl of fast alkaline phosphatase, and 2 µl of exonuclease. Afterwards, the solution was incubated in the Multicycler PTC 200 (Biozym Diagnostik GmbH, Oldendorf, Germany) for 15 min at 37°C to degrade remaining primers and nucleotides by dephosphorylation, and 15 min at 80°C to inactivate ExoSAP-IT reagent. 2.5 µl of a 10 µM forward primer or reverse primer was added to 7.5 µl of PCR product ExoSAP-IT treated, respectively. Samples were sent to GATC Biotech AG (Köln, Germany), where the sequencing was performed.

3.7.7.1 Interpretation of sequences results

Sequencing results were analysed and interpreted with the alignment editor tool BioEdit v7.2.5 (Tom Hall, Ibis Biosciences, Carlsbad, USA) and sequences identities were verified with Blast® nucleotide (National Center for Biotechnology Information, Bethesda, USA).

3.8 Metagenomics

The RNA extracted from 10/35 samples (Horses 4, 6, 7, 11, 16, 18, 22, 31, 32 and 35) was sent to next generation sequencing (NGS), to broaden the identification of agents possibly involved in the lesions observed in the CNS of horses from Brazil. The nucleic acid was extracted with protocol 3 according to Boos et al. (2019) and shipped in dried ice to Arnt Ebinger, Institute of Diagnostic Virology and Dr. Dirk Höper, Laboratory of NGS and Microarray Diagnostics, Friedrich-Loeffler-Institut, Greifswald – Island Riems, Germany. Process of the material, development of protocols, and confection of result reports were carried out by them. In summary, library preparation of the designated brain samples was processed according to the protocol of Wylezich et al. (2018) with the modification for low-quality RNA samples, described by Niller et al. (2020). A 200 bp sequencing run using a 540 chip was performed on an Ion Torrent S5XL instrument (Thermo Fischer Scientific, Carlsbad, USA). The Metagenomic analysis was done using the Reliable Information Extraction of Metagenomic Sequence datasets pipeline (RIEMS 4.0) (Scheuch et al., 2015). Additionally, reads assigned to the super kingdom virus as well as not assigned reads were assembled with the 454 software suite (v3.0; Roche), followed by blastx search versus the virus RefSeq database (download November 2018) (O'Leary et al., 2016) using DIAMOND (Buchfink et al., 2015). Contigs were used as a criterion for pathogen detection.

4. Results

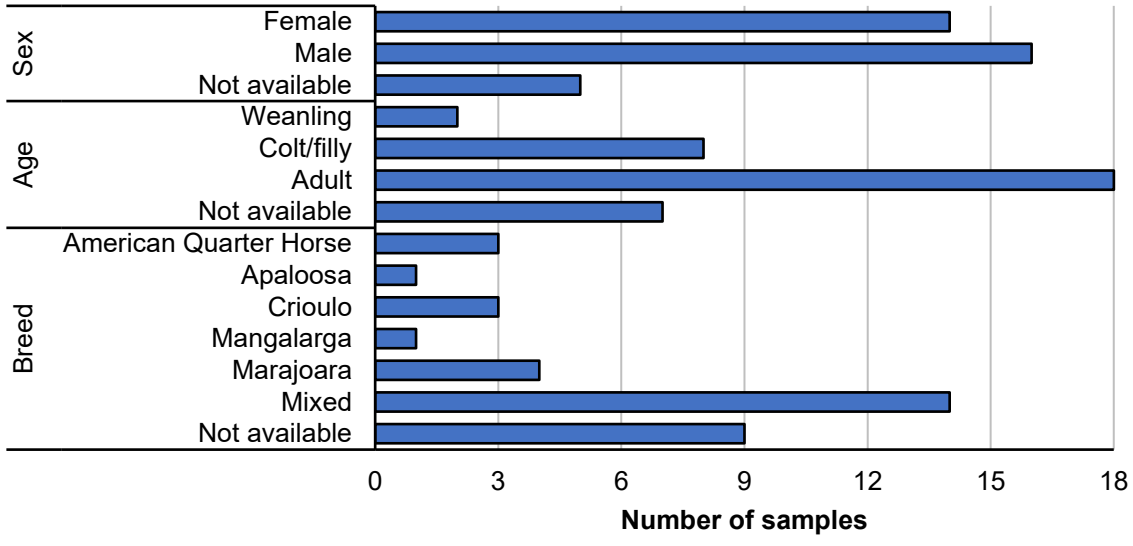
4.1 Samples

The study was carried out with a variety of CNS areas from the 35 horse brains sent from Brazil (**Appendix 4**). Forebrain fragments were present in 25/35 (71%) samples, followed by parts of the midbrain of 14/35 cases (40%), cerebellum of 12/35 (34%) animals, and parts of the spinal cord of 22/35 (63%) horses. Exceptionally from a fragment of the cerebral cortex from horse 4, in all samples, meninges were also present. Additionally, trigeminal ganglia were sent with 3/35 (9%) samples and an unspecified ganglion with 1/35 horse brain (3%). The archived samples consisted of tissues fixed in formalin and embedded in paraffin between the years 2005 and 2014.

4.2 Epidemiology and clinical records

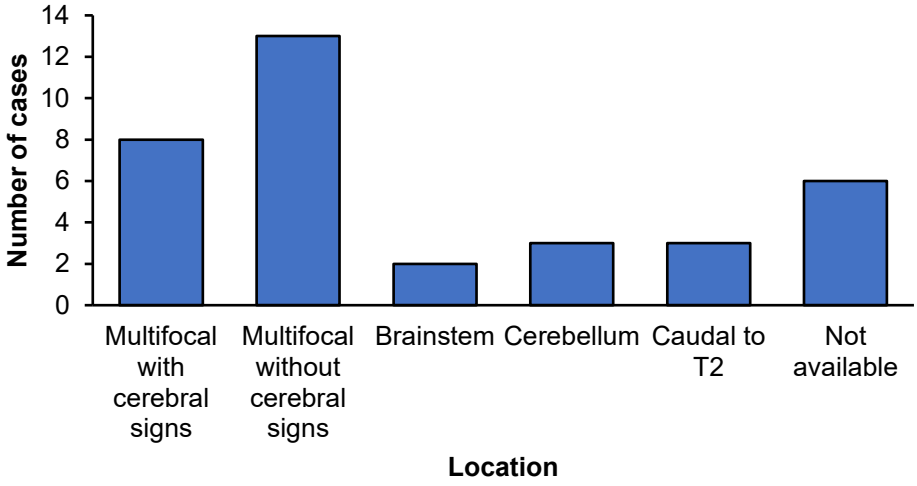
Samples available for the study were retrieved from a well-mixed population of male and female animals, most of them older than four-years (ranging from 6 months to 16 years old), and predominantly from mixed breeds (**Fig. 21**). Clear utility for riding was reported for horses 9-13, 18, and 21, while horse 20 was used for pulling cart and information in this regard was missing for the other 27 animals.

Fig. 21: Distribution of cases regarding sex, age, and breed of 35 horses



Most of the clinical signs pointed to a multifocal distribution of lesions in the CNS (21/35- 60%) especially without cerebral signs (13/21 – 62%) (**Fig. 23**, **Appendix 4**). In the remaining horses, signs were suspicious for brainstem lesions in 2/35 (6%), for cerebellum and caudal to thoracic vertebra T2 in 3/35 (9%) each, and clinical records missing in 6/35 (17%).

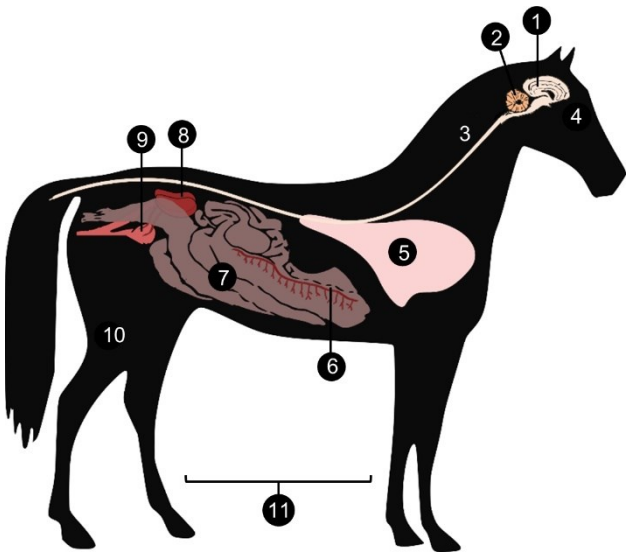
Fig. 23: Presumptive neuroanatomic location of lesions according to the clinical signs reported (N=35)



The animals were referred to the post-mortem examination after euthanasia (7/35, 20%) or found dead in the fields (5/35, 14%), and information concerning death condition was missing for the other 23/35 (66%) animals. The season of death was reported as summer for 6/35 animals (17%), as fall for 20/35 (57%), as spring for 3/35 (8%), and as early winter for 1/35 (3%), while for 6/35 (17%) this information was unavailable. Macroscopic lesions were reported in 18/35 (51%) cases (**Fig. 22**, **Appendix 4**), while unremarkable alterations in 7/35 (20%), and missing information in 10/35 (29%).

Fig. 22: Gross lesions reported in the necropsy of 18/35 horses

❶ **Encephalon:** horse 18 had oedema; horses 19 and 22 had colour alterations; horse 22 had also softening of white matter
❷ **Cerebellum:** horse 22 had a mass with spongy consistency on 4th ventricle
❸ **Spinal cord:** horses 1, 2, and 15 had colour alterations; horse 8 had luxation between C5-C7; horse 21 had a fracture between C5-C6; horses 9, 21, 24 – 27 had haemorrhage
❹ **Eyes:** horses 11-13 had palpebral oedema
❺ **Lungs:** horse 15 had multiple white dots measuring ≈ 0.2cm
❻ **Mesenteric artery:** horses 8 and 18 had parasites (*Strongylus vulgaris*); horse 13 had severe petechial haemorrhage
❼ **Intestines:** horses 8 (*A. perfoliata*, *P. equorum*) and 18 (*A. perfoliata*) had parasites; horses 11-13 had liquefied content, horse 16 had colour alteration and mucous content
❽ **Kidneys:** horse 16 colour alteration
❾ **Bladder:** horse 16 full and multifocal haemorrhage
❿ **Skin:** horse 14 had a superficial lesion in hind limb
⓫ **Body condition:** horse 8 was cachectic

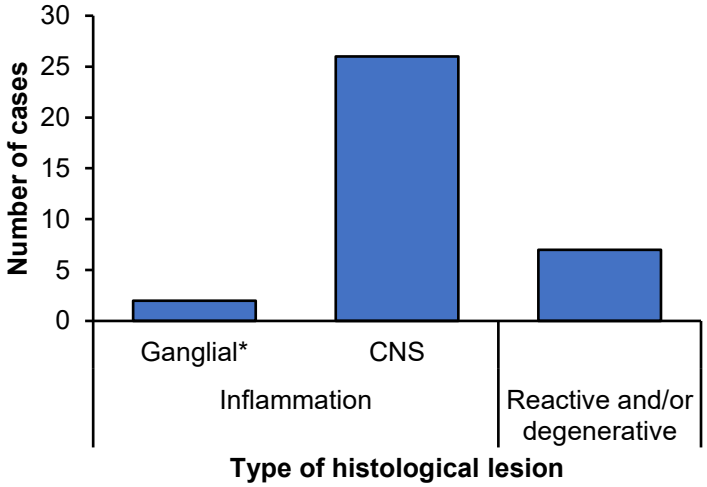


Gross lesions were reported in 18/35 (51%) horses from the cohort. In 13/18 (72%) there were lesions in the CNS (1, 2, 3), and in 3/13 (23%) there were additionally non-CNS lesions (5, 7, 11). The other 5/18 (28%) animals were reported only with non-CNS lesions (4, 6, 7, 8, 9, 10). Illustration prepared on Inkscape version 1.0, available at inkscape.org), horse silhouette "transportation" is licensed under CC0 1.0 creatives common.

4.3 Histology

Histological evaluation was carried out from all CNS areas available of each animal. In general, inflammation was observed in the CNS and ganglia from 28/35 (80%) horses, in the other 7/35 (20%) cases presented reactive and/or degenerative lesions (**Fig. 24**, **Appendix 5**).

Fig. 24: Type of histological lesion observed in the CNS of the 35 horses



* Horses 16 and 21 presented only local inflammatory infiltration in ganglia

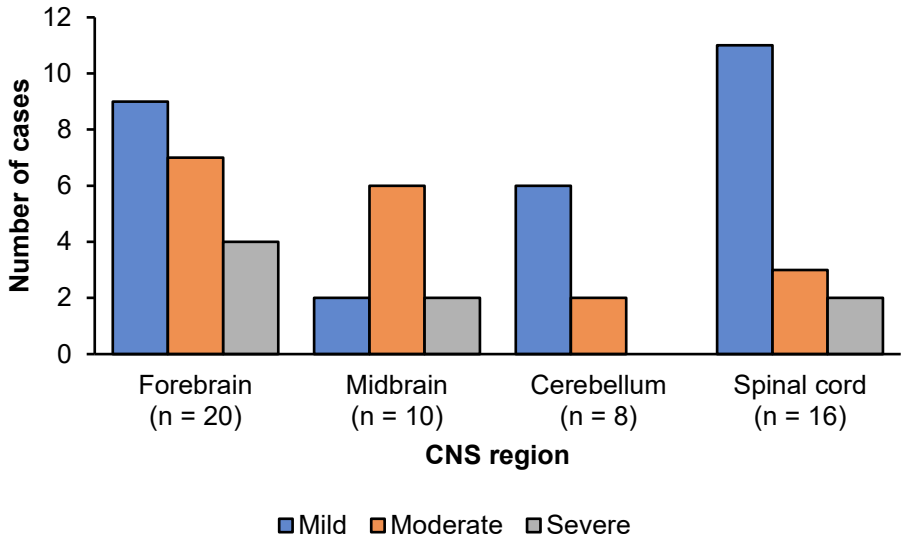
4.3.1 Histological evaluation of samples with inflammation

Histological evaluation of the 28/35 cases that displayed inflammation revealed that 13/28 (46%) of them had multifocal inflammation in the CNS, while the other 15/28 (54%) displayed localized inflammation in the spinal cord of 4/15 (27%), midbrain of 2/15 (13%), forebrain of 7/15 (47%), and ganglia of 2/15 (13%).

4.3.1.1 Degree of inflammatory cell infiltration

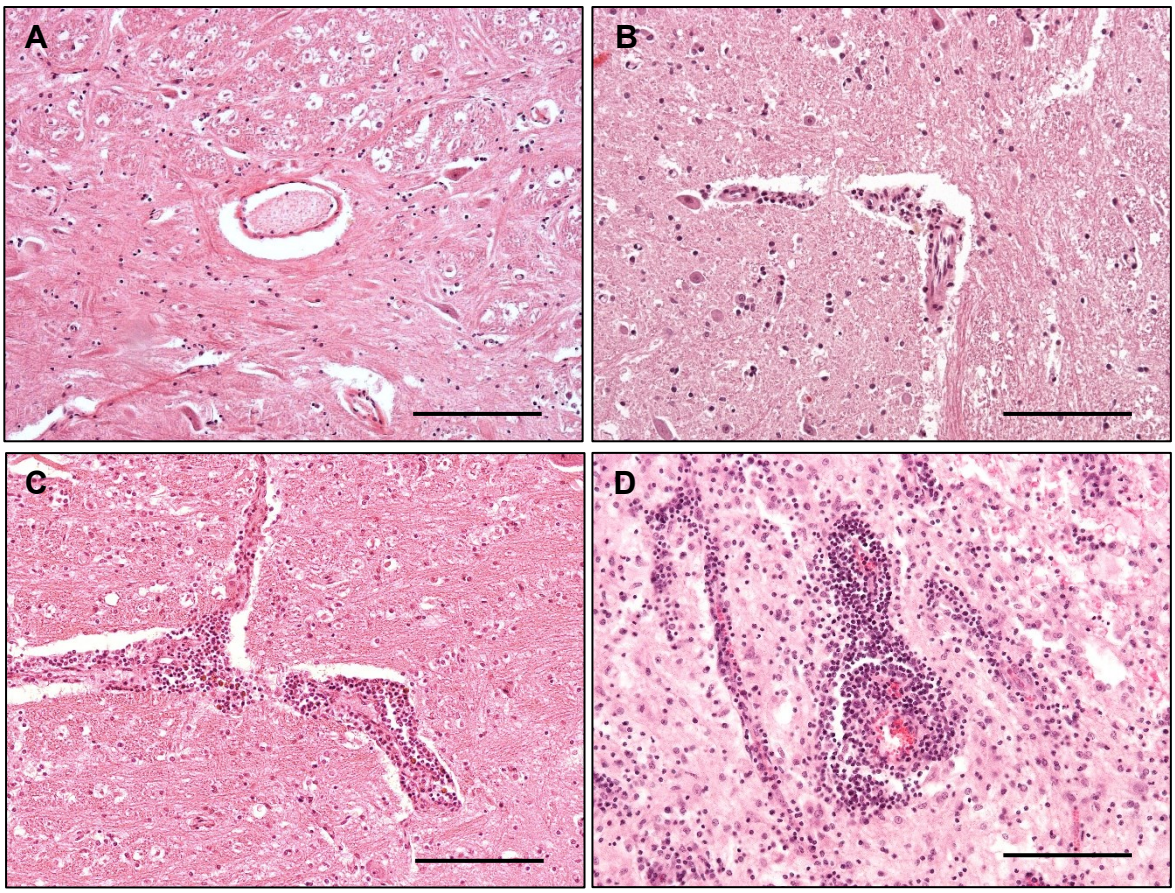
The score system carried out with the 28 samples with inflammation in the CNS (**Appendix 6**). In the forebrain, 20/28 horses displayed inflammatory infiltration, which was characterized as mild in 9/20 (45%), moderate in 7/20 (35%), and severe in 4/20 (20%). Inflammation was noted in the midbrain of 10/28 horses; 2/10 (20%) samples showed mild infiltration, 6/10 (60%) moderate, and 2/10 (20%) severe infiltration. Inflammation was present in the cerebellum of 8/28 horses; 6/8 (75%) samples displayed mild, 2/8 (25%) moderate, and none severe infiltration. In the spinal cord of 16/28 horses inflammation was noted, 11/16 (69%) samples showed mild infiltration, 3/16 (19%) moderate, and 2/16 (12%) samples severe infiltration (**Fig. 25**).

Fig. 25: Degree on inflammation in different areas of the CNS of horses



In general, mild inflammatory infiltration consisted of around 0 to 19 inflammatory cells in perivascular cuffs, with a mean of 1.5 layers of inflammatory cells in these cuffs, and between 19 to 130 inflammatory cells infiltrating the parenchyma (**Fig. 26**B). Moderate infiltration appeared as between 10 to 68 inflammatory cells in perivascular cuffs with a mean of 2 cell layers, and between 61 to 289 inflammatory cells in the parenchyma (**Fig. 26**C). In severe lesions, there were around 37 to 141 cells in perivascular cuffs, at least 3 layers of cells, and from 104 to 494 inflammatory cells in the parenchyma (**Fig. 26**D). These results were not statistically verified due to the high variance among inflammatory infiltration characteristics.

Fig. 26: Severity of inflammation in the brain of 28 horses, hematoxylin-eosin

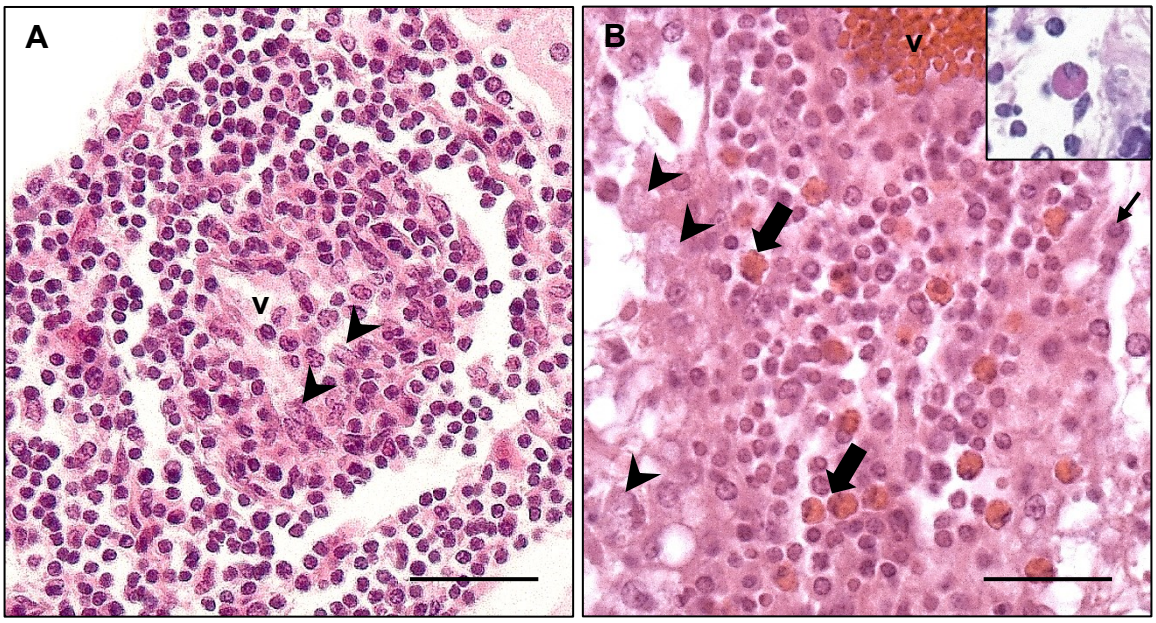


(A) Non-lesioned horse control brain, midbrain. (B) Horse 5, forebrain, mild inflammatory infiltration. (C) Horse 27, forebrain, moderate encephalitis. (D) Horse 9, spinal cord, severe myelitis. Scale bars: 200 µm.

4.3.1.2 Inflammatory cells morphology

In general, 25/28 (89%, **Appendix 5**) CNS samples had +++ of lymphocytes within inflammation in perivascular cuffs and in the parenchyma. In some of these cases, plasma cells (19/28 – 68%), macrophages (12/28 – 43%), and eosinophils (9/28 – 32%) were concurrently present as + (**Fig. 27**A). In 3/28 (11%) samples, inflammation was composed of ++ of lymphocytes and macrophages, and + of plasma cells (horse 9, **Fig. 27**B), ++ of lymphocytes and <+ plasma cells (horse 26), or like the trigeminal ganglion of horse 21, with ++ lymphocytes amidst + of macrophages and <+ of plasma cells. Periodic-acid Schiff (PAS) staining also highlighted Mott cells within perivascular cuffs of horse 9 (**Fig. 27**B-insert).

Fig. 27: Cellular composition of inflammatory lesions in the CNS of horses, hematoxylin-eosin



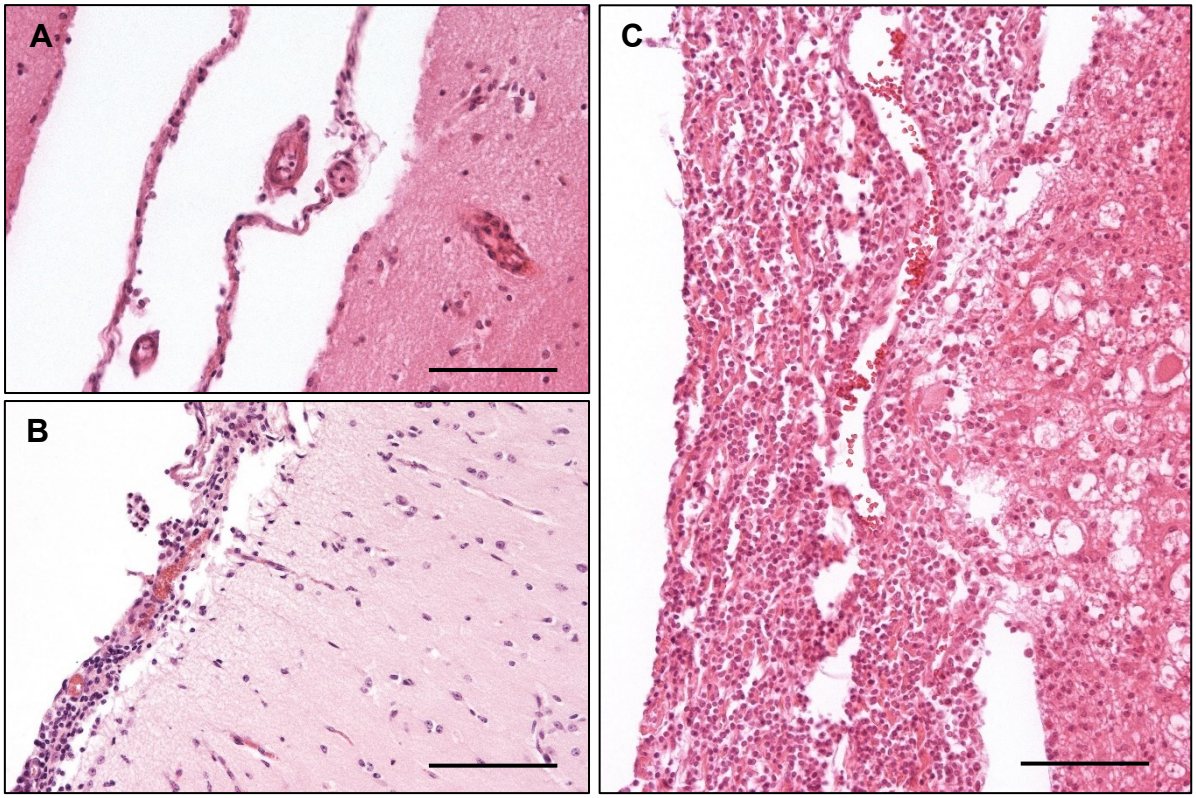
(A) Horse 29, midbrain, predominant lymphocytic inflammatory infiltrate accompanied by macrophages (arrow heads) in the center of the vessel (v). Scale bar: 50 µm. (B) Horse 9, spinal cord, mixed inflammatory infiltrate composed of lymphocytes, plasma cells (arrow), macrophages (arrowhead), and eosinophils (large arrow). Insert shows a PAS-positive Mott cell. (v): blood vessel. Scale bar: 25µm.

4.3.1.3 Meningeal inflammatory infiltrate

Meningitis was observed in 12/35 (34%) cases. It appeared as scattered cellular infiltration in 1/12 samples (8%), as mild infiltration in 4/12 (33%) (**Fig. 28**A), moderate in 5/12 (42%) (**Fig. 28**B), and severe infiltration in 2/12 (17%) cases (**Fig. 28**C).

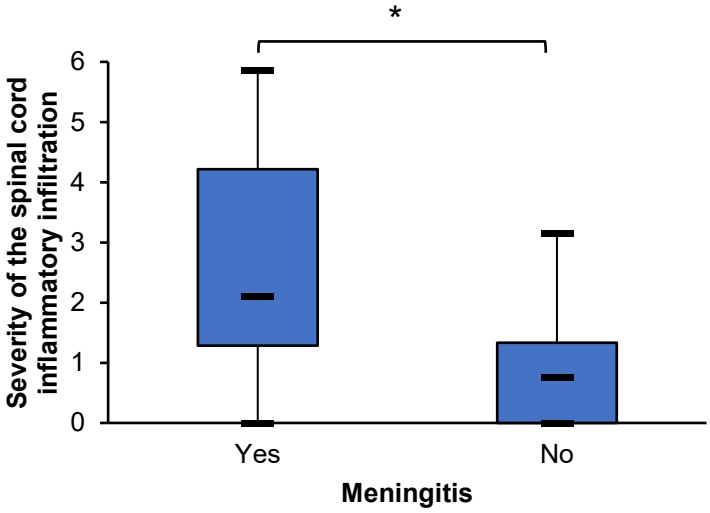
Correlation analysis with Wilcoxon-Mann-Whitney-Test indicated that the severity of inflammation in the spinal cord was higher for cases with meningitis than for cases without meningitis (U= 50.5, $p < 0.05$) (**Fig. 29**). For the other CNS regions, there was no correlation (**Appendix 7-10**).

Fig. 28: Severity of meningeal inflammation in the CNS of horses, hematoxylin-eosin



(A) Horse 27, cerebral cortex, mild meningitis. (B) Horse 35, cerebral cortex, moderate meningitis. (C) Horse 9, spinal cord, severe meningitis. Scale bars: 100 µm.

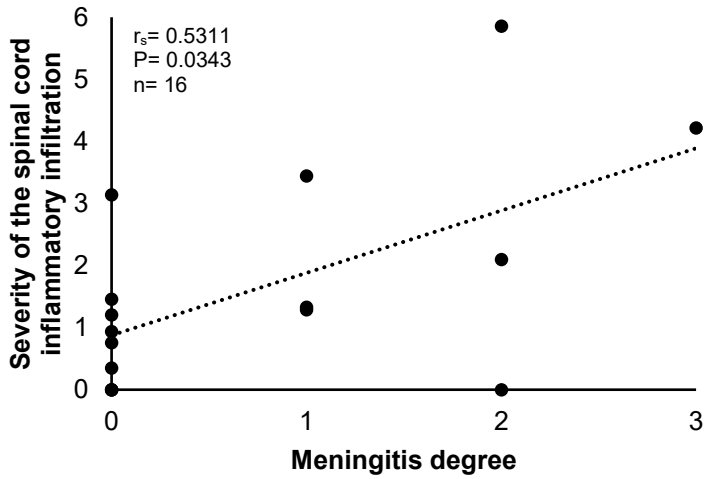
Fig. 29: Wilcoxon-Mann-Whitney-test for spinal cord inflammation and correlation to meningitis



Higher degrees of spinal cord inflammation are statistically correlated to the occurrence of meningitis.
* $p \leq 0.05$.

Additionally, Spearman's rank correlation indicated that there was a significant positive association between the severity of spinal cord inflammation and the meningitis degree ($r_s(14) = 0.53$, $p < 0.05$) (**Fig. 30**, and **Appendix 11** for other CNS regions). There was no correlation between the inflammation severity and the meningitis degree in other regions of the CNS.

Fig. 30: Spearman's rank correlation between the severity of meningitis in spinal cord fragments

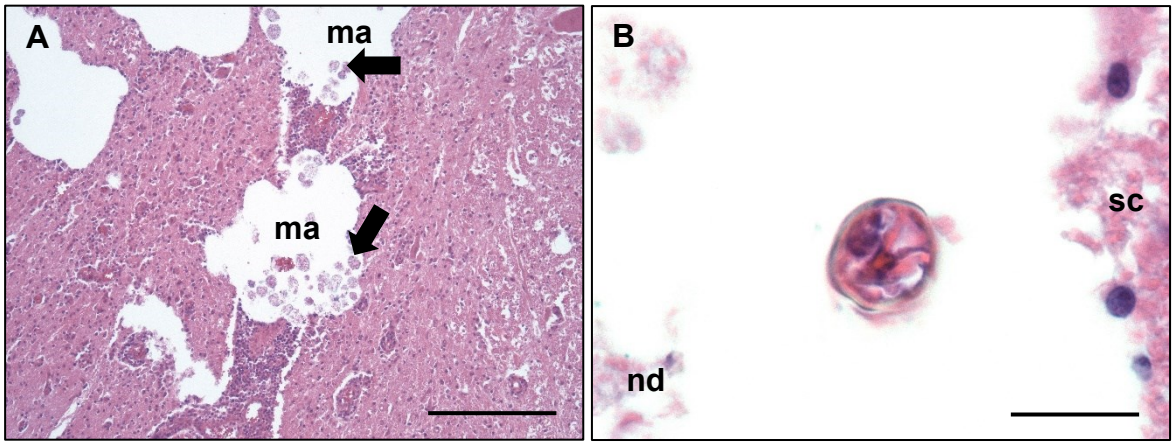


Statistically significant correlation between the severity of inflammatory infiltration in the spinal cord and the meningitis degree in horses. r_s: Spearman's correlation coefficient.

4.3.1.4 Presumptive parasitic infection

In 7/35 (20%) horses, histologic lesions and clinical history were suggestive of a parasitic infection. In the spinal cord of horse 1 (here only spinal cord available), moderate mixed inflammation composed of lymphocytes, macrophages and plasma cells accompanied by few eosinophils was present. In the grey matter, necrotic neurons were multifocally seen and some of them showed neuronophagia. Extensive areas of malacia were seen throughout the spinal cord containing occasional round to oval PAS negative structures measuring 18 x 18µm (**Fig. 31**A) with amorphous light-purple granules, well-defined borders, but irregular surface. A single round structure presented a thin wall and was filled with globular to amorphous light eosinophilic and basophilic content (**Fig. 31**B). In the white matter, there was a focal area of haemorrhage margining the posterior horn. Lesions in the spinal cord from horse 2 were characterized as moderate lymphocytic myelitis, displaying similar oval structures that were scattered throughout the tissue.

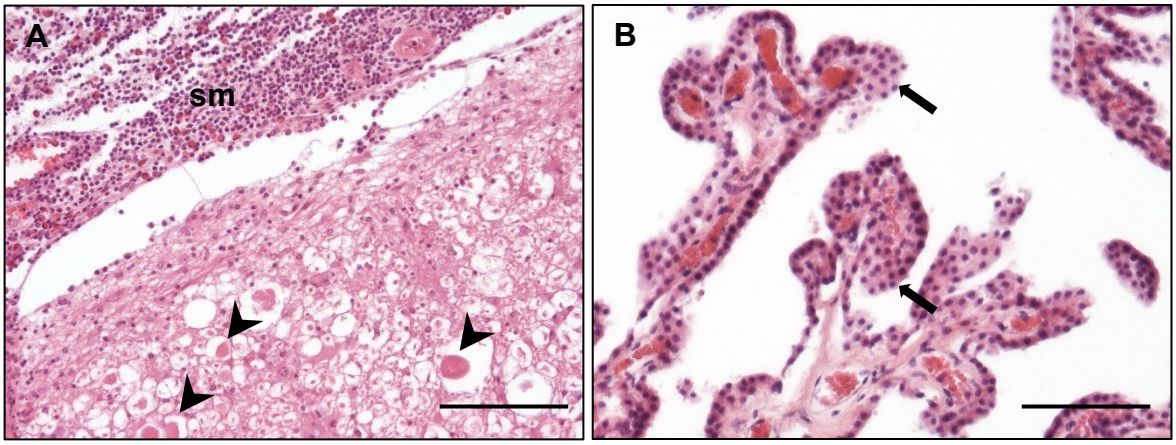
Fig. 31: Suggestive parasitic infection in the spinal cord of horse 1, hematoxylin-eosin



(A) Severe myelitis with malacic areas (ma) filled with oval structures (arrow). Scale bar: 200µm. (B) Round-oval structure found within a malacic area between necrotic debris (nd) and the spinal cord (sc). Scale bar: 25µm.

Horse 9 presented predominantly severe mixed inflammation composed of lymphocytes, macrophages, and plasma cells, followed by eosinophils in the meninges, parenchyma and perivascular cuffs in the lumbar spinal cord (**Fig. 27**B, **Fig. 28**C). There was moderate haemorrhage in the grey matter and axonal spheroids in the white matter. Moderate lymphocytic ganglioneuritis and necrotic neurons in the trigeminal ganglion were additionally observed. Similar, but moderate lesions were observed in the iliac spinal cord and mesencephalon of horse 18. Lesions observed in horse 19 consisted of mild multifocal lymphohistiocytic meningoencephalitis accompanied by necrotic neurons. Horse 20 presented mild lymphocytic meningoencephalitis, occasional axonal spheroids, and mild vacuolization of the white matter. In the cerebral cortex there was mild haemorrhage and the vascular endothelium was sometimes thickened. Horse 22 presented a severe multifocal lymphohistiocytic meningoencephalomyelitis, with moderate number of eosinophils in the spinal cord meninges and atherosclerotic meningeal vessels. In the spinal cord and in the obex, there was multifocal severe vacuolization of the white matter accompanied by spheroids (**Fig. 32**A). In the choroid plexus of the fourth ventricle, there was a choroid plexus papilloma (**Fig. 32**B).

Fig. 32: Suggestive parasitic infection and neoplasia in the CNS of horse 22, hematoxylin-eosin

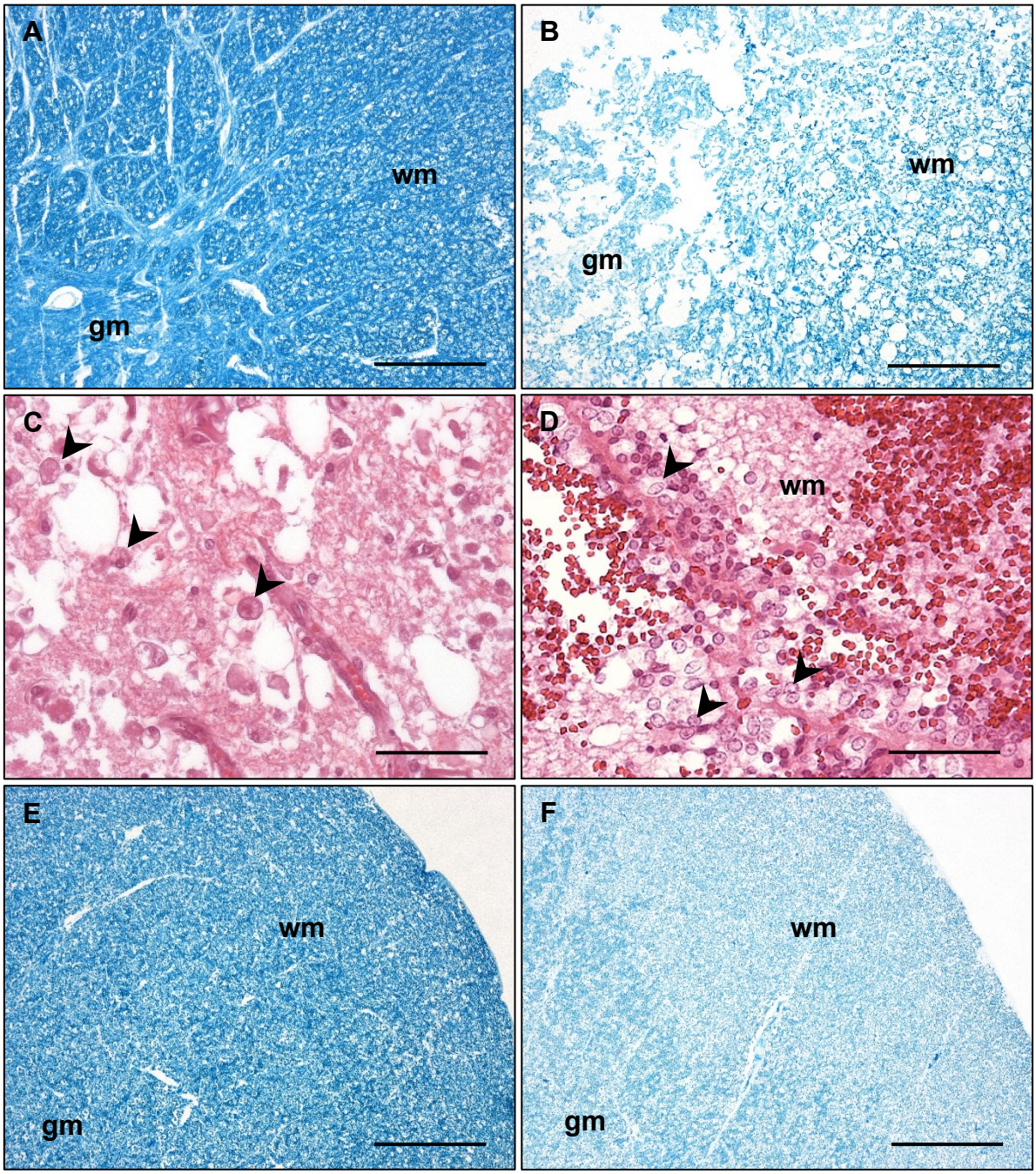


(A) Spinal cord, severe meningitis (sm), spheroids in the white matter (arrow heads). Scale bar: 200 µm.
(B) Third ventricle, choroid plexus papilloma (arrows). Scale bar: 50 µm.

4.3.2 Histological evaluation of samples with reactive and degenerative lesions

The spinal cord of horses 8 and 15 was suggestive of demyelination. When compared to a non-altered tissue (**Fig. 33**A), Luxol-stained sections from horse 8 well preserved cervical intumescence displayed extensive loss of staining (**Fig. 33**B). In the HE staining, there was extensive severe leucomyelomalacia with severe vacuolization of the dorsal cervical horn up to the right-lateral horn, accompanied by myelophages and gitter cells (**Fig. 33**C), and with spheroids at the margin of the lesion. Lesions in the thoracic spinal cord (T5) of horse 15 consisted of extensive areas of haemorrhage especially in the white matter and a focal area infiltrated by macrophages (**Fig. 33**D). Necrotic debris, diffuse moderate parenchymal vacuolization with faint Luxol-fast blue stain (**Fig. 33**E, F), occasional necrotic neurons and axonal spheroids were also observed.

Fig. 33: Reactive and degenerative lesions in the spinal cord of horses

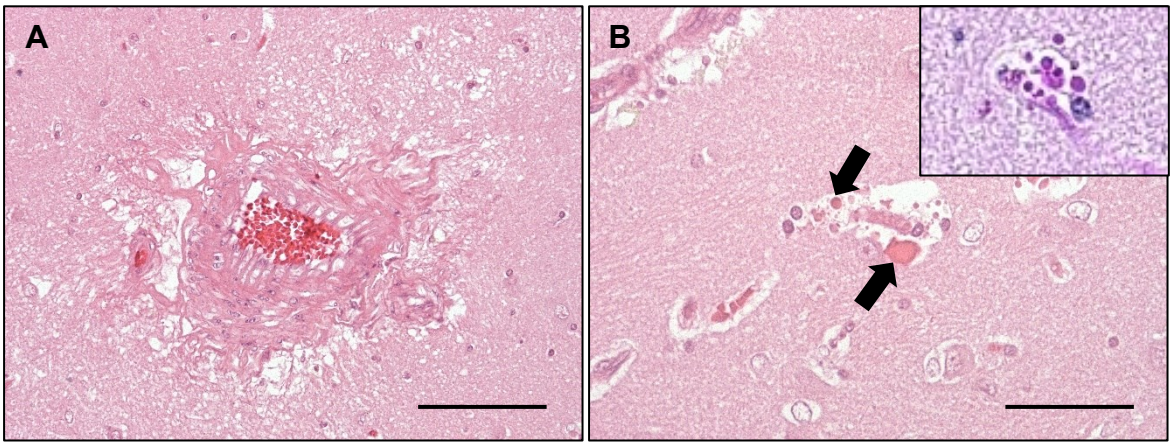


(A) Spinal cord of a control horse, unaffected myelin, Luxol-fast blue staining. Scale bar: 100 µm. (B) Horse 8, spinal cord, loss of staining suggestive of demyelination. Luxol-fast blue. Scale bar: 100 µm. (C) Horse 8, spinal cord, myelophages and gitter cells amidst parenchymal vacuolization. HE. Scale bar: 50 µm. (D) Horse 15, spinal cord, haemorrhage and macrophages/microglia cells amidst vacuolization. HE. Scale bar: 100 µm. (E) Spinal cord of a control horse, unaffected myelin, Luxol-fast blue. Scale bar: 500µm. (F) Horse 15, spinal cord, faint-Luxol staining suggestive of demyelination. Luxol-fast blue. Scale bar: 500µm.

Horses 12 and 13 both from the same herd, presented cerebral cortical laminar necrosis, while horses 14 and 17 presented leucomyelomalacia. In horse 28, degenerative lesions were more present in the hippocampus and cerebral cortex, consisting of disseminated parenchymal vacuolization and reactive endothelium. This might be consistent with hypoxic encephalopathy. In horse 16, a 16-year-old animal, there were vascular alterations, especially in the striatum,

consisting of focal vascular wall calcification, disseminated reactive endothelium of small vessels, and fibrinoid necrosis of vascular wall (**Fig. 34**A), haemorrhage, and accumulation of hyaline droplets around small vessels (**Fig. 34**B, insert shows PAS-positive droplets). Lesions observed in fragments from the spinal cord of horse 21 revealed neuronal necrosis and malacia through the section.

Fig. 34: Degenerative lesions in the striatum of horse 16, hematoxylin-eosin



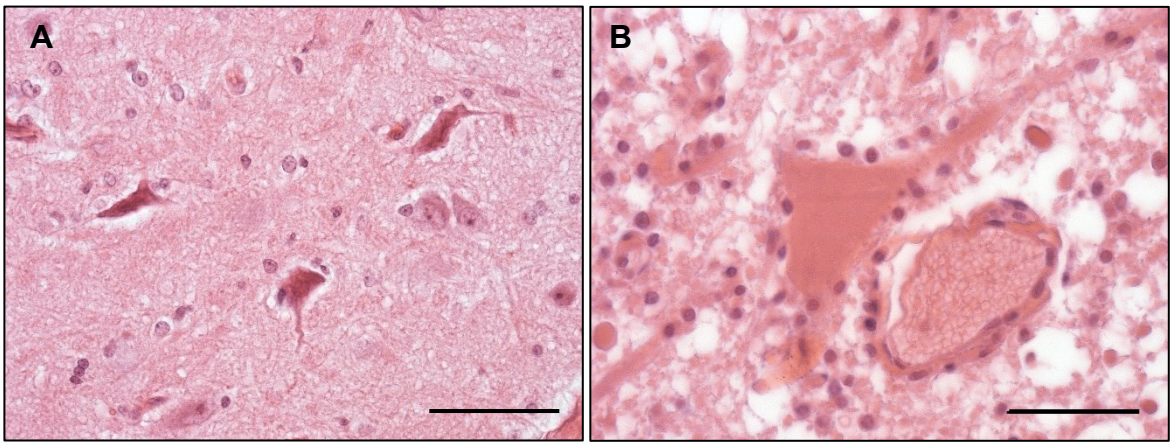
(A) Fibrinoid necrosis of vascular wall and intimal thickening. Scale bar: 200 µm. (B) Droplets of hyaline (arrows) around small blood vessels. Insert shows PAS-positive droplets. Scale bar: 50 µm.

4.3.3 Additional lesions

The following lesions could be present in cases with inflammation and degenerative lesions, and one section might present different alterations simultaneously (**Appendix 5**). Also, there was no statistically significant correlation between the occurrence of these lesions and the occurrence of inflammation (**Appendix 7-10**).

Mild ganglioneuritis in trigeminal ganglion was the only inflammatory lesion in horses 16 and 21. Necrotic neurons were found in 23/35 samples (66%), while satellitosis and/or neuronophagia in 20/35 (57%) (**Fig. 35**A, B). Malacic areas were observed in 8/35 samples (23%), visibly accompanied by macrophages/gitter cells in cases from horses 1, 8, and 9.

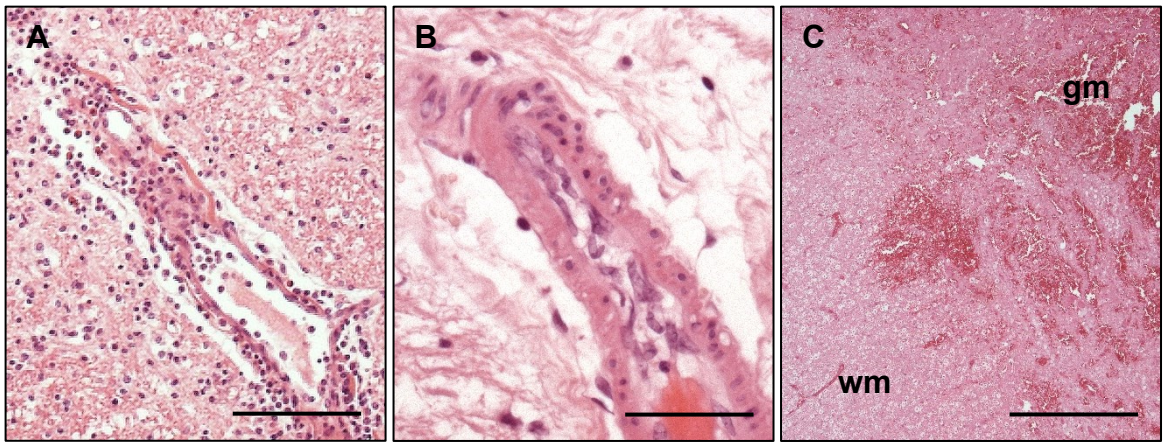
Fig. 35: Neuronal necrosis and satellitosis in the CNS of horses, hematoxylin-eosin



(A) Horse 25, parahippocampal gyrus, neuronal necrosis. Scale bar: 100 µm. (B) Horse 7, spinal cord, satellitosis. Scale bar: 50 µm.

Vascular alterations characterized by vasculitis, reactive endothelium, and haemorrhage were found in 14/35 (40%), 20/35 (57%) and 9/35 (26%) lesioned brains, respectively (**Fig. 36** A-C). Congestion was verified in 4/35 (11%) cases and oedema in 16/35 (46%). Parenchymal vacuolization was found in 17/35 (49%) cases and was always observed along with at least one of the alterations described above.

Fig. 36: Vascular alterations in the CNS of horses, hematoxylin-eosin



(A) Horse 31, midbrain, vasculitis. Scale bar: 100µm. (B) Horse 20, medulla oblongata, reactive endothelium. Scale bar: 50µm. (D) Horse 15, spinal cord, hemorrhage; (gm) gray matter, (wm) white matter. Scale bar: 500µm.

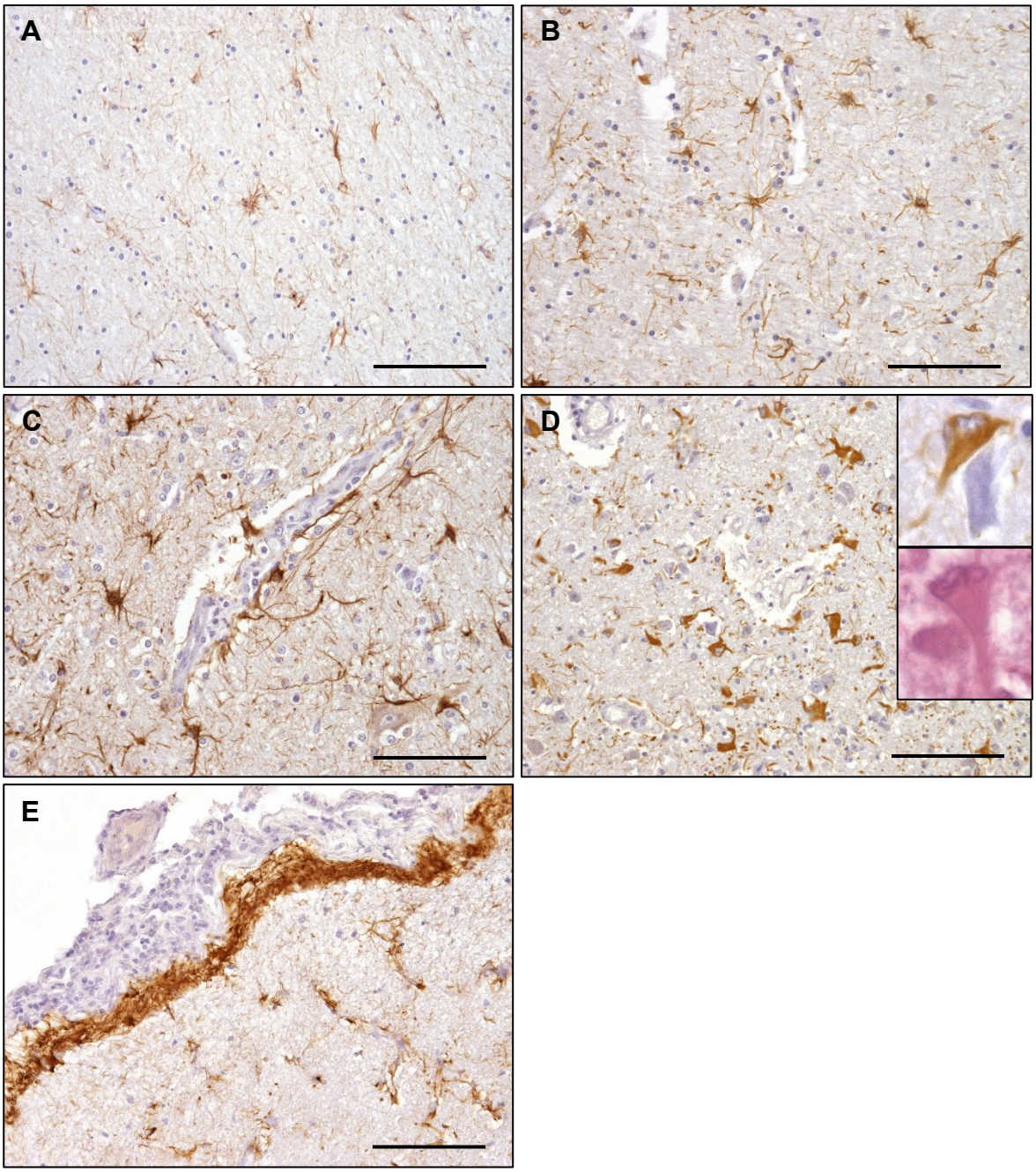
4.4 Immunohistochemistry

4.4.1 Immunohistochemical demonstration of astrocytes

Astrocytes were demonstrated by GFAP immunostaining and alterations were assessed both semi-quantitatively and qualitatively. GFAP positive cells from the four non-altered controls that represented normal astrocytes of horses, displayed light brown cytoplasmic immunostaining, small nucleus, and long, thin processes (**Fig. 37**A). In 9/35 (26%) cases there

was mild astrocytic activation, visible as mild nuclear enlargement and an apparent increase in positive cells (**Fig. 37**B). In 21/35 (60%) cases moderate activation was the highest observed, with variable GFAP staining and general increase in cellular volume, especially in the forebrain, midbrain, and spinal cord (**Fig. 37**C). Severe astrocytic activation observed from 5/35 (14%) cases, included pronounced GFAP immunostaining with hypertrophy of cell bodies and processes, and apparent marked increase of astrocytic cell number (**Fig. 37**D). Gemistocytes were observed in two cases with severe astrogliosis, also already seen in the HE from the forebrain of horse 4 (**Fig. 37**D- inserts). Groups of reactive astrocytes overlapping processes forming compact glial scars were observed in the glia limitans of cases with meningitis (**Fig. 37**E).

Fig. 37: Immunohistochemical demonstration of astrocytic activation by GFAP in the CNS of horses



(A) Control horse 1, forebrain, astrocytes without activation. (B) Horse 13, forebrain, mild astrocytic activation. (C) Horse 31, midbrain, moderate astrocytic activation. (D) Horse 4, severe astrocytic activation. Scale bars: 100µm. Inserts: gemistocytes zoomed in, GFAP-immunostained and HE. (E) Horse 25, forebrain, reactive glia limitans bordering meningitis. Scale bar: 200 µm.

The number of GFAP positive cells in the forebrain (n= 25), midbrain (n= 14), cerebellum (n= 12), and spinal cord (n= 22) available from each case and the mean of astrocytes stained in lesioned and non-lesioned areas is described in **Table 43**.

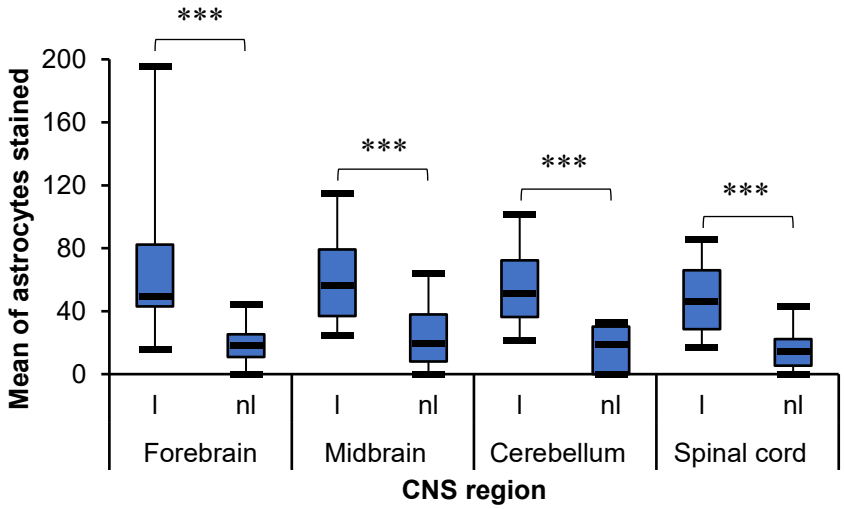
Table 43: Mean of GFAP-positive astrocytes in lesioned and non-lesioned areas in different CNS regions

CNS region	Lesion	n	Mean	St. D	Median	Min	Max
Forebrain	Yes	25	64.9	38.6	49.6	15.6	195
	No	25	18.9	11.8	18.4	0	44.6
Midbrain	Yes	14	59.9	26.9	56.2	24.8	115
	No	14	23.6	19.0	19.6	0	63.7
Cerebellum	Yes	12	54.9	24.9	51.1	21.4	102
	No	12	16.6	13.4	19.0	0	32.8
Spinal cord	Yes	22	48.3	20.0	46.5	16.8	86.0
	No	22	14.7	11.6	14.7	0	42.8

CNS: central nervous system. n: number of fragments available. St. D: standard deviation.

The t-test of the dependent variables showed that there were significant higher number of GFAP positive astrocytes in lesioned areas compared to in non-lesioned areas in the forebrain ($t(24) = 6.3, p < 0.001$), midbrain ($t(13) = 4.22, p = 0.001$), cerebellum ($t(11) = 5.26, p < 0.001$), and spinal cord ($t(21) = 11.01, p \leq 0.001$) (**Fig. 38** and **Appendix 12**).

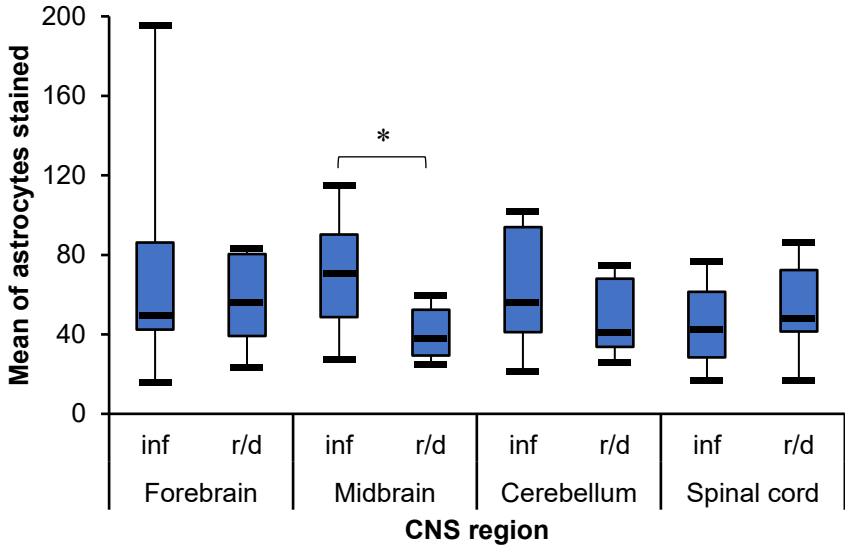
Fig. 38: T-test for dependent variables demonstrates the mean of astrocytes stained in lesioned and non-lesioned areas of different regions from the CNS of horses



l: lesioned. nl: non-lesioned.
***: p≤ 0.001.

The Wilcoxon-Mann-Whitney-test indicated that the mean of astrocytes stained was higher in inflammatory lesions (median= 70.4) than in degenerative lesions (median= 37.8) only from midbrain (U= 38.5, $p < 0.05$) (**Fig. 39** and **Appendix 13**).

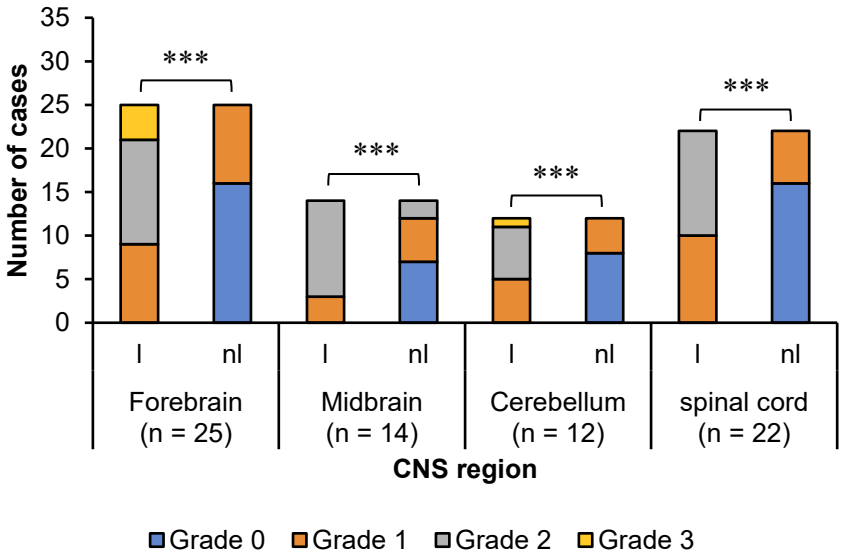
Fig. 39:Wilcoxon-Mann-Whitney-test demonstrates the mean of GFAP positive astrocytes stained according to lesion type in different regions of the CNS of horses



inf: inflammatory. r/d: reactive and/or degenerative lesion.
* $p \leq 0.05$.

Regarding the grade of astrocytic activation, the Wilcoxon Signed-Ranks Test indicated that grades of astrocytic activation (1, 2, or 3) of lesioned areas were statistically significantly higher than non-lesioned areas in all CNS regions ($p \leq 0.001$) (**Fig. 40**).

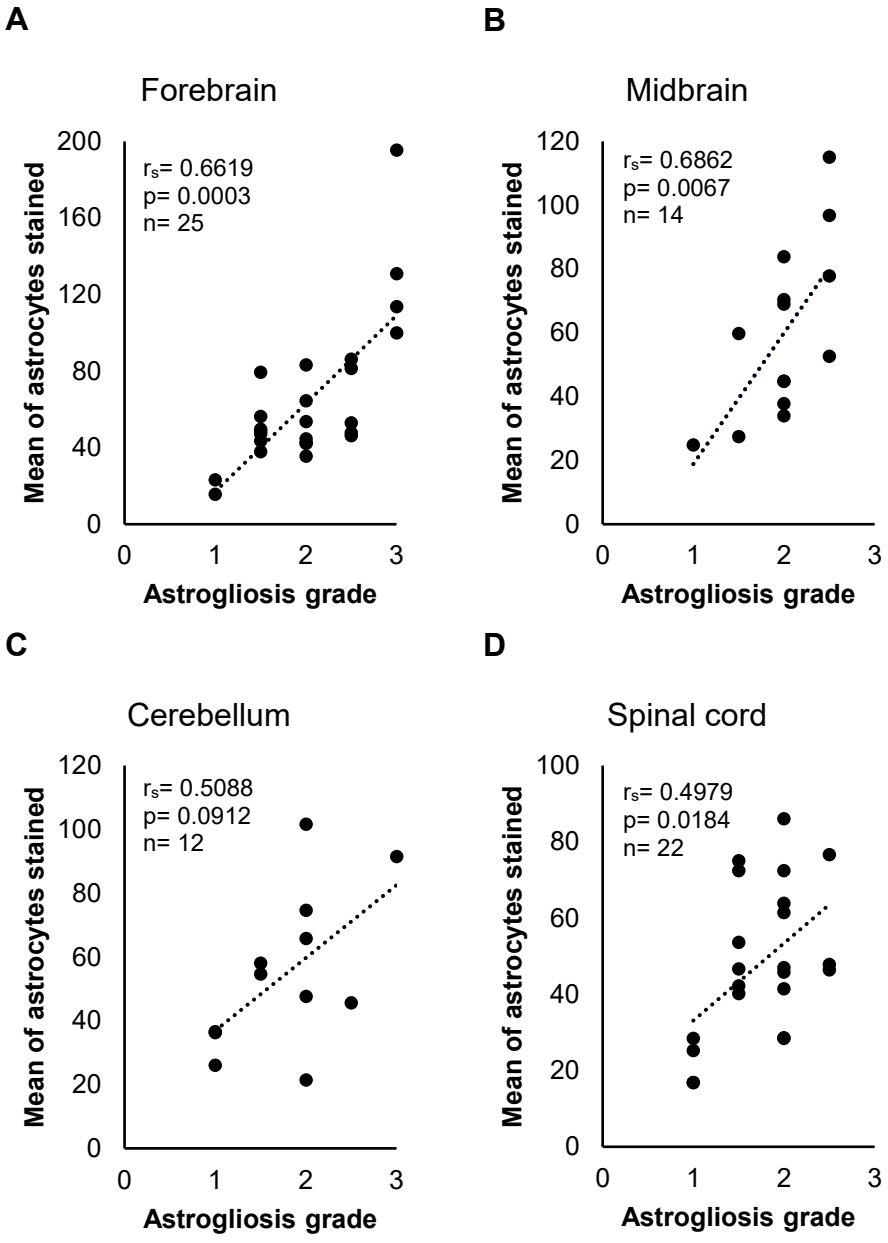
Fig. 40: Wilcoxon Signed-Ranks Test indicates the grade of astroglial activation in lesioned and non-lesioned CNS areas



l: lesioned area. n: number of fragments available. nl: non-lesioned area.
*** $p \leq 0.001$.

Spearman's rank correlation coefficient revealed that there was a positive relationship between the increase in the mean of GFAP positive astrocytes and the increase in grade of astrocytic activation in lesioned brain areas (**Fig. 41**A-D). Stronger correlations were found in forebrain and midbrain areas (**Fig. 41**A, B).

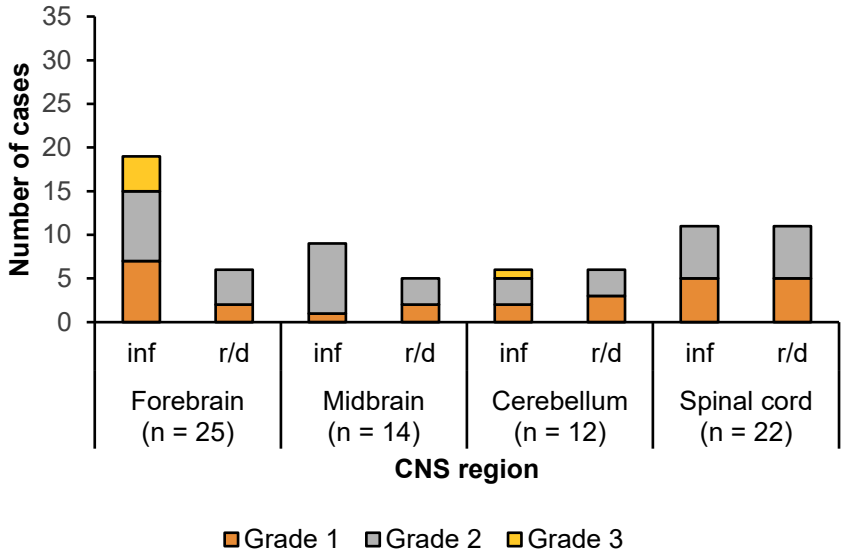
Fig. 41: Spearman's rank correlation coefficient to demonstrate the grade of astrogliosis in different regions of the CNS of horses



n: number of CNS fragments available. r_s: Spearman's correlation coefficient.

Wilcoxon-Mann-Whitney Test with exact inference demonstrated that the grade of astroglial activation was not statistically significantly different between inflammatory and degenerative/reactive lesions ($p \geq 0.05$) (**Fig. 42**).

Fig. 42: Wilcoxon-Mann-Whitney-test with exact inference demonstrates the grade of astroglial activation in inflammatory and reactive/degenerative lesions of different CNS areas



inf: inflammatory. n: number of fragments available. r/d: reactive and/or degenerative lesions.

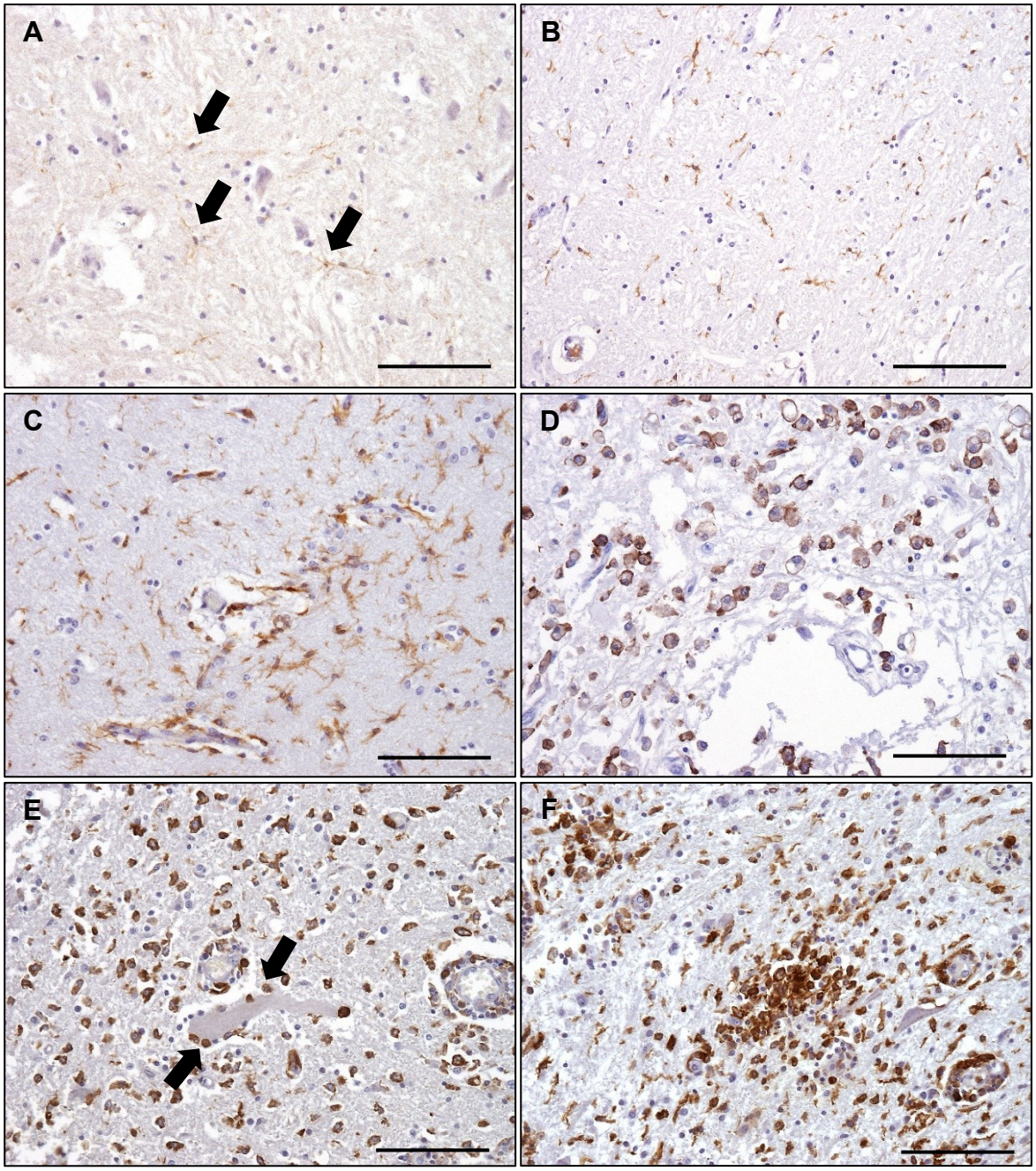
4.4.2 Immunohistochemical demonstration of microglia

Microglial activation was assessed both semi-quantitatively and qualitatively. Resting microglia were demonstrated by Iba1 expression in the four horses used as control and in 6/35 (17%) cases from Brazil, as lightly immunostained cells, with ramified processes, small, oval or elongated nucleus (**Fig. 43**A). Mild activation was the highest grade in 17/35 (48%) cases and appeared as apparent increase in the number of immunostained cells, whose cell body was also mildly increased (**Fig. 43**B). Bushy cells were characterized by an enlarged cells body and thickened; ramified processes were observed in cases with moderate microglial activation. Densely "packed" and intensely immunostained cells were also observed mixed to bushy cells (**Fig. 43**C). These features of moderate microglial activation were observed in 5/35 (14%) cases. Severe microglial activation was recognized in 7/35 (20%) cases (**Fig. 43**D). There was in general, retraction of ramifications and immunostained cells presented not rarely ameboid shape, consistent with Gitter cells morphology (horses 1, 7, 9, 22), although in this stage it is not possible to differentiate microglia cells from macrophages recruited from the periphery. Their bulky cytoplasm stained strongly on the borders, decreasing in intensity when reaching

the cell centre, while the nucleus, when visible, was not immunostained. The previously described large oval structures within malacic areas of the spinal cord from horse 1 remained unstained. Rod cells on the other hand, were observed in this case and in the spinal cord from horse 22, and appeared as elongated cells, with bipolar processes.

Microglia accompanying satellite cells and neuronophagia among the samples of 22/35 (63%) horses were also seen (**Fig. 43**E), as well as the formation of microglial nodules in 7/35 (20%) horses (Horses 1, 9, 24, 26, 27, 29, 32) (**Fig. 43**F). These were usually formed by large and compact groups of strongly stained microglia cells.

Fig. 43: Immunohistochemical demonstration of Iba1 for assessment of microglial activation in the CNS of horses



(A) Control horse 2, spinal cord, immunostained cells with no alterations (arrows). (B) Horse 18, midbrain, mild activation. (C) Horse 22, forebrain, moderate activation and increase in number. (D) Horse 7, forebrain, severe activation consistent with Gitter cells morphology. (E) and (F) Horse 1, spinal cord, Iba1-positive cells around neuron (arrows) and compact group of immunostained cells, compatible with microglial nodule, respectively. Scale bars: 100µm.

The number of Iba1 positive cells in the forebrain (n= 25), midbrain (n= 14), cerebellum (n= 12), and spinal (n= 22) available from each case and the mean of Iba1 positive cells in lesioned and non-lesioned areas are described in **Table 44**.

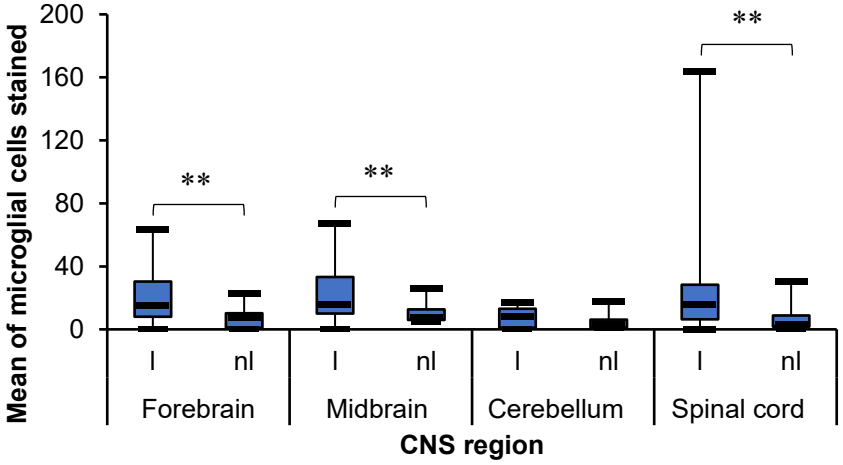
Table 44: Mean of Iba1-positive microglia in lesioned and non-lesioned areas in the different CNS areas of horses

CNS region	Lesion	n	Mean	St. D	Median	Min	Max
Forebrain	Yes	25	21.2	20.4	15	0	63.2
	No	25	7.1	5.7	7.2	0	22.6
Midbrain	Yes	14	24.2	20.5	16	0	67.4
	No	14	10.0	6.4	7.1	3	25.6
Cerebellum	Yes	12	7.9	6.0	8.1	0	17.0
	No	12	4.7	4.9	3.2	0.4	17.6
Spinal cord	Yes	22	27.2	38	15.5	0	163.8
	No	22	6.3	6.9	3.4	0	30.4

CNS: central nervous system. n: number of fragments available. St. D: standard deviation.

The t-test for dependent variables showed that there were significant higher number of Iba1 1 positive microglia in lesioned areas compared to non-lesioned areas of the forebrain ($t(24) = 3.87$, $p = 0.007$), midbrain ($t(13) = 3.31$, $p = 0.0056$) and spinal cord ($t(21) = 3.02$, $p = 0.0065$). There was no significance between the mean of Iba1 positive microglia in the cerebellum ($t(11) = 1.25$, $p = 0.2365$) (**Fig. 44** and **Appendix 14**).

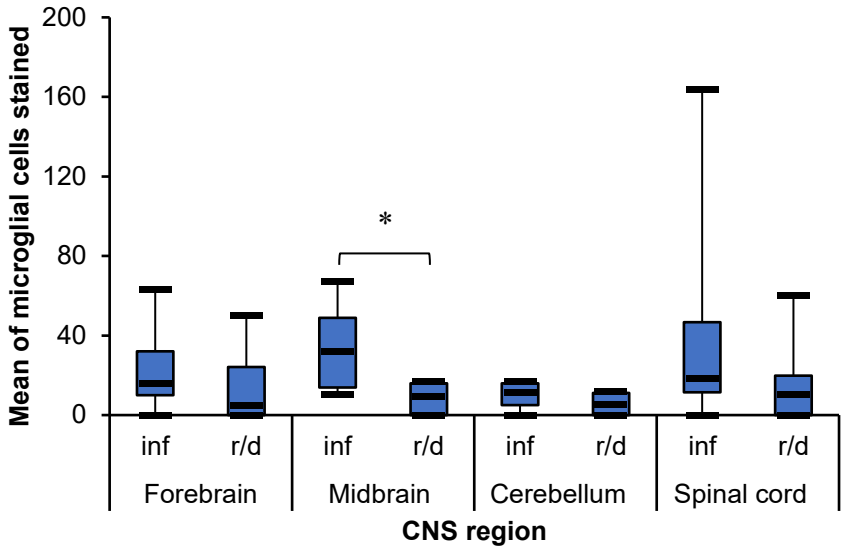
Fig. 44: T-test for dependent variables demonstrates the mean of microglia stained in lesioned and non-lesioned areas of different regions from the CNS of horses



l: lesioned. nl: non-lesioned.
** p≤0.01

The Wilcoxon-Mann-Whitney-test indicated that the mean of microglial cells stained was higher in inflammatory lesions (median = 32) than in degenerative lesions (median = 9.4) only from midbrain ($U = 39.5$, $p = 0.0230$) (**Fig. 45** and **Appendix 15**).

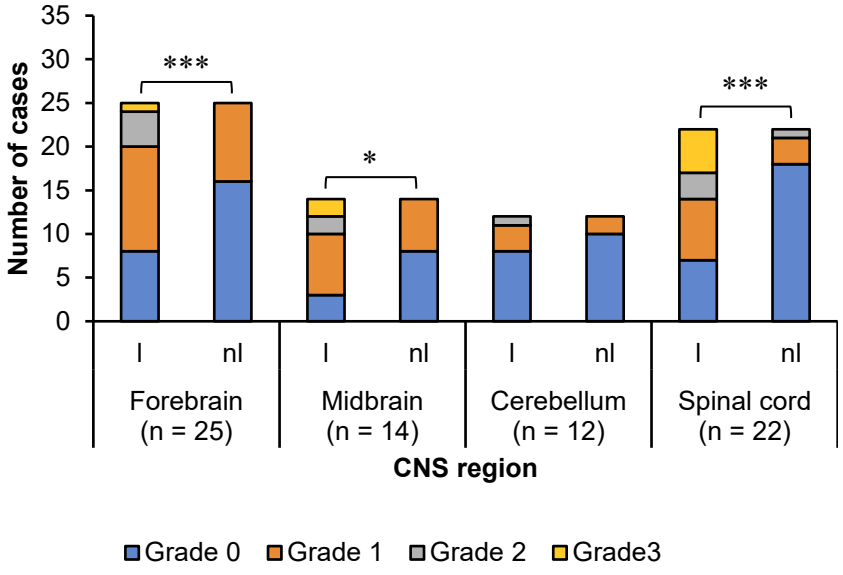
Fig. 45: Wilcoxon-Mann-Whitney-test demonstrates the mean of Iba1 positive microglia stained according to the lesion type in different regions of the CNS of horses



inf: inflammatory. r/d: reactive and/or degenerative lesion.
* $p \leq 0.05$.

Regarding the grade of microglia activation, the Wilcoxon Signed-Ranks Test indicated that grades of microglia activation (1, 2, or 3) of lesioned areas were statistically significantly higher than non-lesioned areas in forebrain ($p < 0.001$), midbrain ($p = 0.0020$), and spinal cord ($p < 0.001$), but not in the cerebellum ($p = 0.5313$) (**Fig. 46**).

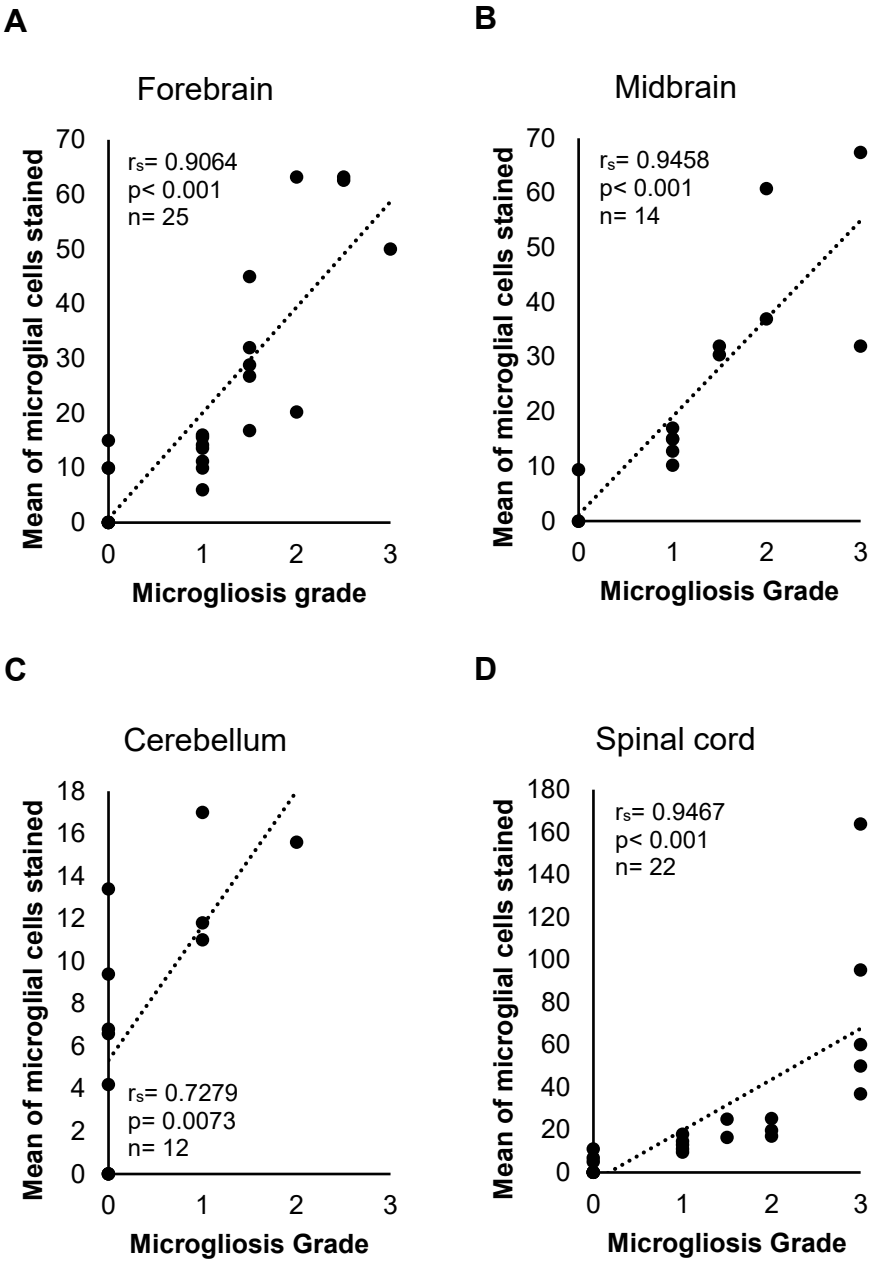
Fig. 46: Wilcoxon Signed-Ranks Test indicates the grade of microglial activation in lesioned and non-lesioned CNS areas



l: lesioned. n: number of CNS fragments available. nl: non-lesioned area.
* $p \leq 0.05$. *** $p < 0.001$.

Spearman's rank correlation coefficient revealed that there was a positive relationship between the increase in the mean of Iba1 positive cells and the increase in grade of microglial activation in the lesioned areas of the CNS (**Fig. 47**).

Fig. 47: Spearman's rank correlation coefficient to demonstrate the grade of microgliosis in different regions of the CNS of horses

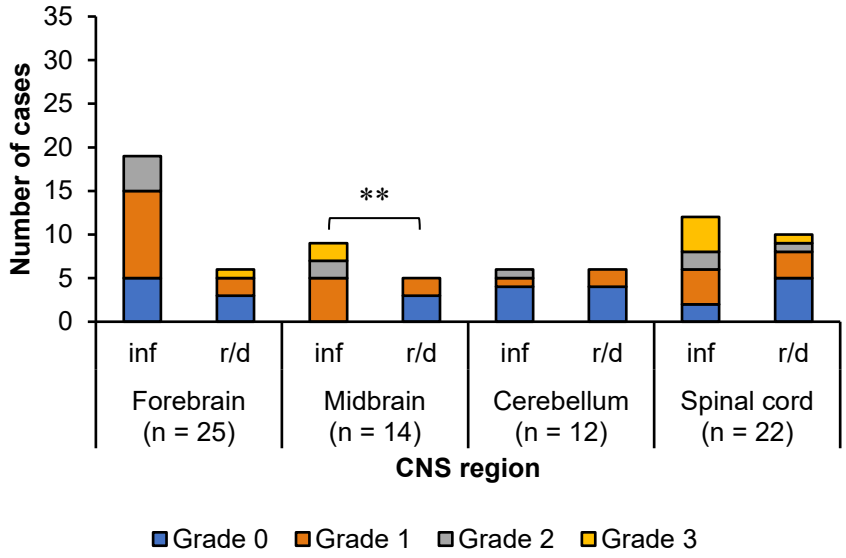


n: number of CNS fragments available. r_s: Spearman's correlation coefficient.

Wilcoxon-Mann-Whitney Test with exact inference demonstrated that the grade of microglial activation was statistically significantly higher only in inflammatory lesions from midbrain fragments compared to degenerative/reactive lesions (p = 0.007). In spinal cord

fragments, the inflammatory lesions also showed higher activation grades, but this difference did not reach statistical significance (**Fig. 48**).

Fig. 48: Wilcoxon-Mann-Whitney-test with exact inference demonstrates the grade of microglial activation in inflammatory and reactive/degenerative lesions of different CNS areas



inf: inflammatory. n: number of fragments available. r/d: reactive and/or degenerative lesion.
** $p \leq 0.05$.

4.4.3 Immunohistochemical demonstration of infectious agents

In 7/35 horses (20%) viral or protozoal antigens were detected by IHC (**Table 45**). Positive controls adopted for each antigen demonstrated positive immunostaining while all negative controls did not display any signal.

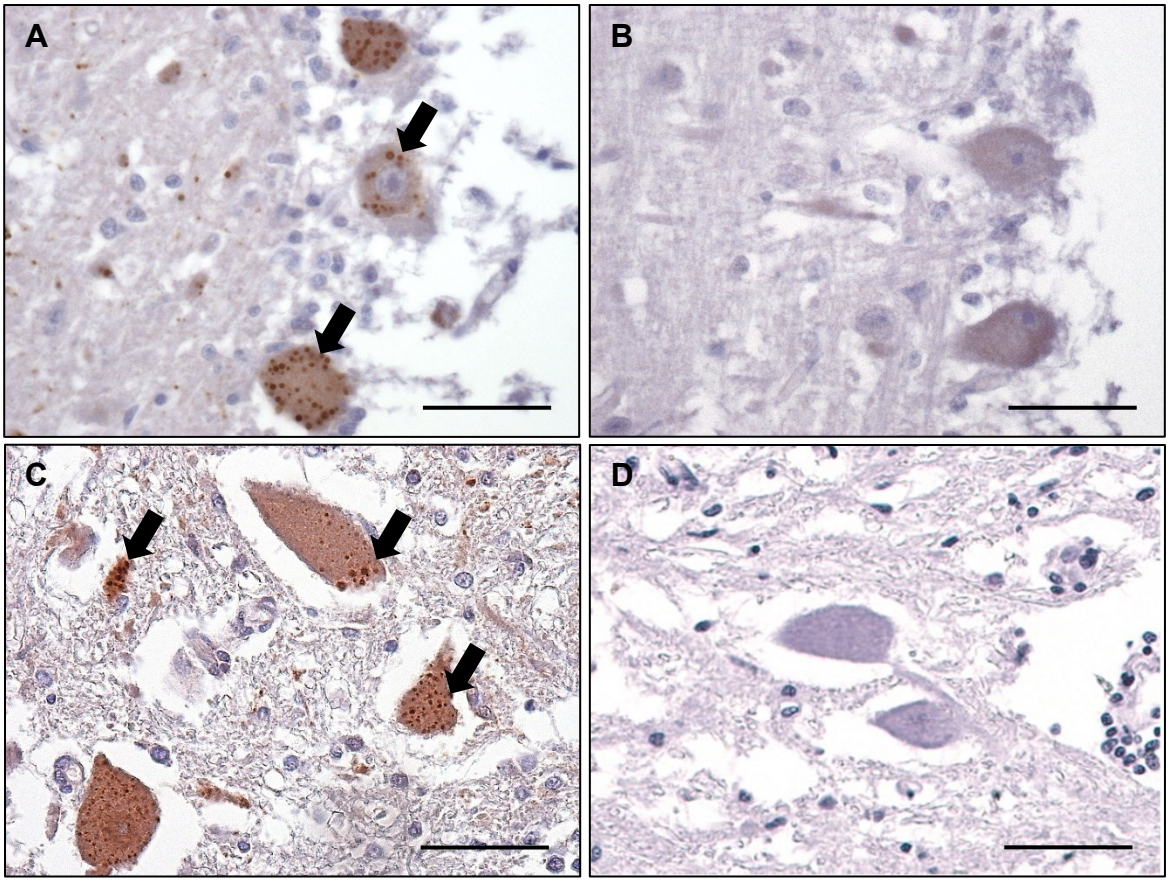
Table 45: Immunohistochemical demonstration of infectious agents in the CNS of the 35 horses

Antigen	Positive cases/ samples tested	Horse ID	Antigen distribution
Rabies virus Antigen not stated	1/35	2	++
Borna disease virus Nucleoprotein (Bo-18)	0/35	*	*
Borna disease virus Phosphoprotein p24	0/35	*	*
Equine herpesvirus-1 Antigen not stated	0/35	*	*
Double-stranded RNA Large gene from unspecified agent	3/35	1, 3, 17	+, +, +
Alphavirus group Whole virus lysate	2/35	26, 31	+, +
Flavivirus group Envelope protein	0/35	*	*
Listeria monocytogenes Antigen not stated	0/35	*	*
Neospora caninum Antigen not stated	0/35	*	*
Toxoplasma gondii Antigen not stated	0/35	*	*
Sarcocystis neurona Cultured merozoites	5/35	1, 2, 9, 19, 20	+, +, +, +, +

* no positive staining. + weak/focal staining. ++ moderate/2 to 5 foci with positive signal.

For rabies virus (**RABV) antigen**, the positive control displayed cytoplasmic positive staining of Purkinje cells, characterized by multiple large-rounded aggregates (**Fig. 49**A) consistent with Negri bodies. Similar intracytoplasmic staining within neurons of the spinal cord was found in **horse 2** appearing as multiple large-rounded strongly stained aggregates (**Fig. 49**C).

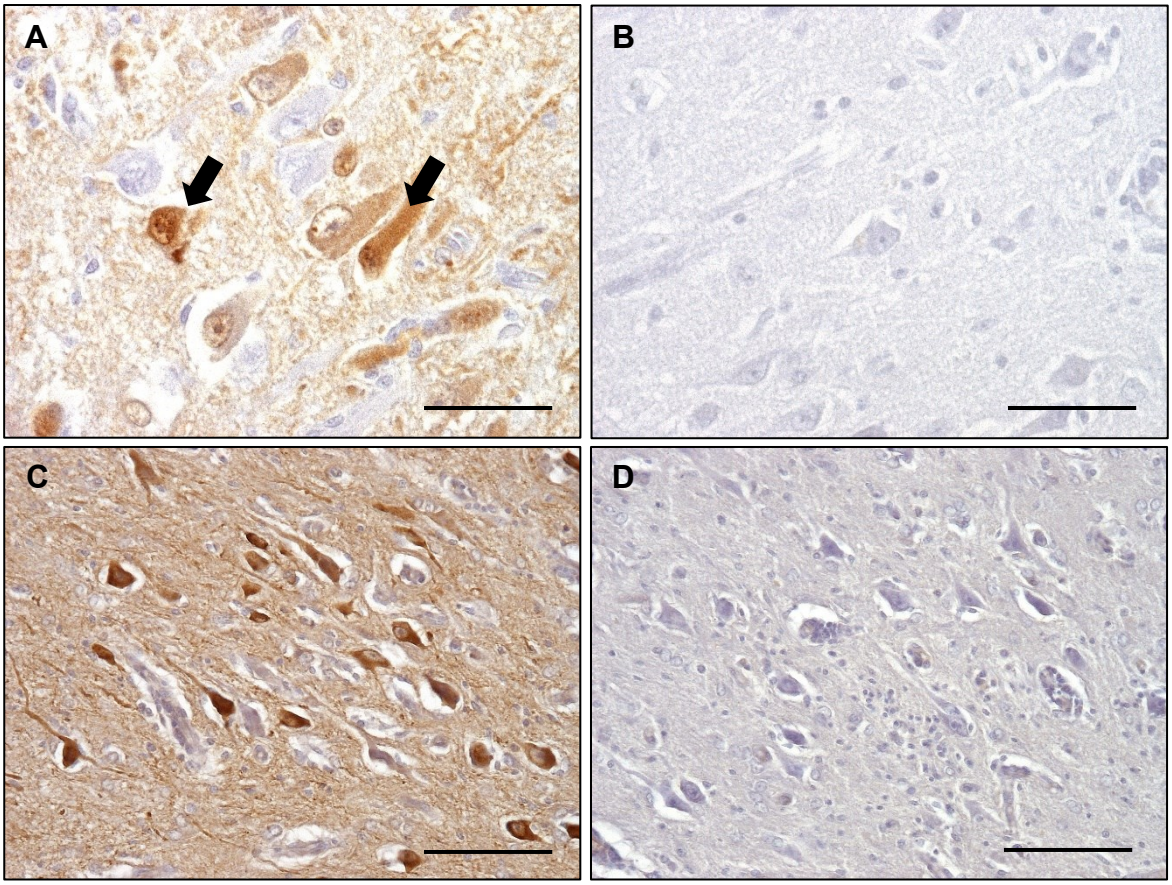
Fig. 49: Immunohistochemical demonstration of rabies virus antigen



(A) Mouse, cerebellum demonstrating cytoplasmic viral antigen aggregates in Purkinje cells (arrows). (B) Mouse, cerebellum, rabbit-control serum, no staining. (C) Horse 2, spinal cord, viral intracytoplasmic aggregates (arrows). (D) Horse 2, spinal cord, rabbit-control serum, no staining. Scale bars: 50 µm.

Regarding the immunostaining obtained for **bornaviruses**, although none of the samples from Brazil were positive, the controls displayed the expected immunostaining well. Demonstration of the viral nucleoprotein by the monoclonal antibody Bo18 (**Fig. 50**A) and the viral phosphoprotein by the polyclonal p24 (**Fig. 50**C) was possible in the nucleus and cytoplasm of neurons in the hippocampus of a naturally BoDV-1- infected horse.

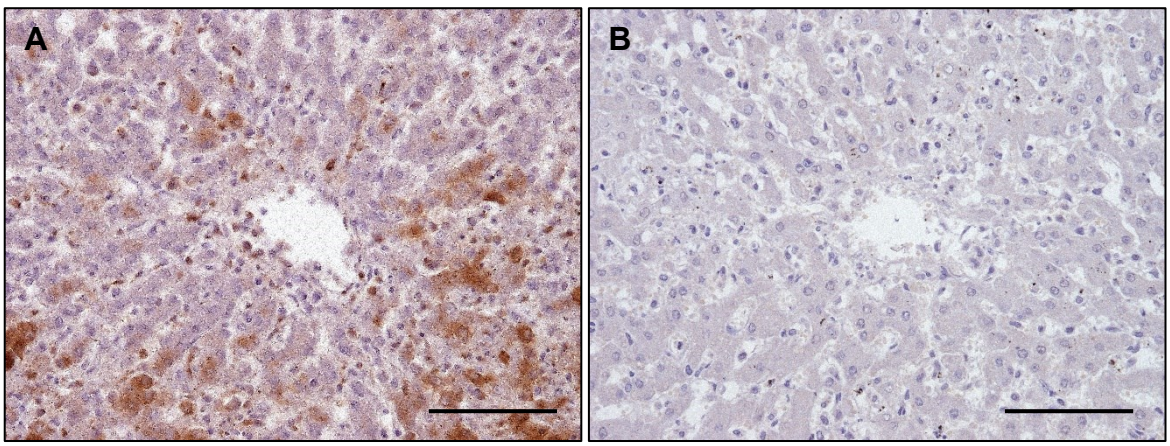
Fig. 50:Immunohistochemical demonstration of Borna disease virus antigens



(A) Horse, hippocampus, BoDV-1 nucleoprotein 18. Viral antigen was present in the nucleus and cytoplasm of neurons (arrows). (B) Horse, hippocampus, rabbit-control serum, no staining. Scale bars: 50 µm. (C) Horse, hippocampus, BoDV phosphoprotein 24. Viral antigen was present in the nucleus and cytoplasm of neurons (arrows). (D) Horse, hippocampus, rabbit-control serum, no staining. Scale bars: 100µm.

Detection of **equine herpesvirus type 1** (EHV-1) was not possible in the 35 horses. The positive control displayed the expected immunostaining correctly as predominantly cytoplasmic, sometimes highlighting degenerated cells (**Fig. 51**).

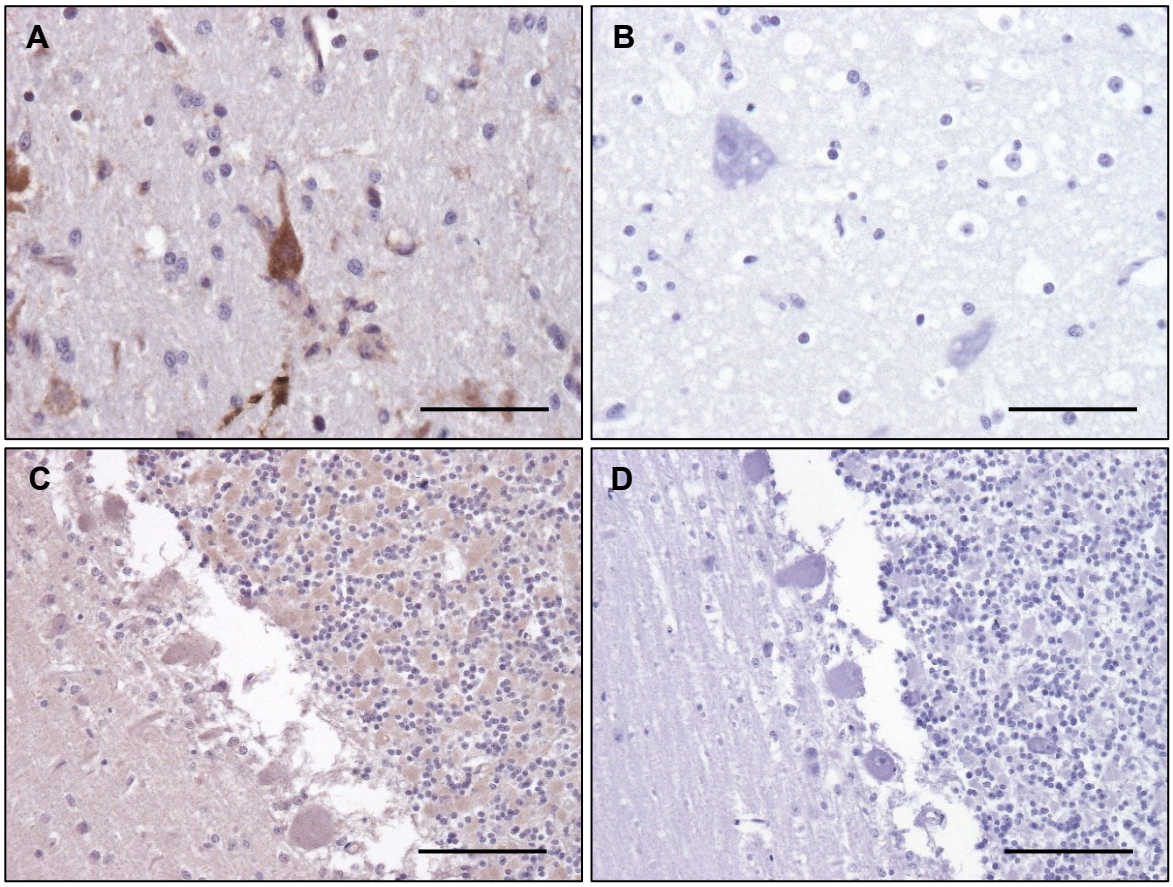
Fig. 51: Immunohistochemical demonstration of equine herpesvirus type 1 antigen



(A) Horse, liver. Cytoplasmic positive staining within hepatocytes. (B) Horse, liver, rabbit control-serum, no staining. Scale bars: 100µm.

Demonstration of **dsRNA** in controls infected by RNA viruses was only observed in the brain tissue of a pelican infected by the positive single-stranded RNA virus WNV, showing positive staining in the cytoplasm and processes of neurons (**Fig. 52**A). There was no staining in a rabies virus control, as a negative sense single-stranded RNA virus (**Fig. 52**C).

Fig. 52: Immunohistochemical demonstration of double-stranded RNA antigen in RNA virus infections

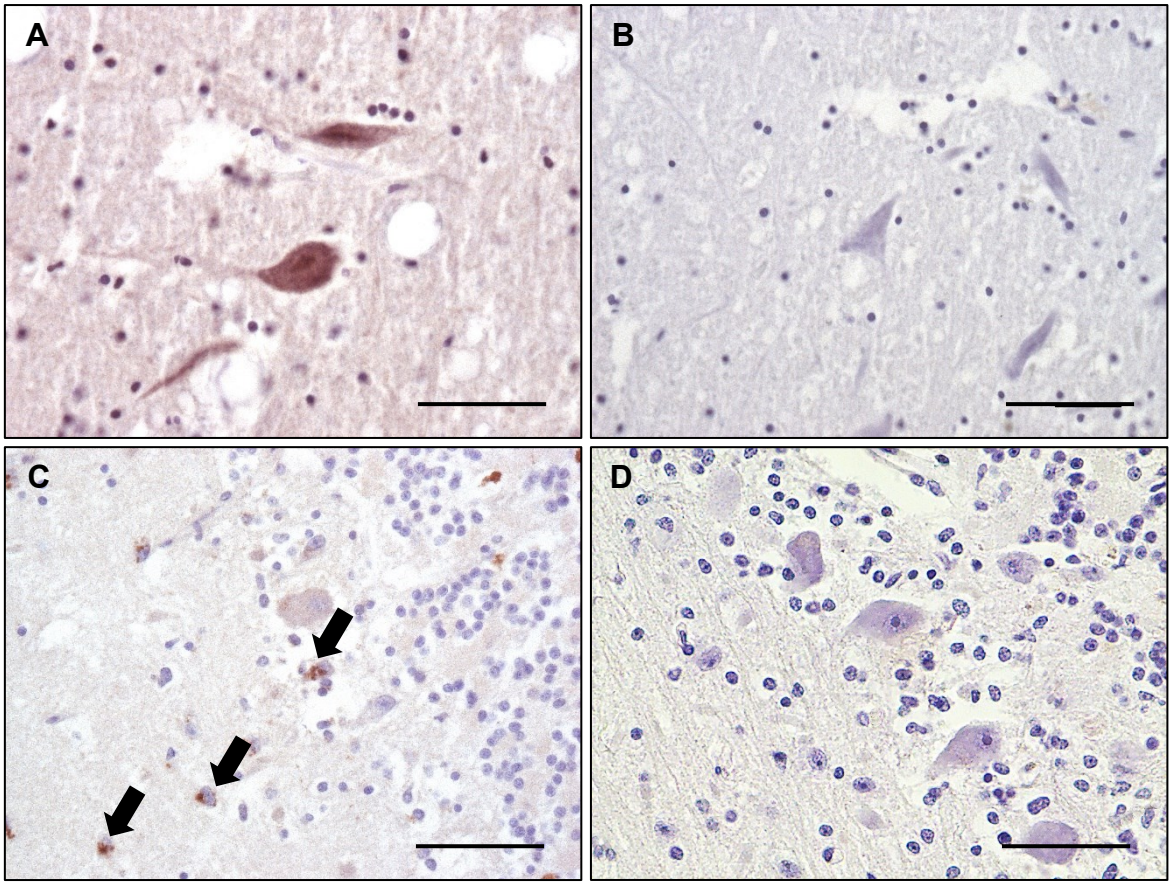


(A) Pelican, brain, West Nile virus-infected. Positive cytoplasmic immunostaining within neurons. (B). Chicken T-lymphocytes control, no immunostaining. Scale bars: 50 µm. (C) Mouse, brain, rabies virus infected. No specific immunostaining. (D) Mouse, brain, rabies virus infected. Incubated with chicken T-lymphocytes antibody, no immunostaining. Scale bars: 100µm.

Double-stranded RNA antigen was demonstrated in dsDNA virus infection as shown for swine herpervirus-1 infected brain tissue, as cytoplasmic fine-granular staining within neurons (**Fig. 53**A), and for EHV-1 infected cerebellum there was cytoplasmic coarse granular staining of glial cells (**Fig. 53**C).

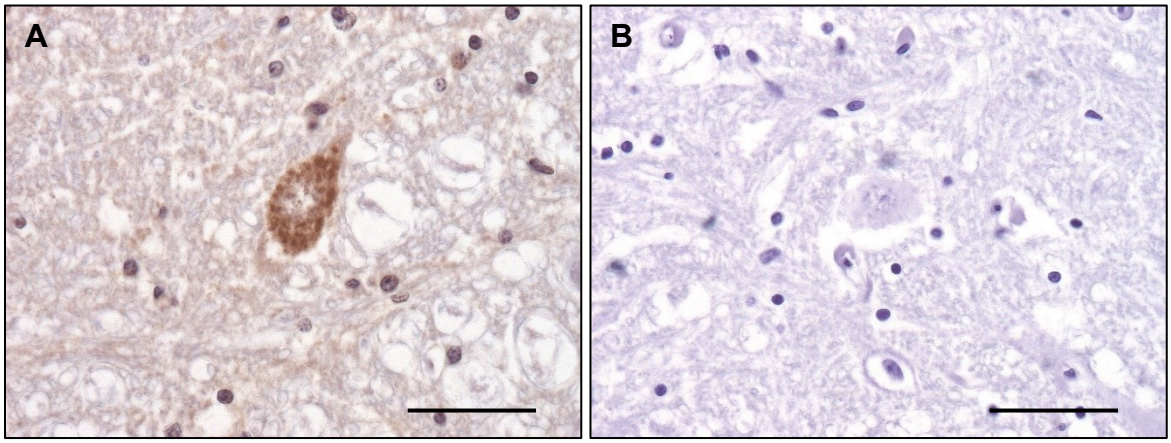
Horses 1, 3, and 17 (3/35, **8%**) showed a positive immunostaining for dsRNA. The immunostaining was confined to the neurons, characterized by multiple strongly stained aggregates within the cytoplasm (**Fig. 54**).

Fig. 53: Immunohistochemical demonstration of double-stranded RNA antigen in DNA virus infections



(A) Swine, brain, swine herpesvirus-1. Positive cytoplasmic staining within neurons. (B) Swine, brain, swine herpesvirus-1. Chicken T-lymphocytes antibody, no immunostaining. Scale bars: 50 µm. (C) Horse, brain, EHV-1. Positive cytoplasmic coarse granular immunostaining of glial cells (arrows). (D) Horse, brain, EHV-1. Chicken T-lymphocytes antibody, no immunostaining. Scale bars: 100 µm.

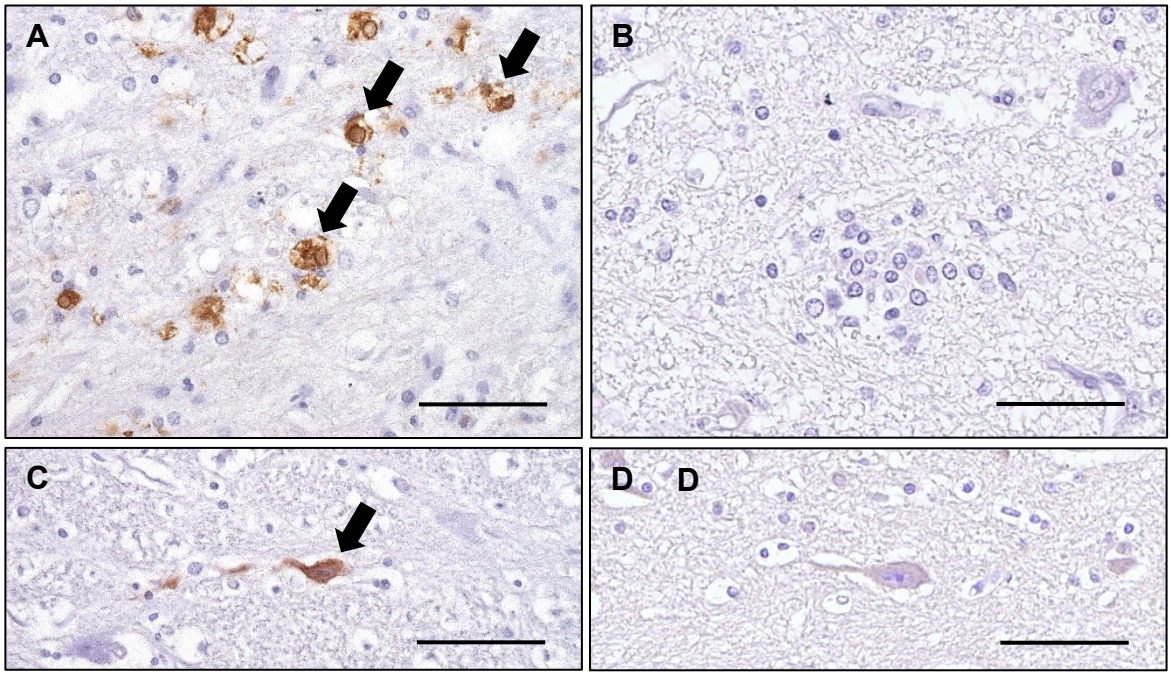
Fig. 54: Immunohistochemical demonstration of double-stranded RNA antigen in the CNS of horse 17



(A) Positive staining within neuronal cytoplasm. (B) Brain tissue incubated with chicken T-lymphocytes antibody, no immunostaining. Scale bars: 50 µm.

Immunohistochemical detection of **alphavirus proteins** was observed in the control horse 31 as immunostaining of astrocytes in the mesencephalon, and in the midbrain of horse 26 as moderate immunostaining within the cytoplasm of a neuron (**Fig. 55**).

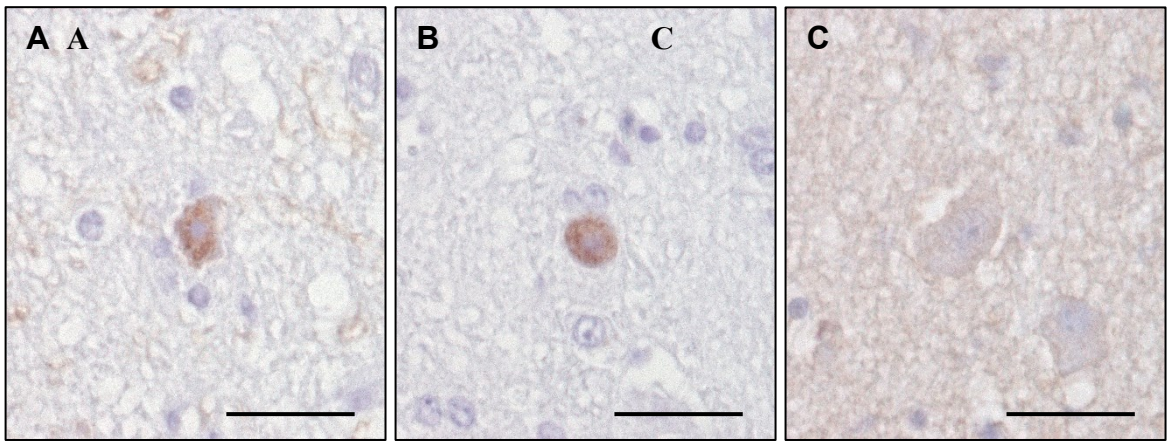
Fig. 55: Immunohistochemical demonstration of alphavirus lysate antigen



(A) Horse 31, midbrain displaying staining of glial cells (arrows). (B) Horse 31, midbrain, incubated with chicken T-lymphocytes antibody, no immunostaining. Scale bars: 50 µm. (C) Horse 26, midbrain, moderate staining within neuronal soma. (D) Horse 26, midbrain, incubated with chicken T-lymphocytes antibody, no immunostaining. Scale bars: 50 µm.

Flavivirus antigen detection although not observed in the samples from Brazil was characterized by few positively stained neurons as roughly granular cytoplasmic immunostaining (**Fig. 56**) in the control consisting of the CNS of a WNV-infected pelican.

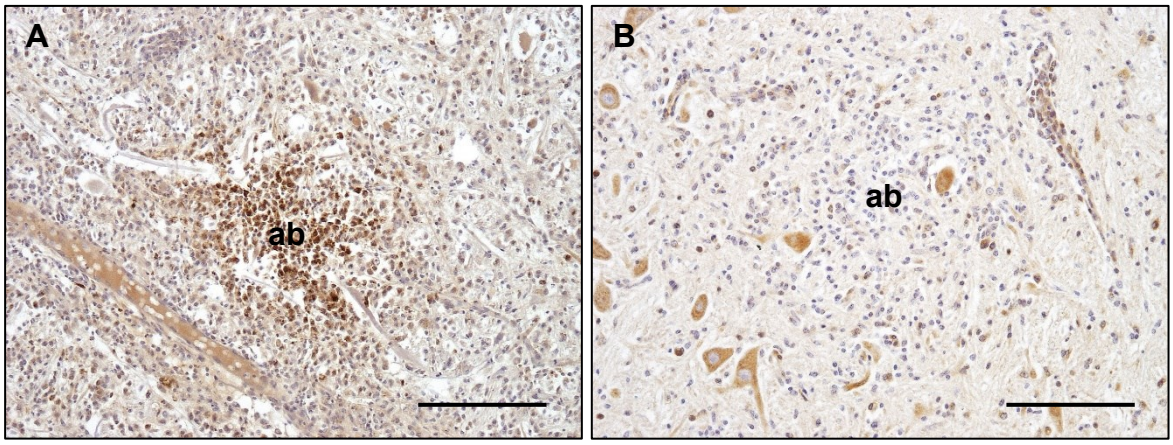
Fig. 56: Immunohistochemical demonstration of flavivirus envelope antigen



(A) and (B) pelican, brain, WNV-infected. Light-granular staining within neurons. (C) Pelican, brain, WNV-infected. Incubated with chicken T-lymphocytes antibody, no immunostaining. Scale bars: 50 µm.

Immunohistochemical demonstration of ***Listeria* sp.** in the positive control was found within inflammatory cells in the microabscesses (**Fig. 57**) of the control sheep, but not in the samples from Brazil.

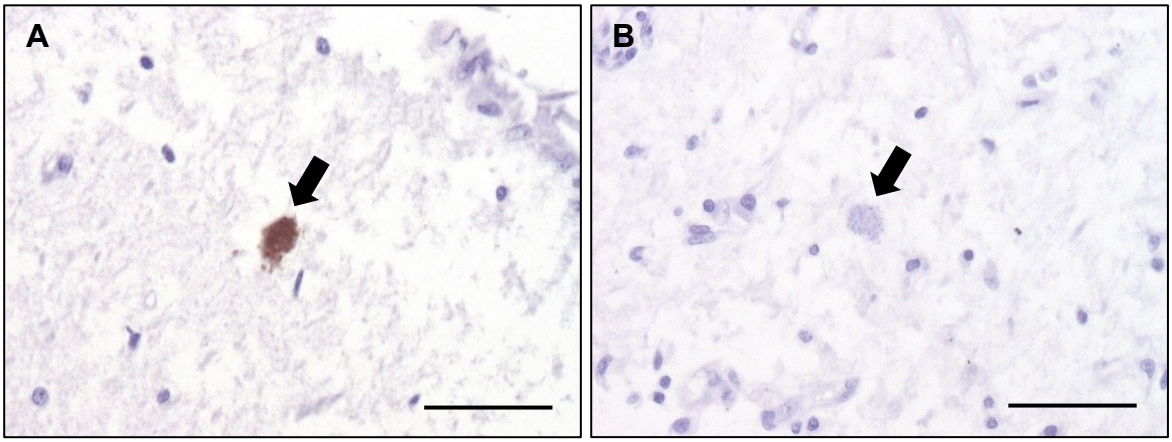
Fig. 57: Immunohistochemical demonstration of *Listeria* sp. antigen



(A) Sheep, obex, *Listeria* sp. infection. Inflammatory cells within the microabcsess (ab) are strongly stained. (B) Sheep, obex, *Listeria* sp. infection, rabbit-control serum. Lack of specific immunostaining. Scale bars: 200µm.

Immunohistochemical demonstration for antigens of the three **Apicomplexa protozoa** tested revealed scattered parasitic structures in tissues of the controls. *N. caninum* antigen was detected as round to oval structures with rough granular immunostaining (**Fig. 58**) in the cerebellum of the dog control. Samples from Brazil were negative.

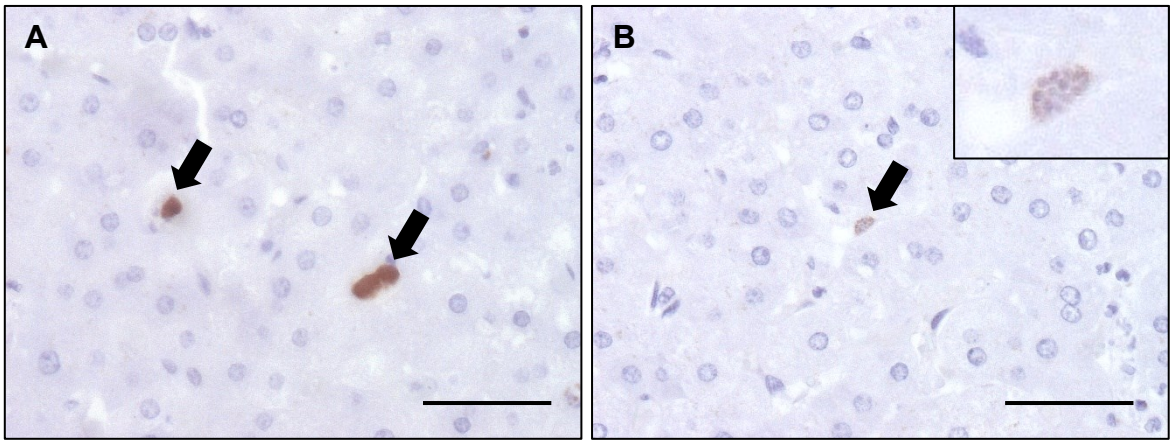
Fig. 58: Immunohistochemical demonstration of *Neospora caninum* antigen



(A) Dog, cerebellum, *Neospora caninum* infection. Staining of parasitic structure (arrow). (B) Dog, cerebellum, chicken T-lymphocytes antibody. No immunostaining (arrow). Scale bars: 50 µm.

Similarly, by immunohistochemical demonstration of ***T. gondii*** **antigen** appeared in the parasitic structures containing tachyzoites in the positive control consisting of a kangaroo's liver. (**Fig. 59**). All samples from Brazil remained unstained.

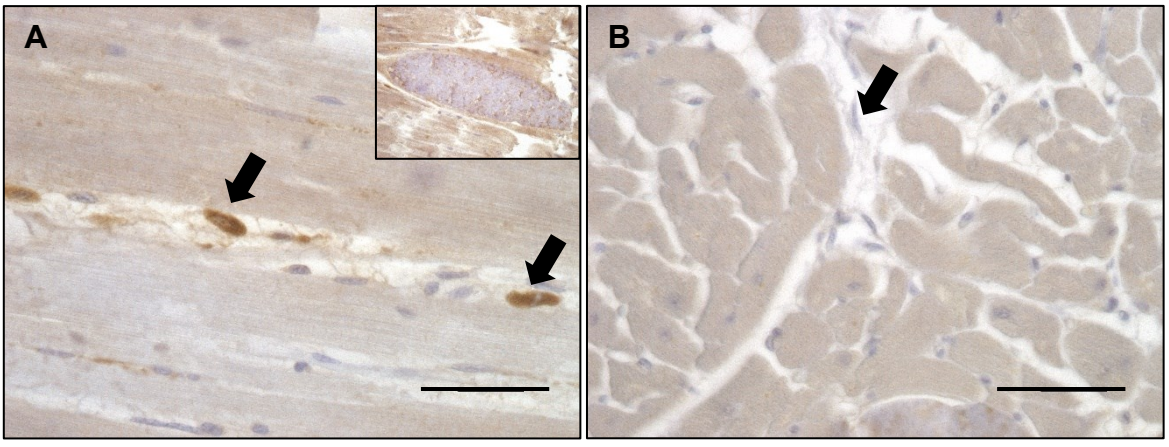
Fig. 59: Immunohistochemical demonstration of *Toxoplasma gondii* antigen



(A) Kangaroo, liver, *Toxoplasma gondii* antibody. Staining of scattered parasitic structures containing tachizoites (arrows). (B) Kangaroo, liver, rabbit-control serum. No immunostaining (arrow and parasite highlight). Scale bars: 50 µm.

Regarding ***S. neurona*** immunostaining in the bovine control muscle, schizonts appeared with rough granular immunostaining and measured approximately 16 x 6 µm (**Fig. 60**A). The sarcocyst, on the other hand, was demonstrated as irregularly stained in between the merozoites and measured 60 x 150 µm (**Fig. 60**A, insert).

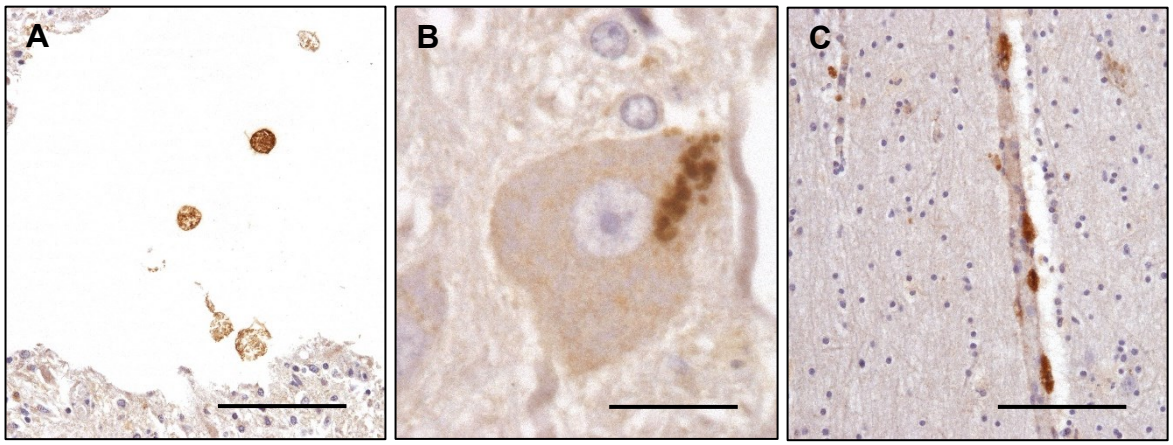
Fig. 60: Immunohistochemical demonstration of *Sarcocystis neurona* antigen



(A) Bovine, muscle, *S. neurona* infection. Schizonts are strongly stained (arrows), but the sarcocyst (insert) was irregularly immunostained. (B) Bovine, muscle, *S. neurona* infection, incubation with rabbit-control serum. No immunostaining. Scale bars: 50nµm.

In **5/7 horses** (57%) that presented lesions and history suggestive of parasitic infection there was a distinct immunostaining for *S. neurona*. In the lumbar spinal cord of horses 1 and 2, there was staining of big (18 x 18 µm), round structures located within malacic areas, resembling degenerated macrophages (**Fig. 61**A), and rarely, rough-granular staining within glial cells (not shown). Within the soma of a few neurons of horse 20 medulla oblongata, there was strong immunostaining of granular to round structures forming clusters, sometimes arranged in a circle resembling merozoites (**Fig. 61**B). In the medulla oblongata of horse 20, in the lumbar spinal cord of horse 9, and in the cerebral cortex of horse 19, there were foamy-like staining of oval to elongate perivascular structures resembling schizonts, measuring approximately 16 x 8 µm (**Fig. 61**C).

Fig. 61: Immunohistochemical demonstration of *S. neurona* antigen in the CNS of horses



(A) Horse 1, spinal cord. Staining of structures resembling degenerate macrophages within malacic area. Scale bar: 200µm. (B) Horse 20, medulla oblongata. Numerous granular-stained structures within neuronal soma. Scale bar: 25 µm. (C) Horse 19, cerebral cortex. Strong foamy staining of oval to elongate perivascular parasitic structures. Scale bar: 100 µm.

4.5 Molecular detection of equine housekeeping gene and pathogens

4.5.1 Optimization of RNA isolation from FFPE material

RNA measurements from the 18 test samples firstly tested to optimize an RNA isolation method for FFPE material was depicted in detail in Boos et al. (2019). There was increase in the RNA yield in protocols using longer periods of proteinase K incubation (protocol 2 and 3) when compared to protocol 1. These samples also showed higher purity rates of isolated material concerning proteins (260/280 nm) and inorganic contaminants (260/230 nm) (**Table 46**).

Table 46: RNA yields obtained with 3 different isolation protocols (n=18)

	Protocol 1			Protocol 2			Protocol 3		
	RNA ng/µl	260/ 280	260/ 230	RNA ng/µl	260/ 280	260/ 230	RNA ng/µl	260/ 280	260/ 230
n	18	18	18	18	18	18	18	18	18
Mean	12.7	1.6	1.2	63.1	1.7	1.1	268.1	1.8	1.8
SEM	2.8	0.04	0.08	26.7	0.04	0.1	54.1	0.02	0.03
St. D	10.9	0.2	0.3	113.5	0.2	0.5	229.5	0.08	0.1
Range	44.9	0.7	1.5	455.5	0.6	1.6	761.4	0.3	0.6
Min	1.3	1.2	0.2	4.4	1.3	0.4	24.6	1.6	1.4
Max	46.2	1.9	1.7	459.9	1.9	1.9	786	1.9	2.0

Max: maximum. Min: minimum. SEM: standard error of mean. St. D: standard deviation. 260/280: protein absorbance ratio. 260/230: inorganic contaminants absorbance ratio.

The extraction parameter for 260/280 nm at protocols 1 and 3 had moderate correlation ($r(16) = 0.551, p = 0.018$), but not between the other protocols. Analysis of variance (ANOVA) with repeated measures indicated that there were significant differences in parameters obtained with protocol 3 compared to protocols 1 and 2. In overall, protocol 3 had the best RNA yield ($F(2) = 41.12, p < 0.001$), protein absorbance ratio ($F(2) = 11.18, p = 0.0002$), and inorganic contaminants absorbance ratio ($F(2) = 19.85, p < 0.001$). For details in the performance of protocol 3 see Boos et al. (2019).

4.5.1.1 RNA isolation from FFPE material of control and equine samples

After optimization of the RNA extraction, protocol 3 was chosen due to its increased performance. The RNA yields from the reference FFPE materials are listed in **Table 47**.

Table 47: RNA yields obtained from the CNS from FFPE controls

Control identification	RNA (ng/µl)	260/280	260/230
Horse RNA1[a]	267.6	2.03	1.97
Horse RNA2[b]	314.5	2.05	1.9
Cat[c]	134.9	1.86	1.95
Sheep[d]	225.7	1.98	2.08
Squirrel[e]	56.4	1.95	1.88
Rabies virus	21.2	1.48	0.74
Borna disease virus 1	17.1	1.84	1.31
Alphavirus (Horse 31)	162.3	1.81	1.88
Flavivirus (WNV)	142.5	1.99	2.03
Paramyxovirus (CDV)	50	1.72	1.58

260/280: RNA purity concerning proteins. 260/230: RNA purity regarding inorganic contaminants. CDV: canine distemper virus. WNV: West Nile virus. [a] and b: positive controls for GAPDH PCR. [c] : negative control for equine GAPDH 64bp. [d] : negative control for equine GAPDH 103bp. [e] : negative control for equine GAPDH 170bp – 517bp.

For all 35 horses CNS samples comprising this study, RNA isolation was carried out in duplicate to obtain a final aliquot of 48µl each (**Table 48** and **Appendix 16**).

Table 48: RNA yields obtained from the CNS from FFPE samples of 35 horses

Variable	Mean	St. D	SEM	Median	Min	Max
RNA ng/µl	245.44	186.54	31.53	181.7	19	786
260/280	1.83	0.082	0.014	1.815	1.65	1.95
260/230	1.76	0.178	0.030	1.81	1.27	2

Max: maximum value. Mean: mean of yield values obtained after extraction in duplicate (N=70). Min: minimum value. SEM: standard error of mean. St. D: standard deviation.

4.5.2 Genomic DNA isolation from FFPE material of control and equine samples

The gDNA isolation from reference FFPE materials is listed in 3.7.3 (Materials and Methods). Values obtained with the DNA extraction of FFPE material from the 35 CNS equine samples are shown in **Table 49** (**Appendix 16** for detail).

Table 49: Genomic DNA yields obtained from the CNS from FFPE samples of 35 horses

Variable	Mean	St. D	SEM	Median	Min	Max
DNA ng/µl	60.129	10.247	7.744	51.2	9.4	201.30
260/280	1.6791	0.1107	0.0187	1.7	1.42	1.85
260/230	1.290	0.410	0.069	1.36	0.370	2.10

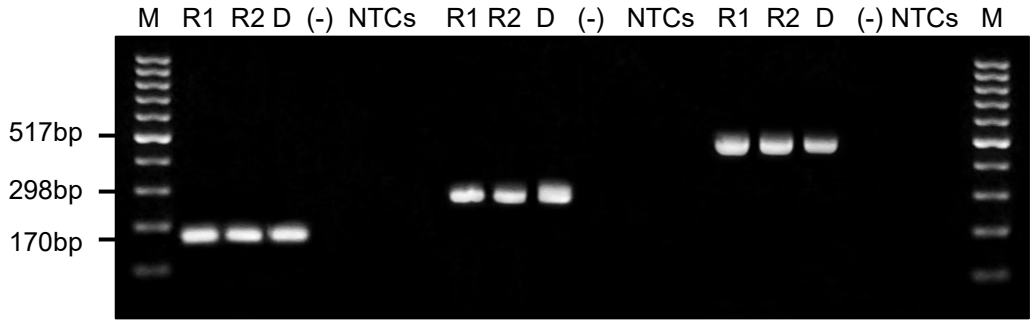
Max: maximum value. Min: minimum value. SEM: standard error of mean. St. D: standard deviation.

4.5.3 Optimization of PCR amplification for FFPE material

4.5.3.1 Amplification of equine GAPDH mRNA

Equine GAPDH mRNA amplification was carried out according to Boos et at. (2019). In summary, when the 18 FFPE brain samples were tested for the 3 extraction protocols (3.7.1), the obtained cDNA from RNA extraction protocols 1 and 2 could not produce visible bands superior to 64bp. Using protocol 3, the quality of the obtained RNA/cDNA was sufficient to obtain amplicons ranging from 46bp to 298bp. Thus, protocol 3 was further used for all 35 equine samples of this study. Using positive controls from horse FFPE material products lengthening 170bp, 298bp, and 517bp could be amplified (**Fig. 62**).

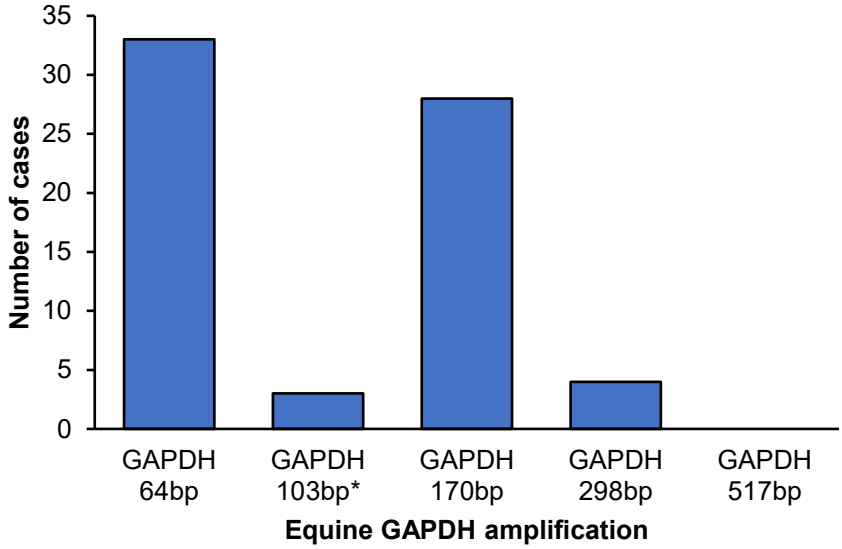
Fig. 62: Equine glyceraldehyde-3-phosphate dehydrogenase mRNA amplification of 170bp, 298bp and 517bp in 2% agarose gel



(M) marker GR100. (R1) Horse control 1. (R2) Horse control 2. (D) Horse DNA control. (-) negative control. (NTCs): 2x no-template control.

Equine GAPDH amplification using the 35 Brazilian samples was possible up to 298 bp (**Fig. 63**). Samples from horses 3 and 4 were only tested in case of positive results with pathogens due to material scarcity. The 64 bp GAPDH amplicon was generated using 33/35 samples tested, while for 27/33 samples (82%), there was also amplification of the 170 bp product. For the samples that were negative for the 170 bp PCR, but positive for 64 bp, PCR for amplification of a 103 bp product was carried out and demonstrated visible bands from 3 horses (32-34). For 4/33 (12%) of the samples an amplicon of 298 bp was generated, while from none of them 517 bp product was amplified (**Appendix 17-20**).

Fig. 63: Equine glyceraldehyde-3-phosphate dehydrogenase mRNA amplification obtained with the cDNA of equine FFPE brains (n=33)



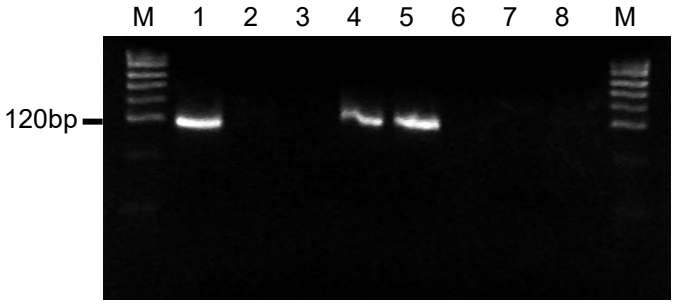
* tested with samples that amplified 64bp products, but not 170bp. GAPDH: glyceraldehyde phosphate dehydrogenase.

4.5.3.2 Pan-lyssavirus RT-PCR

Pan-lyssavirus semi-nested PCR, L protein

Positive lyssavirus RNA controls produced the desired 120 bp product when RNA derived from cells was used concentrated (69.6 ng) and diluted at 1:10. Proper amplification conditions were confirmed by amplicon sequencing demonstrating high identities with Rabies virus strains (i.e. Accession JQ685925.1). The desired 120bp product of the lyssavirus was detected in sample of horse 2 (**Fig. 64**) but nor for the other 34 horses.

Fig. 64: Amplification of 120bp from pan-lyssavirus L gene in 4% agarose gel



(M) pUC19 marker. Lane (1) RABV infected cells. (2) and (3) empty. (4) and (5) horse 2. (6) horse, brain, negative control. (7) and (8) NTCs.

From the PCR product obtained from horse 2, BLAST nucleotide analysis of the 122 bp sequence obtained, revealed 92% identities with the rabies virus strain 633 polymerase (L) gene, accession JN897322.1 (**Fig. 65**).

Fig. 65: Nucleotide sequence alignment of lyssavirus L gene fragment

```
                    10        20        30        40        50        60        70        80
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
JN897322.1          --TTTTTCGCGTT-GATGTCTTGGAATTTGAGGTTGTACTTTGTCATCACAGAAAAGCTCCTCGCCAATTATATCTTGCC
Horse 2             TT..C.....T..AT.....A........A.....................................................

                    90        100       110       120       130       140       150       160
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
JN897322.1          ACTCTTTGACGCATTGACTATGACAGACAACTTGAACAAGGTGTTTAAGAAGTTGATCGACAGAGTCACCGGACAGGGGC
Horse 2             ......C................T.....T..........A--------------------------------
```

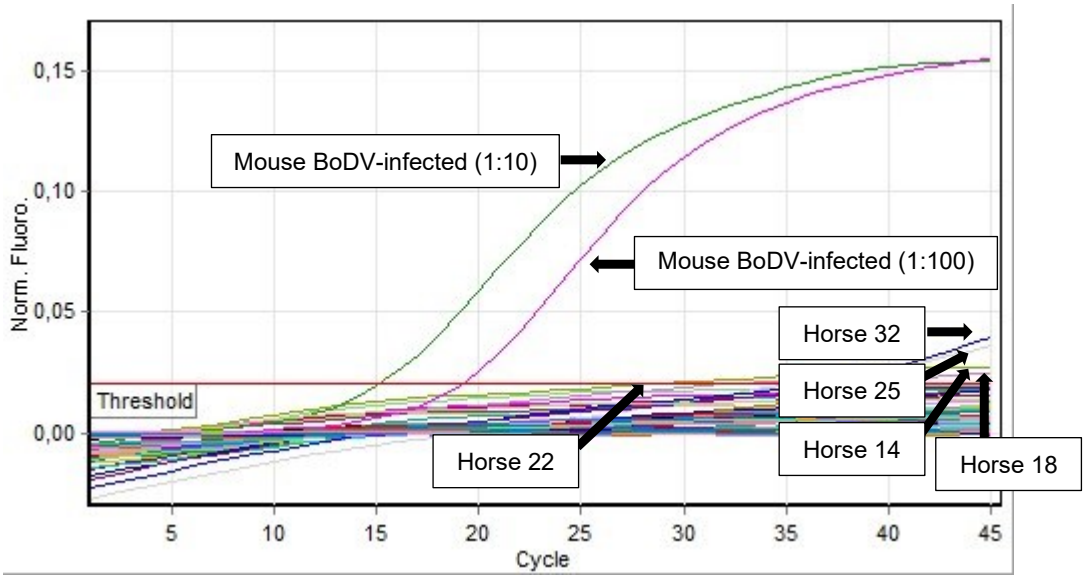
Sequence alignment of Rabies virus strain 633 polymerase (L) gene (JN897322.1) and horse 2, similarities plot in dots.

4.5.3.3 Pan-Borna disease virus RT-PCRs

Pan-Borna disease virus One Step RT-qPCR, X/P genes

The Ct values obtained from the positive controls (fresh tissue from the frontal cortex of mice infected with BoDV-1, diluted at 1:10 and 1:100) varied from 15.11 to 19.16, respectively (**Fig. 66**). Among the horses tested, 5/35 (horse 14, 18, 22, 25, 32) demonstrated weakly detectable amplification, presenting Ct values from 27.69 to 44.48. All other horses were tested negative.

Fig. 66: Pan-Borna disease virus X/P genes amplification curves with Real-time RT-PCR

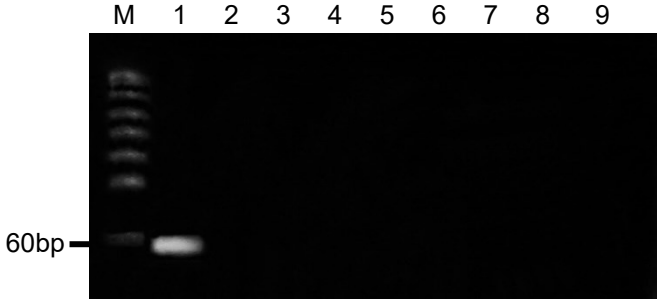


Amplification curves observed for the mouse brain positive controls diluted at 1:10 (Ct 15.11) and 1:100 (Ct 19.16).

Pan-Borna disease virus two-step RT-PCR

Using the positive control material as described above, the desired amplification product of 60 bp was observed (**Fig. 67**). There was no amplicon using the samples from horses that presented Ct values over the range of the positive controls in the RT-qPCR using the two step RT-PCR.

Fig. 67: Amplification of 60bp from pan-Borna disease virus X/P genes in 4% agarose gel



(M): marker pUC19. Lane (1) mouse, brain, BoDV-1 infected. (2) horse 14. (3) horse 18. (4) horse 22. (5) horse 25. (6) horse 32. (7) horse, negative control. (8) and (9) NTCs.

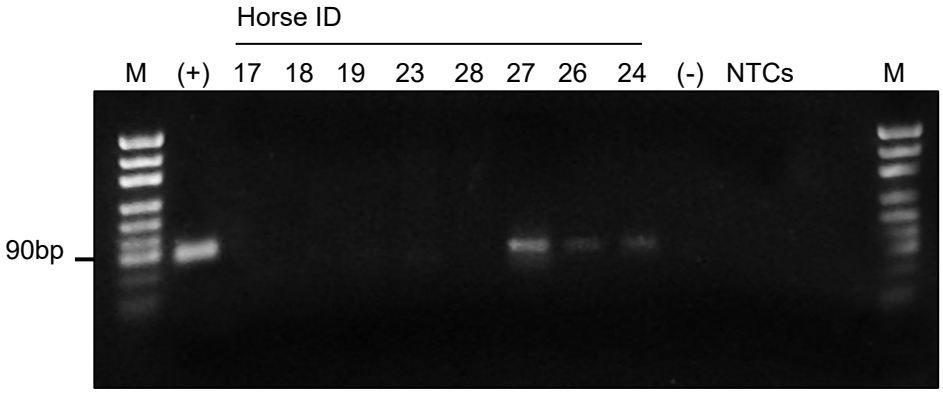
4.5.3.4 Equine herpesvirus-1 PCR

Equine herpesvirus-1 PCR, ORF33

Amplification of the 90 bp EHV-1 specific product was successful using the equine controls with fresh and FFPE brain tissues. Sequence analysis from these controls demonstrated similarities up to 100% with EHV-1 strains (i.e. accession MG732975.1). This 90 bp amplicon was amplified from 3/35 samples (horses 24, 26, 27) (**Fig. 68**). Sequence analysis of the PCR

product from the brain of horse 27 produced a sequence with 89% homology to EHV-1 (accession KP975078.1, **Fig. 69**). Sequence analyses was not possible from the amplicons of horses 24 and 26.

Fig. 68: Amplification of 90bp from equine herpesvirus-1 ORF33 in 2% agarose gel



(M) marker pUC19. (+) horse, brain, EHV-1 infected, FFPE. Horse ID: identification of each animal tested. (-) horse, brain, negative control, FFPE.

Fig. 69: Nucleotide sequence alignment of equine herpesvirus-1 ORF33 fragment

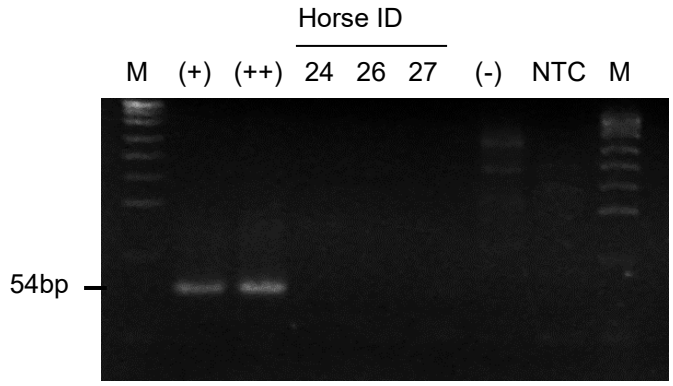
```
                61790       61800       61810       61820       61830       61840
                .|....|....|....|....|....|....|....|....|....|....|....|....|..
KP975078.1      CGCCTAC-CGGATCCACCGTCGTACGCAT-CGAACCACCTAGAACTTGC-CCCAAGTTT
Horse 27        T..G.T.G..C.--..TA.....G.....AA..................G.....TAA.
```

Sequence alignment of Equine herpesvirus type 1, accession KP975078.1 and horse 27, similarities plot in dots.

Equine herpesvirus-1 PCR, ORF30

An amplification product of 54 bp of the DNA polymerase catalytic subunit ORF 30 was yielded with the positive controls. Sequence analyses demonstrated high similarities with neuropathogenic (i.e. Accession KU206466.1) and non-neuropathogenic (i.e. Accession KY852346.1) EHV-1 strains (**Fig. 70**). There was no amplification possible using the 35 horse brain samples.

Fig. 70: Amplification of 54bp from equine herpesvirus type 1 ORF30 in 2% agarose gel



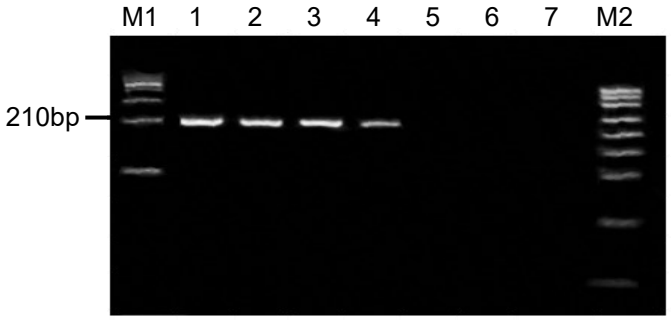
(M) marker pUC19. (+) horse, brain, EHV-1 infected, fresh tissue. (++) horse, brain, EHV-1 infected, FFPE tissue. Horse ID: animals tested. (-) horse, brain, negative control. (NTC) non-template control.

4.5.3.5 Pan-alphavirus RT-PCR

Pan-alphavirus One Step nested RT-PCR, non-structural protein 4 gene

Serial dilutions of the CHIKV positive control demonstrated strong visible bands in dilutions ranging from 1:10 up to 1:10000 (**Fig. 71**). No amplification of the expected 210 bp product was observed with the 35 Brazilian samples.

Fig. 71: Amplification of 210bp from pan-alphavirus non- structural protein gene 4 in 4% agarose gel



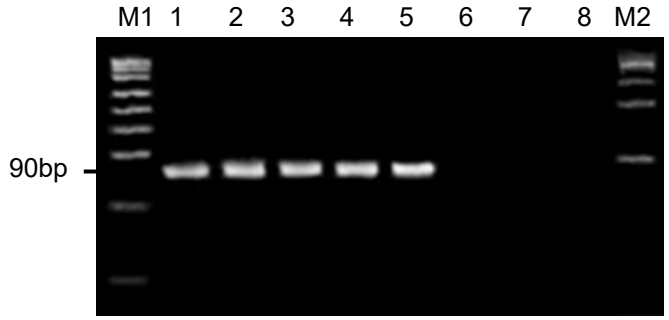
(M1) marker GR100. Lane (1) CHIKV infected cells, 1:10 RNA dilution. (2) CHIKV 1:100. (3) CHIKV 1:1,000. (4) CHIKV 1:10,000. (5) horse, brain, negative control. (6) and (7): NTCs. (M2) marker pUC19.

Pan-alphavirus semi-nested PCR, non-structural protein 4

With a semi-nested approach, an amplification product of 90 bp was observed using the serial dilutions using 189.6 ng (concentrated) RNA up to dilutions of 1:10,000 from CHIKV-infected cells (**Fig. 72**). Sequence analysis from this control demonstrated 96% similarities with CHIKV strains (accession MF773569.1). Amplification of this product was also possible using samples from horse 31 and 32 (**Fig. 73**). Horse 31 was previously described in an outbreak of EEEV by Silva et al. (2011) by using fresh CNS tissue. Sousa et al. (2015) also demonstrated horse 32 as alphavirus positive by IHC. Horses 24-27, 29, 30, and 33 that were previously tested positive for alphaviruses, whether by using fresh material for PCR or fixed tissues for IHC,

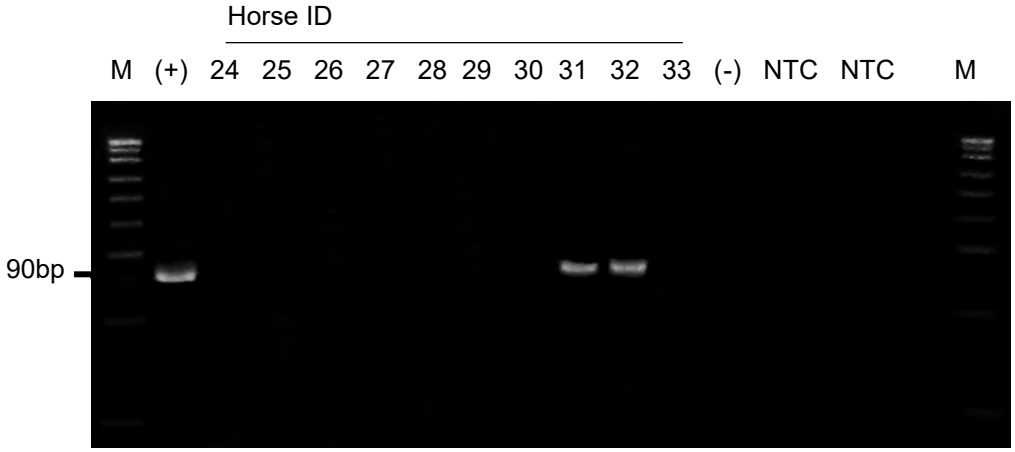
showed however, no amplification products using this protocol (**Fig. 73**), although they amplified at least a 170bp GAPDH product.

Fig. 72: Amplification of 90bp from pan-alphavirus non-structural protein gene 4 in 4% agarose gel



(M1) marker pUC19. Lane (1) CHIKV infected cells, 189.6ng RNA. (2) CHIKV 1:10. (3) CHIKV 1:100. (4) CHIKV 1:1,000. (5) CHIKV 1:10,000. (6) horse, brain, negative control. (7) and (8): non-template controls. (M2) marker GR100.

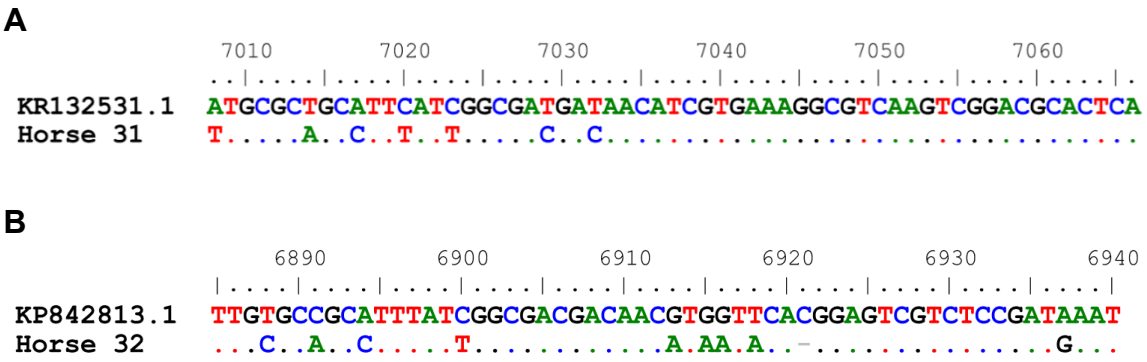
Fig. 73: Amplification of 90bp from pan-alphavirus non-structural protein 4 gene in 4% agarose gel



(M) marker pUC19. (+) CHIKV infected cells 1:10,000. Horse ID: animals tested. (-) horse, brain, negative control. (NTC) non-template control.

Sequence analysis from both positive horses demonstrated high similarities with viruses of the *Togaviridae* family. For horse 31, the obtained sequence showed, by BLAST nucleotide analysis, 90% identities with Madariaga virus isolate MADV/crossbred horse/BR/PR/01/2009 (Accession KR132531.1) (**Fig. 74**A). For horse 32, 82% identities with Mayaro virus strain FPY0046 Nsp1-3, Nsp4, and structural polyprotein genes (accession KP842813.1) were found (**Fig. 74**B).

Fig. 74: Nucleotide sequence alignment of pan-alphavirus non-structural protein 4 gene fragment



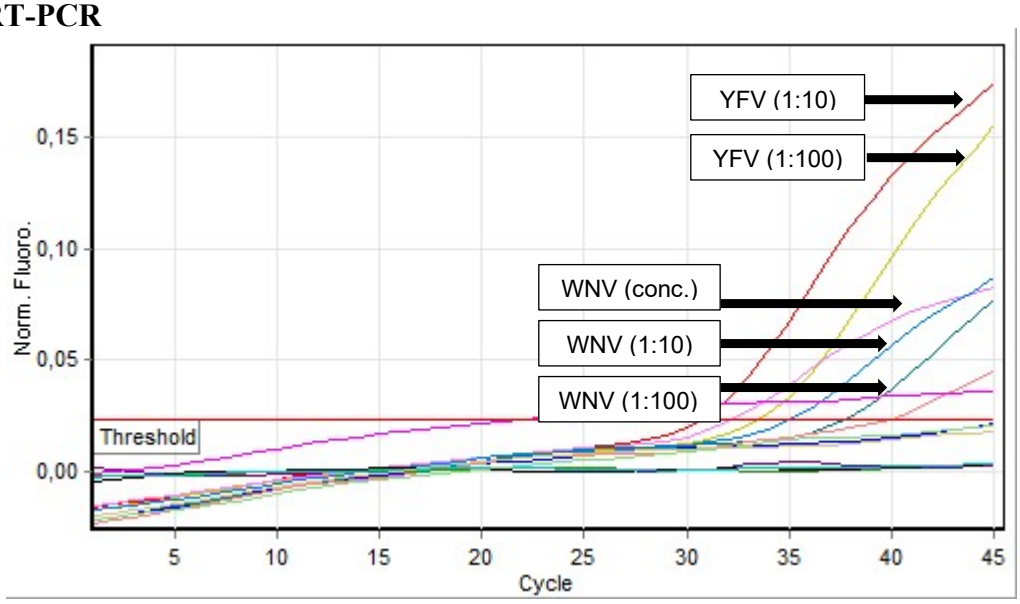
(A) Sequence alignment of MADV (KR132531.1) and horse 31. (B) Sequence alignment of MAYV (KP842813.1) and horse 32. Similarities plot in dots.

4.5.3.6 Pan-flavivirus RT-PCRs

Pan-flavivirus One Step RT-PCR Real-time, non-structural protein 5 gene

The Ct values obtained with the positive controls (cells infected with YFV and brain FFPE infected with WNV) ranged from 30.71 to 39.93 (**Fig. 75**). None of the FFPE samples from the CNS of the 35 horses displayed any amplification curves.

Fig. 75: Pan-flavivirus non-structural protein 5 gene amplification curves with Real-time RT-PCR



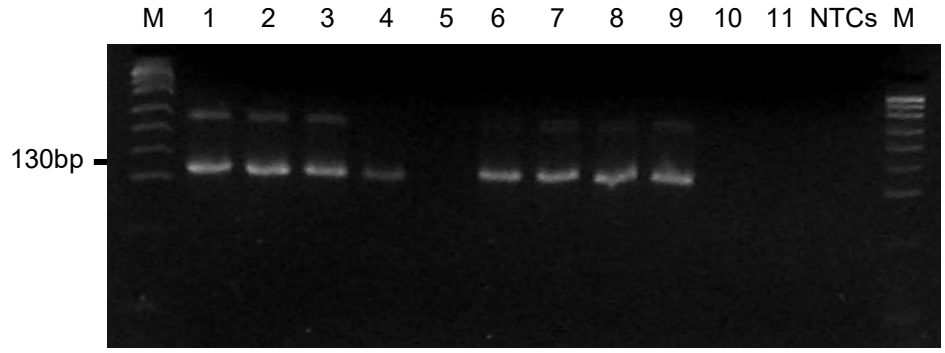
Conc.: template concentration of 1000ng. WNV: West Nile virus. YFV: Yellow fever virus. Amplification curves observed for the positive controls. YFV 1:10 (Ct 30.7), YFV 1:100 (Ct 33.5), WNV concentrated (Ct 32.25), WNV 1:10 (Ct 35.1), WNV 1:100 (Ct 37.8), WNV 1:1000 (Ct 39.93).

Pan-flavivirus semi-nested RT-PCR, non-structural protein 5 gene

Positive flavivirus RNA controls produced the desired 130bp product when RNA derived from YFV-infected cells was used concentrated (3.4 ng) and diluted up to 1:1,000, and

from FFPE material infected with WNV with RNA concentrated (1,000 ng) up to a dilution of 1:1,000 (**Fig. 76**). Amplification curves obtained with the controls by applying a SYBR Green version of this protocol can be assessed in the **Appendix 21**.

Fig. 76: Amplification of 130bp from pan-flavivirus non-structural protein 5 gene in 4% agarose gel

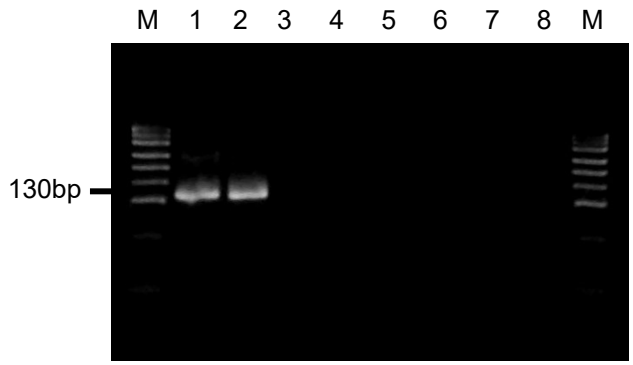


(M) marker pUC19. (1) YFV-infected cells, concentrated RNA. (2) YFV-infected cells, 1:10. (3) YFV-infected cells, 1:100. (4) YFV-infected cells, 1:1,000. (5) YFV-infected cells, 1:10,000. (6) WNV FFPE, concentrated RNA. (7) WNV FFPE, 1:10. (8) WNV FFPE, 1:100. (9) WNV FFPE, 1:1,000. (10) WNV FFPE, 1:10,000. (11) horse, brain, FFPE negative control. NTCs: 2x no-template control.

Proper amplification conditions of the control material were confirmed by amplicon sequencing demonstrating high similarities with YFV isolate YFV-FNV (Accession: MG051218.1) and with West Nile virus isolate WNV-1/US/BID-V4383/2006 (Accession: HQ671745.1).

The desired amplification was observed with brain sample from horse 4 (**Fig. 77**, **Appendix 22**). Sequence analysis of the amplicon demonstrated high similarities with Rocio virus isolates, the highest query recovery was of 96%, and 76/90 (90%) identities with the Rocio virus strain SPH 34675 (Accession: AY632542.4) (**Fig. 78**).

Fig. 77: Amplification of 130bp from pan-flavivirus non-structural protein 5 gene in 4% agarose gel



(M) marker pUC19. (1) pelican, brain, WNV infected FFPE, 1:1,000 RNA dilution. (2) horse 4, brain, 228ng RNA input. (3) horse 5, brain. (4) horse 6, brain. (5) horse 7, brain. (6) horse, brain, negative control. (7) and (8) NTCs.

Fig. 78: Nucleotide sequence alignment of pan-flavivirus non-structural protein 5 gene fragment

```
                9010        9020        9030        9040        9050        9060
                 |....|....|....|....|....|....|....|....|....|....|....|....
AY632542.4      TTTACAACATGATGGGAAAGCGAGAGAAGAAGCTAGGAGAGTTTGGGAAAGCTAAGGGCA
Horse 4         -..................A............GTC..............A.....A.

                9070        9080        9090        9100
                 |....|....|....|....|....|....|
AY632542.4      GCCGCGCCATTTGGTATATGTGGCTGGGGGC
Horse 4         ....T.....A..............A..
```

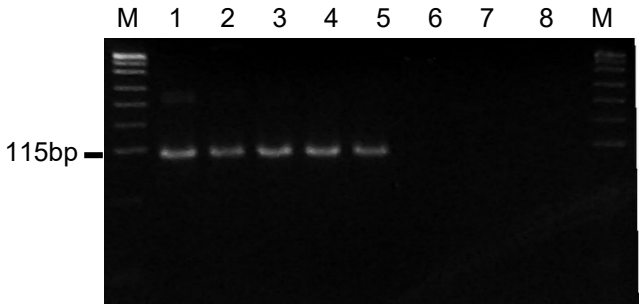
Sequence alignment (93bp) of Rocio virus strain SPH 34675 (AY632542.4) and horse 4, similarities plot in dots.

4.5.3.7 Pan-bunyavirus RT-PCR

Pan-bunyavirus semi-nested PCR, S segment

Using the positive controls, the desired 115 bp product of Orthobunyaviruses was generated in a RNA concentration of 1000 ng until the last dilution tested of 1:10,000 (**Fig. 79**).

Fig. 79: Amplification of 115bp from pan-bunyavirus S segment in 4% agarose gel



(M) pUC19 marker. (1 to 5) serial dilution of LaCrosse virus infected cells, from 1,000ng to 1:10,000. (6) horse, brain, negative control. (7) and (8) non-template controls.

Sequencing analysis from the positive control demonstrated high similarities with La Crosse virus strains (Accession: KP271104.1). There was no amplification from the 35 samples of the CNS of the horses from Brazil.

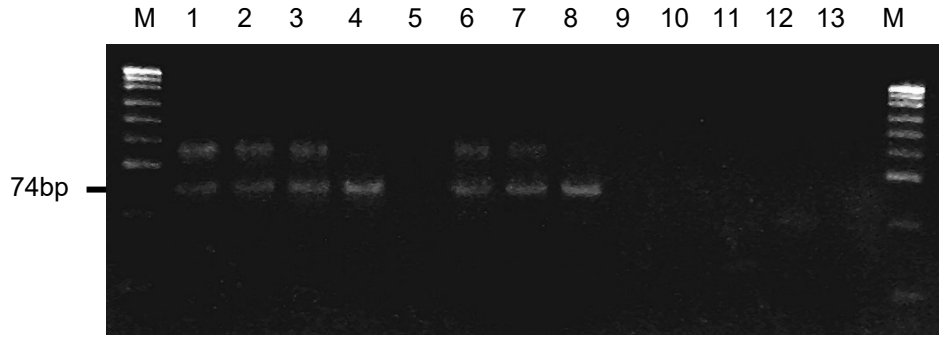
4.5.3.8 Pan-paramyxovirinae RT-PCR

Pan-paramyxovirinae semi-nested PCR, L gene

Using the positive controls, the designed 74 bp long product of paramyxoviruses was generated. Positive controls consisted of NiV RNA concentrated from 61.2 ng up to a dilution of 1:1,000, and of a CDV FFPE control in a concentration of 600 ng until a dilution of 1:100 (**Fig. 80**). Sequence analysis of PCR product amplified by the NiV control showed 89% identities with several strains of the same virus (Accession: KY295932.1), and with the CDV

control, 100% identities with several strains of the respective virus (Accession: MH337872.1). There was no amplification from the 35 CNS samples of the horses from Brazil.

Fig. 80: Amplification of 74bp from pan-paramyxovirinae L gene in 4% agarose gel



(M) pUC19 marker. (1 to 5) serial dilutions of Nipah virus infected cells, from 61.2ng to 1:10,000. (6 to 10) serial dilution of CDV FFPE material, from 600ng to 1:10,000. (11) horse, brain, negative control. (12) and (13) NTCs.

4.5.3.9 Pan-coccidia PCR

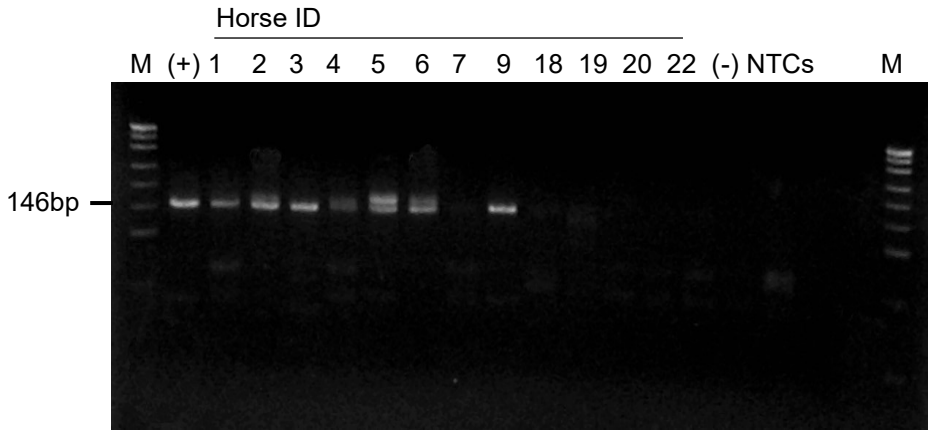
Pan-coccidia nested PCR

Universal nested coccidia PCR was carried out at the Institute of Parasitology, JLU Giessen, by Dr. Joerg Hirzmann. No amplification of the 382 bp or 500-1000 kb products was possible from the horses suggestive of parasitic infection in the histology and clinical records (horses 1, 9, 18, 19, 20, 22).

Pan-Coccidia semi-nested PCR, 18S ss rRNA

Using the positive controls, the designed 116 bp long product of coccidia was amplified with a DNA concentration of 30 ng and proper working conditions were confirmed by successful sequencing of *Eimeria arloingi* (accession MF356556.1). Amplification of this product was possible in 7/35 samples (20%), some of them suggestive of parasitic infection in the histology and clinical records (horses 1, 2, and 9, **Fig. 81**).

Fig. 81: Amplification of 146bp from pan-coccidia 18S ss rRNA in 4% agarose gel



(M) pUC19 marker. (+) goat, jejunum, infection by *Eimeria arloingi*. Horse ID: identification number of animals tested. (-) horse, brain, negative control. (NTCs) 2x non- template controls.

Sequence analysis of the amplification products indicated that *Sarcocystis* spp. were detected in the samples from horses 1, 2, and 9. Sequences obtained from horse 4 produced unreadable, low quality signals. Sequences from horses 3, 5, and 6 were considered highly contaminated by fungi, which were also detected with the coccidia PCR. Other findings with these three samples consisted of detection of *Babesia* sp. (Accession: KT895089.1) sequences with 96% query recovery and 95.45% to 96.33% identities.

4.5.3.10 Pan-mamastrovirus RT-PCR

There was no amplification of the 160 bp product with a RT-qPCR to detect *Mamastrovirus* in samples from the 35 horses from Brazil. The technique was carried out at the Friedrich-Loeffler-Institut and was kindly coordinated by Dr. Bernd Hoffmann.

4.6 Metagenomic screening for pathogens and metagenome

The metagenomic screening from the CNS of the 10/35 horses tested (horses 4, 6, 7, 11, 16, 18, 22, 31, 32, 35) generated a range of ~ 2 million to ~ 6 million reads per sample, which were classified into taxonomic assignments, excluded due to low quality or short size, or remained unclassified (**Table 50**).

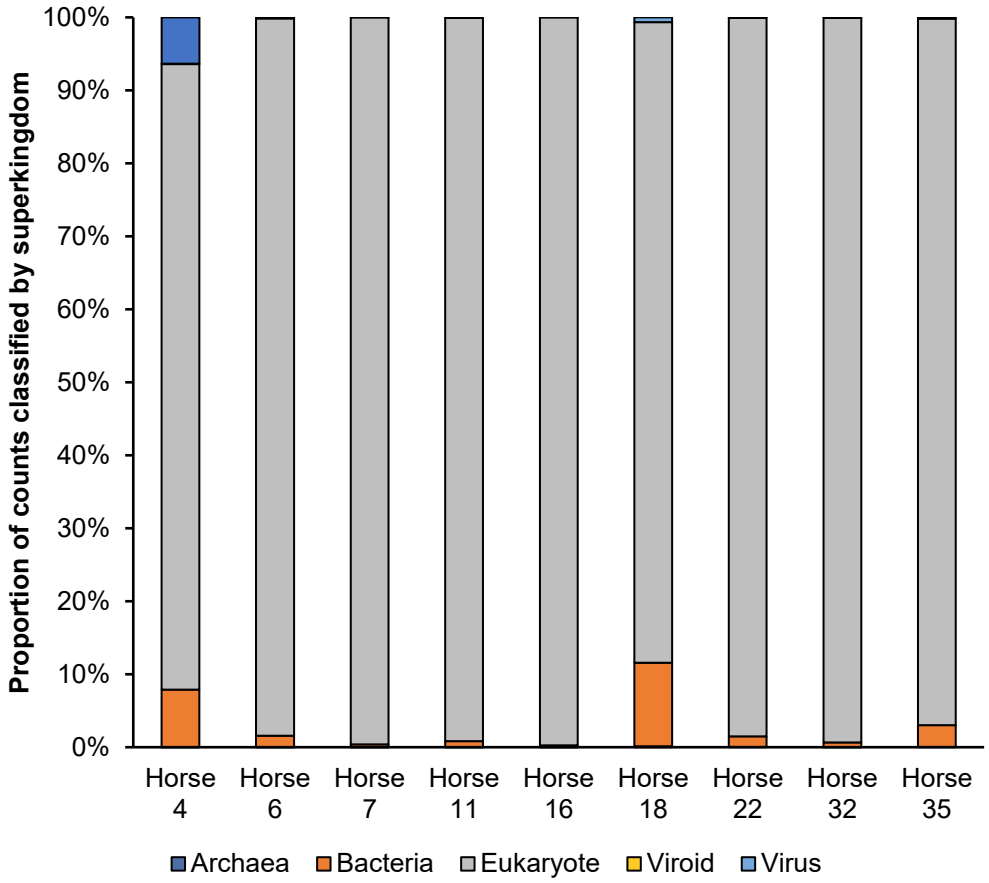
Table 50: Summary of reads obtained in the metagenomic screening of the CNS of horses

Horse ID	Total number of reads	Number of classified reads	Number of low-quality reads [a]	Number of unclassified reads
Horse 4	2,948,362	2,477,689	52,163	418,510
Horse 6	5,133,476	4,926,638	103,190	103,648
Horse 7	5,003,430	4,902,418	91,031	9,981
Horse 11	4,515,387	4,168,017	163,990	183,380
Horse 16	6,075,657	5,801,132	138,853	136,672
Horse 18	2,326,914	1,844,591	200,913	281,410
Horse 22	5,336,966	4,791,367	204,668	340,931
Horse 32	4,415,708	3,565,718	211,792	638,198
Horse 35	4,099,533	3,749,187	230,242	120,104

[a] number of excluded reads (low quality and/or too short)

For **horse 31**, the metagenomic screening produced several contigs of up to 645 nt of **Madariaga virus** isolates (accession KR132531.1), which confirmed the results obtained in this study (4.4.3 and 4.5.3.5) with the results reported by Silva et al. (2015). The metagenome from the other 9 cases tested revealed large amounts of microorganisms present in the samples. Although a pathogen could not be determined from their CNS material, a taxonomical approach from each sample revealed that eukaryotic reads were predominant in all samples, followed by bacterial and viral reads, with archaea and viroid reads in much lesser degree (**Fig. 82**).

Fig. 82: Relative abundance of reads classified by super kingdom found in datasets generated from FFPE CNS of horses



Abundance of reads is given as percentage from complete datasets.

Eukaryotic screening

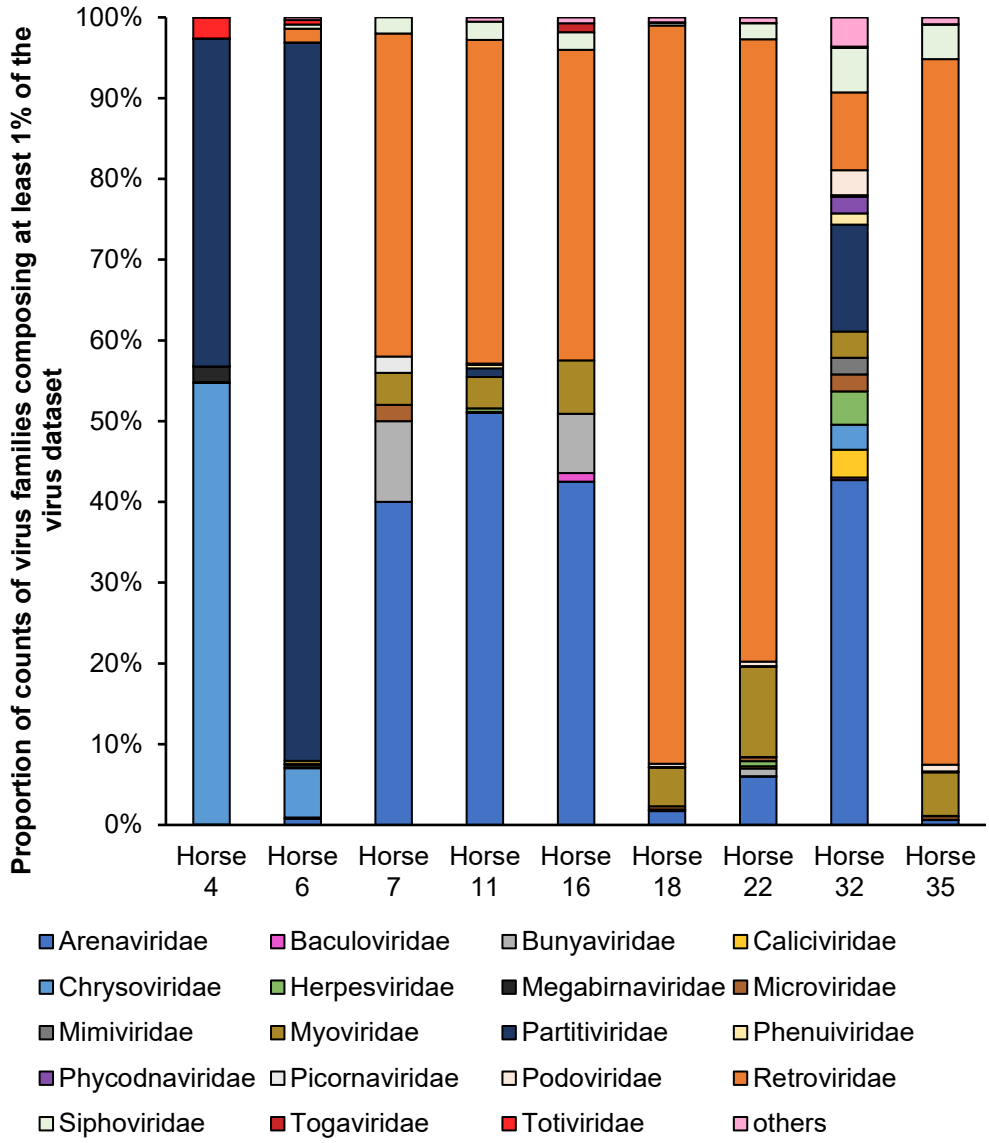
Higher abundance of eukaryotic reads found in datasets from the animals tested consisted of counts from Animalia sequences. The most relevant findings for the neuropathology of horses consisted of reads for parasites and fungi, even though respective families appeared usually under 1% of the eukaryotic data sets. Some exceptions were observed, like the frequent assemblies of the *Ascarididae* - *Parascaris equorum* with the samples from 6/9 cases (horses 7, 11, 16, 22, 32, 35). For **horse 22**, this finding supported by the histological and clinical findings as well as negative results for the techniques tested in this study could match with an uncommon migration by ***P. equorum*** to the CNS. For other cases, sample contamination might hinder the correct assignment of the parasite as a true pathological event. Apart from *P. equorum*, other helminths were identified, and included the families *Ascarididae*, *Onchocercidae*, *Anisakidae*, *Strongyloididae*, *Oxyuridae*, *Angyostrongylidae*, and *Rhabditidae* in all samples investigated. Further reads from parasites consisted of coccidia that are significant for equine health such as *Sarcocystidae* (*Neospora caninum* and *Toxoplasma gondii* found in horses 4, 6, 11, 16, 22, and 35, *Sarcocystis neurona* in horse 22, *Besnoitia* spp.

in horse 6, *Eimeridae* in horse 4, *Cryptosporidiidae* [*Cryptosporidium parvum* – horse 4]) were detected. Likewise, the blood parasites from *Trypanosomatidae* and *Plasmodiidae* families were detected in all samples. *Babesiidae* were detected in all, except in horse 4, and *Theileriidae* except in horse 4 and 35. Amplification by PCR of *Babesia* spp. sequences in the CNS of horse 6 match with the NGS findings that included reads from *Babesia* sp. XXB-HangZhou (accession KT895089.1), along with *B. microti*, *B. divergens*, and *B.* sp. *spanish dog,* and can suggest this animal as carrier of the cited parasites to other animal species. Fungi were also identified in all samples, including the neuroinfectious *Cryptococcaceae*, *Aspergillaceae*, and *Nectriaceae*, with emphasis in *Fusarium verticillioides* counts (horses 4, 6, 11, 35).

Viral screening

Besides the confirmation of MADV infection in horse 31, ~ 350 nt contigs for enzootic nasal tumour virus of goats, a retrovirus, was found in horse 22 sample, which had a choroid plexus papilloma in the third ventricle. Samples from horses 4 and 32 that were positive for flavivirus and alphavirus PCR, respectively, had no reads for these pathogens. Up to 53 virus families were found among the 9 cases, with the most commonly detected eukaryotic viral reads belonging to the families in **Fig. 83**.Viruses known to cause CNS disease in horses and that make at least 1% of the virus data set, although not assembled, belong to the families *Bunyaviridae* (*Bunyamwera virus*, *Tete virus*, and *Uukuniemi virus* in horses 7, 16, 22), *Togaviridae* (*Sindbis virus* in horses 16, 18, 22, 35), and *Herpesviridae* (EHV-1 only on horse 32). Other viruses like *Bornaviridae* (horses 32 and 35) and *Rhabdoviridae* (horse 18 and 35) also appeared but in less than 1% of the respective samples data sets und unassembled. Unclassified viruses were also screened, but likewise, did not generate significant results. *Arenaviridae* and *Retroviridae* appeared in abundance in all samples (**Fig. 83**) because they are used as control for the system. There were still reads for other viruses, emphasizing several plant viruses and mycoviruses (i.e. *Chrysoviridae*, *Partiviridae*, and *Totiviridae*) considered environmental contaminants.

Fig. 83: Relative abundance of counts from the virus families found in datasets generated from FFPE CNS of horses



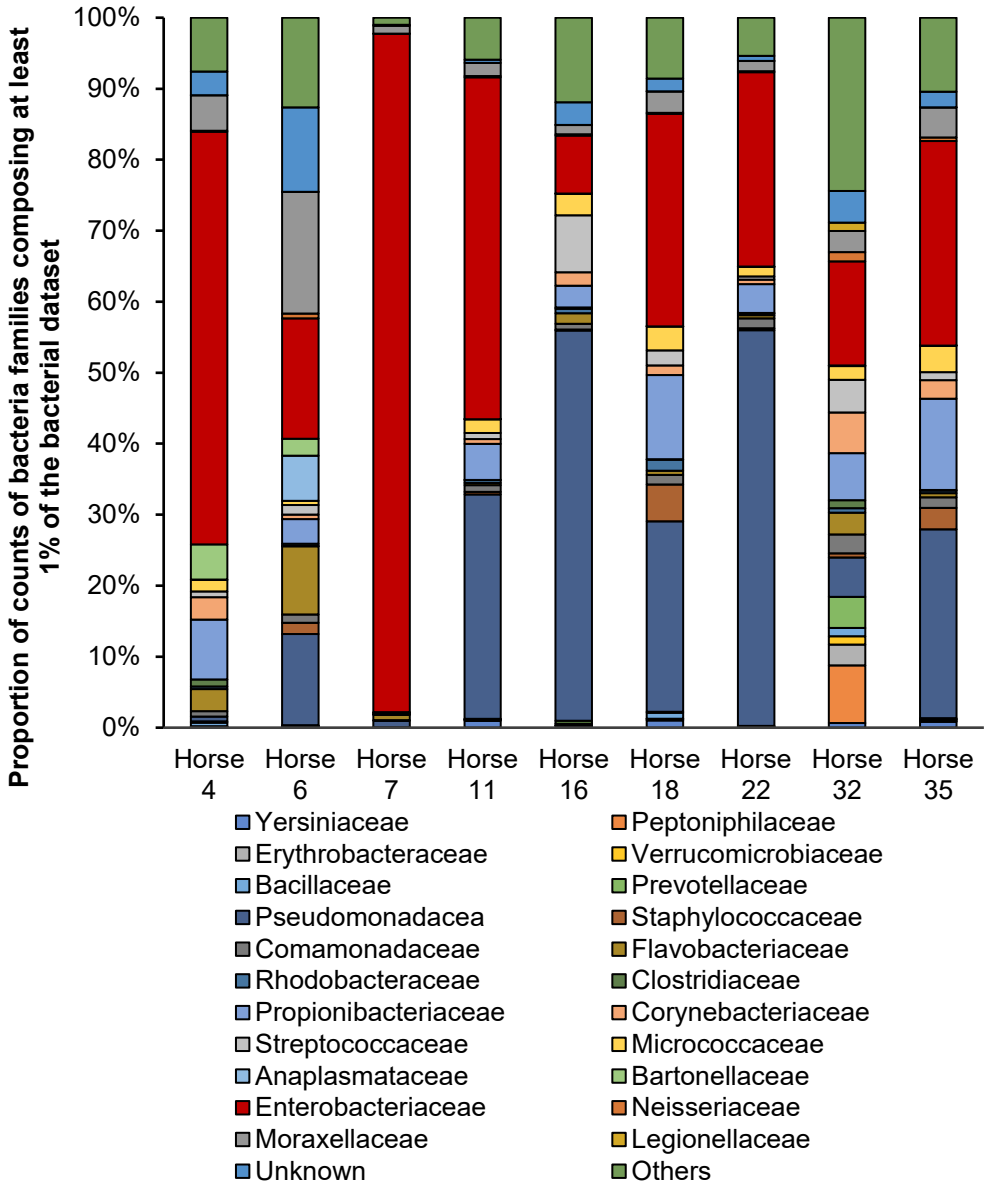
Virus families were displayed when they comprised at least 1% of total viral datasets. Unknown viruses are not displayed. Others: summary of virus families that make less than 1% of the viral dataset.

Bacterial screening

Bacteria commonly found in the ambient were predominant in the screening, but there were no significant assemblies except for **horse 7**, producing contigs of up to 837 nt from the bacteria ***Elizabethkingia anophelis*** (Accession: CP015068.2). *E. anophelis* was found in other samples but did not produced significant results. For the samples from horses 6, 11, and 16 was practically impossible to distinguish between contamination and a potential real cause of infection. Up to 305 families were found in the 9 samples (horse 31 excluded). **Fig. 84** demonstrates the primary families that account for over 1% of the population of at least one sample. Similar as for the viral screening, bacteria that might cause non-suppurative

encephalitis in horses were within the screening, like *Listeria monocytogenes* (horses 6, 11, 16, 22, and 35), *Borrelia burgdorferi* (horses 11, 16, 22, and 35) but represent less than 1% of the bacterial dataset in every sample tested, alongside bacteria responsible for toxic-infections like the ***Clostridiaceae*** (horses 6, **11**, 16, 22, and 35).

Fig. 84: Relative abundance of counts from the bacteria families found in datasets generated from the FFPE CNS of horses

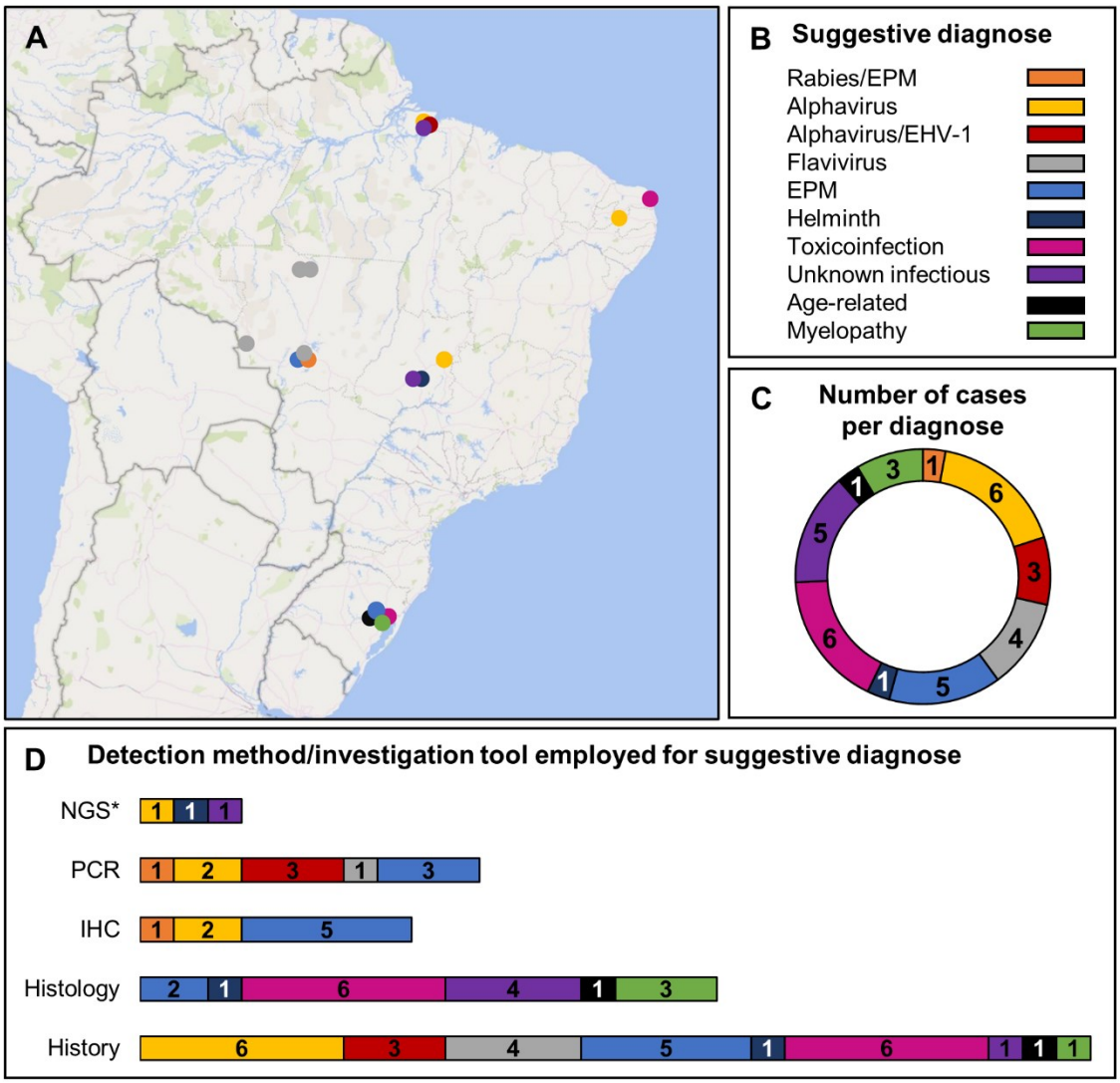


Bacterial families were displayed when they comprised at least 1% of total bacterial datasets. Others: summary of bacteria families that make less than 1% of the bacterial dataset.

4.7 Summary of etiologic diagnose

With the methods provided, in 20/35 cases (57%), the infectious agent detected could be related/suggested to the disease displayed by the cases (**Fig. 85**A-D). For other 15/35 (43%), an unknown infectious etiology was suggested, or degenerative lesions could have mimicked an infection (**Fig. 85**A-D). Concurrently, 4/35 (19%) were also considered pathogen carriers of *Babesia* spp. (horses 3, 5, 6) and *E. anopheles* (horse 7), by PCR and NGS, respectively.

Fig. 85: Summary of detected pathogens and suggestive diagnose for the 35 CNS of horses from Brazil



* 10/35 samples were tested. IHC: immunohistochemistry. NGS: next-generation sequencing. PCR: polymerase-chain reaction

Furthermore, the most frequent findings observed for each suggested/confirmed diagnose is demonstrated on **Table 51**.

Table 51: Summary of findings of the investigations with detected/suggested infectious agents in the CNS of horses (N=35)

Suggestive diagnose	Epidemiology	Clinical signs	Gross lesions	Histo. lesions	Morphology	Glial pattern
Inflammation						
Rabies/EPM (horse 2)	Male, 8 years, Appaloosa, early summer	Posterior paralysis and weakness	Spinal cord – colour alteration	Moderate, spinal cord	Myelitis, lymphoplasmacytic	Mild astro- and microgliosis
Flavivirus (horses 3, 4, 5, 6)	Male, 3-10 years, mixed breed, late summer	Behaviour alteration, sialorrhea, depression, circling, ataxia, tremors	Not available	Moderate to severe, cerebral cortex	Encephalitis, lymphoplasmacytic	Severe astrogliosis and mild microgliosis in cerebral cortex
Alphavirus (horses 25, 29-33)	Female ~ male, 6 months to 5 years old, mixed breed, fall	Circling, ataxia (and hypermetria), recumbency	Not observed	Mild to severe, forebrain > midbrain	Encephalitis, lymphoplasmacytic, eosinophils and few macrophages	~ moderate astrogliosis, ~ mild-moderate microgliosis
Alphavirus/EHV-1 (horses 24, 26, 27)	Female, 6 months to 5 years old, Marajoara, fall	Sternal recumbency, circling, tongue paralysis	Meningeal haemorrhage	Severe in forebrain, mild to mod. others	Meningoencephalo(myelitis) lymphoplasmacytic, eosinophils and few macrophages	Mod. to severe astrogliosis, mild to mod. microgliosis
EPM (horses 1, 9, 18, 19, 20)	Male, ~ 6 years old, mixed breed, fall > summer	Posterior paralysis/ paresis, weakness, ataxia	Spinal cord: colour alteration	Mild to severe, spinal cord	Meningoencephalomyelitis, mixed (lymphocytes, macrophages, plasma cells, eosinophils)	Moderate astrogliosis, mild-moderate microgliosis
Helminth larvae migration (horse 22)	Male, 10 years old, American Quarter Horse, early winter	Circling, vestibular syndrome, ataxia	Encephalon: meningeal vessels stuffed, brainstem: softening, colour alteration, fourth ventricle: mass	Severe in spinal cord, cerebral cortex, moderate in cerebellum	Meningoencephalomyelitis, mixed (lymphocytes, macrophages, eosinophils, plasma cells)	Moderate astrogliosis, severe microgliosis
Unknown agent (horses 7, 23, 34, 35)	Horse 7 only: female, 4 years, American Quarter Horse, fall	Horse 7 only: circling, vestibular syndrome, blindness, ataxia	Horse 7: not observed	Mild to moderate	Encephalo(myelitis) lymphocytic	Mild-moderate astrogliosis, mild-moderate microgliosis

Suggestive diagnose	Epidemiology	Clinical signs	Gross lesions	Histo. lesions	Morphology	Glial pattern
Inflammation and degenerative						
Suggestive of *C. botulinum* neurotoxin (horses 10-13)	Female, > 4 years old, mixed breed, fall	Recumbency, tetraparesis, ataxia, pedalling	Palpebral oedema	Mild	Encephalitis, lymphocytic (horses 10, 11), cerebral cortical laminar necrosis (all)	Mild to severe astrogliosis and microgliosis
Suggestive of *C. tetani* neurotoxin (horses 14, 17)	Female = male, > 2 years old, fall	Stiffness, tremors, weakness	Skin lesion (horse 14)	Mild to moderate	Leucomyelomalacia	Mild to moderate astrogliosis, max. mild microgliosis
Local inflammation (ganglioneuritis)						
Age-related vasculopathy (horse 16)	Female, 16 years old, spring	Not available, but clinical record suggested rabies	Small intestine, kidneys: colour alteration, bladder: replenished, haemorrhagic mucosa	Moderate in striatum, mod. in trigeminal ganglion	Encephalopathy (necrosis), fibrinoid Ganglioneuritis, lymphohistiocytic	Moderate astrogliosis, mild microgliosis
Post-traumatic myelopathy (horse 21)	Male, 6 years old, Crioulo, spring	Felt when mating, blindness, head rotation, no balance	Fracture C5-C6 with focal haemorrhage	Mild in spinal cord and ganglion	Myelomalacia Ganglioneuritis, lymphohistiocytic	Mild astrogliosis, resting microglia
Degenerative						
Degenerative myelopathy (horse 8)	Male, 2.5 years old, mixed breed, fall	Recumbency	Cachexia, cervical spinal cord: luxation C5-C6, mesentery artery: parasites	Severe in spinal cord, mild forebrain, and cerebellum	Poliomyelomalacia, macrophages	Moderate astrogliosis, severe microgliosis
Post-traumatic myelopathy (horse 15)	Female,7 years old, AQH, fall	Recumbency, caudal insensibility, pain in atlas region	Spinal cord: colour alteration T5-T12	Mild to moderate	Myelomalacia, haemorrhage	Moderate astro- and microgliosis
Unknown agent (horse 28)	Not available	Not available	Not available	Mild to moderate	Polioencephalomyelomalacia	Moderate astrogliosis, mild microgliosis

AQH: American Quarter Horse. Histo.: histological. Mod.: moderate.

5. Discussion

In this retrospective study, diagnostic tools were established and/or optimized for the use of formalin-fixed paraffin-embedded (FFPE) material to analyse known infectious and zoonotic pathogens of horses from Brazil. Horses were used as study model because they are in close contact to humans and are susceptible to several similar zoonotic agents as humans so that they represent suitable sentinels for respective risk assessments (Bender & Tsukayama, 2004; Gossner et al., 2017; Timoney, 2000).

A thorough epidemiological and pathological re-evaluation of 35 equine cases suffering from non-suppurative encephalitis and encephalopathies was carried out regarding a possible infectious aetiology. The 35 CNS samples were used for morphologic assessment of inflammation, immunohistochemical pattern of astrocytic and microglial activation, and for antigen detection of 9 possible infectious agents (5 viruses, 3 protozoa, 1 bacterium) and double stranded RNA (dsRNA) that, when positive, indicates a possible viral infection. The equine cases were also tested for pan-RT-PCR assays to detect the nucleic acid of 9 families/class of pathogens (8 viruses, 1 coccidia). Additionally, the nucleic acid of 10/35 cases were sent to research partners, Arnt Ebinger and Dr. Dirk Höper, at the Friedrich Loeffler Institute, Greifswald - Riems Island, who performed the metagenomic screening for known, unexpected, or newly emerging pathogens.

There are studies that examine the aetiology of unresolved CNS diseases in horses, that study a specific pathogen, or that use fresh clinical samples to screen the metagenome of sick horses (Altan et al., 2019; Cunha et al., 2016; Delcambre et al., 2016). However, a comprehensive study, carried out signally with FFPE material was not yet carried out.

5.1 Complementary diagnostic tools optimized for FFPE material

5.1.1 Optimized histological evaluation

Histologically, a panel of different lesions were found in the 35 cases. This variance is experienced by studies that investigate (and find) different species of pathogens infecting the cohorts studied, already demonstrated in cats and dogs (Schwab et al., 2007), and cattle (Theil et al., 1998). Also, cases of natural infection can display different lesions induced by the same pathogen because of the different clinical stages, like in the early infection by alphavirus vs. later course of infection (Cantile & Youssef, 2016) or in unusual reactions of a known pathogen, like demonstrated by Algermissen (2010) in a study of Borna disease (BD) in horses.

For proper analyses, a score system for the severity of inflammation in the CNS of horses was established including the mean of cuffed perivascular inflammatory cells, the number of layers in these cuffs, and inflammatory cells in the parenchyma. A systematic demonstration of

inflammation in different regions of the CNS allows to observe where lesions were more severe, or where lesions occurred, to establish a possible predictive pattern that could be associated with a certain pathogen detection. Ideally, the clinical signs presented by the animal should match with the localization of the histological lesions. In a retrospective study, however, not all areas of the CNS could be available. Therefore, the score system was established based on four main regions in the CNS: forebrain, midbrain, cerebellum, and spinal cord according to Kaufmann et al. (2012). In 28/35 horses, inflammation was present either locally or multifocally in the CNS. Horses that had a suggestive viral infection presented more pronounced infiltrates in the forebrain and midbrain, usually accompanied by mild inflammation in other areas of the CNS. This observation complements the findings of other researches, which suggest a viral predilection site, like alphavirus, to midbrain (horses 24, 25, 26, 29, 31) and forebrain (horses 24, 27, 29) (Campos et al., 2013; Del Piero et al., 2001; Sousa et al., 2015). Flaviviruses on the other hand, usually present more caudal lesions (Toplu et al., 2015), but the correspondent regions were missing in the suggestive cases (horses 3, 4, 5, 6). Furthermore, the case of a coinfection between rabies virus and *Sarcocystis* sp. (horse 2) displayed moderate-severe infiltrates in the spinal cord, considered an optimal location for identification of the virus in horses (Bassuino et al., 2016). Inflammation tended to be moderate to severe in CNS areas where also parasite sections were observed, like in the forebrain of horse 20 and spinal cord of horses 1, 2, and 9; the latter displayed the highest *degree of inflammatory cell infiltration* (DICI) observed among all samples and fragments. These findings support that parasites induce pronounced local inflammatory reaction, although relatively little is known on the immune response to coccidia infection in horses (Liang et al., 1998; Spencer et al., 2004). Cerebellar fragments, however, were underrepresented in this study, and could have influenced the characterization on inflammatory infiltrates in this area.

Most of the cases with inflammation (25/28) had a predominant lymphocytic infiltration, with other cells, like plasma cells, macrophages, and eosinophils amidst. Although this usually suggests a viral infection, other pathogens like protozoa (lesions demonstrated in horses 1, 2, 18, 19, 20) and bacteria such as *Listeria monocytogenes* (Cantile & Youssef, 2016) can also display a similar pattern of inflammatory cells. In the other three cases (3/28), there was a mixed inflammation due to protozoa infection (horse 9), and balanced inflammatory infiltration of lymphocytes and plasma cells (horse 26) added to macrophages (horse 21 trigeminal ganglion).

Meningitis was observed in 12/35 cases, with severe lesions observed in spinal cord sections from horses 9 and 22, related to parasitic infections and concurrent inflammation. The degree of inflammatory infiltration in the spinal cord correlated significantly to the occurrence

of meningitis and its severity. Also, meningitis in viral encephalitis is seldom as severe as in lesions produced by parasites (Cantile & Youssef, 2016).

The parasitic structure found in the spinal cord of horse 1 could not be morphologically identified as a protozoon. A parasitic co-infection could be suggested, as in Schale et al. (2018), but the agent in this study is yet to be identified. Additional findings like neuronal necrosis and satellitosis/neuronophagia tend to be related to neurotropic viruses, like rabies virus (RABV) in horse 2, and alphaviruses in horses 24-27, 29-33, but can also be found associated to EPM and in cases of an unknown infectious cause. Vasculitis was present in viral and parasitic infections in this study, while reactive endothelia and haemorrhage appeared regardless of agent. Malacia was heterogeneously observed among samples presenting viral, parasitic, and degenerative lesions and was more often associated with severe lesions, like in horse 2 and 9 (rabies/EPM) and 8 (degenerative). Although the nature of the malacic process was not specific for cause, there is some specificity in the localization of lesions and in their pattern of distribution. These features allowed the recognition of chronic poliomyelomalacia (horse 8), leucomyelomalacia (horses 14, 17), acute myelomalacia (horse 15), and polioencephalomalacia (horse 28). Parenchymal vacuolization was predominantly observed in lesions suggestive of viral infection (6/17), tied with degenerative lesions.

5.1.2 Optimized immunohistochemical studies

5.1.2.1 Immunohistochemical demonstration of astrocytes

Astrocytic alterations were assessed with the score system established based on the morphological and quantitative alterations observed in the 35 equine CNS cases from Brazil and in four non-altered equine brains. Immunostaining with the polyclonal antibody glial fibrillary acid protein (GFAP) was chosen for the study as it has been demonstrated to be adequate for equine samples and it is widely available in veterinary laboratories (Delcambre et al., 2016; Meneses et al., 2017).

In the literature no reports on grading from equine astrocytes IHC were available so that an own grading scheme was specifically developed for the equine samples. Normal astrocytes (grade 0 of astrocytic alteration) appeared as staining of a range of 0 to 30 astrocytes/200x high-power field (HPF) that were well-ramified with minimal contact between cells and tightly connected to blood vessels as also described by Meneses et al. (2017). Other authors described at least the increase in number of GFAP-stained cells in brain areas undergoing an injury when compared to normal horse brains (Delcambre et al., 2017; Lemos et al., 2008). In mild astrogliosis (grade 1), there were slight astrocytic morphologic alterations consisting of mild nuclear enlargement and apparent increase in positive cells, that ranged from 16 to 60/200x

HPF. These features were also described by Campos et al. (2013) and Sousa et al. (2015), in the astrocytes from horses infected with eastern equine encephalitis virus (EEEV) and match with the findings in this study from the forebrain, midbrain, and cerebellum from horses 24-27 and 32. Meanwhile, mild astrocytic activation was observed in at least one region of the CNS of cases with degenerative lesions (like in horses 12, 14, 16, 17, 28), associated to more severe alterations in other brain areas. This feature was already reported in neurotoxic events, like induced by mycotic, plant, and botulinic toxins, and uremic/hepatic encephalopathy (Bandarra et al., 2010; Bouchard et al., 1994; Frye et al., 2001; Giannitti et al., 2011; Jenkinson et al., 2017). Grade 2 stained around 40 to 100 cells/200x HPF, accompanied by a more prominent cellular hypertrophy, and astrocytic processes overlapped. With this configuration, suggested parasitic infections like displayed by horses 1, 9, 18, 19, 20, and 22, are supported by the findings demonstrated in mice experimentally infected with *Toxocara canis* (Eid et al., 2015; Liao et al., 2008) and pigs with neurocysticercosis (Sikasunge et al., 2009). Grade 3 astrogliosis was associated with pronounced GFAP immunostaining and more than 77 astrocytes/200x HPF with severe alterations as found in forebrain and cerebellum in 5/35 horses. Like in human severe astrogliosis, in horses there was pronounced cellular hypertrophy of cell body and processes. There was substantial intermingling and overlapping of neighbouring astrocytes processes with blurring and disruption of individual astrocyte domains as described by Sofroniew & Vinters (2010). In the literature, this severe diffuse reaction pattern is described in severe focal lesions, infections, and chronic neurodegeneration (Sofroniew & Vinters, 2010). Comparably, all horses that had grade 3 astrogliosis had also some degree of inflammatory lesions that were suggested as being caused by a virus (horses 4, 24, 27, 33) or a toxin (horse 11). Occasionally also gemistocytes were present as seen in the forebrain of horse 4 and midbrain of horse 29, which occur when there is lethal injury towards astrocytes, and in these cases associated to viral infections (Cantile & Youssef, 2016).

Another interesting feature was the reactive glia limitans bordering i.e. the severe meningitis in the forebrain of horse 25 (viral infection suggested). This scar and barrier formation, is composed of highly GFAP-reactive astrocytes amidst milder astrocytic alterations in the same tissue, with the intent to enclose a necrotic lesion and/or an infection also demonstrated in experiments with mice (Anderson et al., 2016).

Furthermore, the statistical analysis carried out with the equine samples supported the morphological astrocytic alterations translated into the score system established. Correlation tests indicated that lesioned areas have higher number ($p \leq 0.05$) of GFAP-positive astrocytes than non-lesioned areas, as well as higher astrocytic alteration grades, independently if an

inflammatory or degenerative lesion triggered the response. The positive correlation obtained with a Spearman's rank correlation coefficient showed that the astrogliosis grades were correctly designated: the higher the number of GFAP-positive cells, the more severe were the morphological alterations.

The statistical model established for equine samples addressed some of the missing aspects regarding astrocytic reaction in horses. Like observed in humans, astrogliosis in horses occurs in a spectrum of heterogenous changes, translated into the grades of alteration (1, 2, 3) in this study. Besides, astrogliosis is widely used as a hallmark of diseased CNS tissue, and therefore, the correct designation of morphologic and quantitative astrocytic alterations, which was missing for horses, can also help to relate certain pathogens/conditions in unresolved cases of encephalitis and encephalopathies.

5.1.2.2 Immunohistochemical demonstration of microglia

To assess microglial activation in the CNS of horses, the score system established was based on the morphological and quantitative alterations observed of 35 equine cases from Brazil and four non-altered equine brains. Immunostaining with the ionized calcium-binding adaptor molecule 1 (Iba1) was chosen for the study as it has been demonstrated to be sensitively upregulated during microglial response to injury in human and animal studies (Delcambre et al., 2017; Dénes et al., 2006; Ide et al., 2011; Imai & Kohsaka, 2002; Jager et al., 2017).

Like in the astrocytic alterations, there are no reports on grading microglial alterations from the brain of horses, hence a specific scheme was developed. Resting microglia (grade 0) were characterized by faintly stained cells that exhibited ramified and slender processes, with inconspicuous, small, hyperchromatic, and wedge-shaped nucleus, also observed by other authors (Delcambre et al., 2016). Resting microglia were found in all four non-altered equine brains used as controls, as well as in non-lesioned areas bordering lesions and in CNS regions completely lesion-free (but from cases with alterations in other sections). Similar morphology of resting microglia was described in early studies of microglia phenotyping (Kreutzberg, 1996) and in the equine non-altered controls from an experimental study with *Trypanosoma evansi* infection (Lemos et al., 2008). A mild microglial activation (grade 1) was characterized by a range of 6 to 30 Iba1-positive cells per 200x HPF and the morphological alterations were discrete, inclined to the beginning of cellular processes retraction. Grade 1 was the highest alteration observed in 17/35 cases and although there are no reports of solely mild microgliosis as the main microglial alteration in horses, resting microglia are progressively activated in traumatic injuries, ischemic stroke, demyelination, and neurodegenerative diseases (reviewed by Li & Zhang, 2015). In other 4/35 cases (horses 10, 24, 26, 27), this mild activation was

associated to more severe grades in other CNS regions. In the moderate level of activation (grade 2) observed in 5/35 cases (horses 15, 23, 24, 26, 29), a range of 17 to 60 cells/200x HPF were Iba1-positive, there was distinguishable cellular processes retraction, and a general hypertrophic reaction with increased number of Iba1-positive cells, also described by other studies (Cantile & Youssef, 2016; Cartier et al., 2014; Li & Zhang, 2015). Grade 3 microglia activation was observed in 7/35 cases and were associated with Gitter cell occurrence. These bulky cells, peripherally stained with Iba1, were observed concurrently with other smaller, densely packed, and intensely stained microglia. Gitter cells were observed within brain areas of horses displaying inflammatory lesions suggested to be caused by parasites (horses 1, 9, 22), viruses (horses 7, 27), and toxins (horse 10), and in a case with degenerative spinal cord of unknown origin (horse 8). Also observed in other investigations, these cells are the ultimate scavengers directed to phagocyte diffuse lesions produced by protozoan (Lemos et al., 2008; MacKay et al., 2000; Witonsky et al., 2014), helminthic (Vasconcelos et al., 2007), and viral infections (Bassuino et al., 2016), and as a local response in lesions due to *Trema micrantha* intoxication (Lorenzett et al., 2018; Pavarini et al., 2013), ischemic encephalopathy due to placental insufficiency (Wilcox et al., 2009) and leucoencephalomalacia by mycotoxicosis (Giannitti et al., 2011). There was also the increase in Iba1-positive cells in grades 2 and 3 accompanied by lesser grades in the periphery of lesions or adjacent areas of the CNS. This could indicate the presence of proliferating microglia and/or migrating cells from the bloodstream, but such differentiation is not possible with Iba1 (Lassmann et al., 2012; Lemos et al., 2008; reviewed by Li & Zhang, 2016). On the other hand, moderate to severe microgliosis were statistically associated to inflammatory infiltrates in the midbrain, with a tendency to occur in suggestive viral (horses 24, 27, 29) and parasitic infections (horse 22).

The statistical analysis also supported the morphological aspects attributed in the score system. Correlation tests indicated that lesioned areas from forebrain, midbrain, and spinal cord have higher number ($p \leq 0.05$) of Iba1-positive cells than non-lesioned areas, as well as higher microglial activation grade. Cerebellum sections were underrepresented in the study and this could have influenced the lack of correlations in the statistical analysis. The positive correlation obtained with a Spearman's rank correlation coefficient showed that the microglia activation grades were correctly designated: the higher the number of Iba1-positive cells, the more severe were the morphological alterations.

Other interesting features of microglial activation in the CNS of horses and that could help in the assessment of an etiology underlying the alterations observed, are microglial nodules; observed in 7/35 cases that presented non-suppurative inflammatory infiltration. In

five of those cases (horses 24, 26, 27, 29, 32), a viral infection could support the presence of microglial nodules, as frequently described in the literature (Bassuino et al., 2016; Bielefeldt-Ohmann et al., 2017; Cantile et al. , 2001; Delcambre et al., 2017; Little et al., 1985). For other 2 cases (horses 1 and 9), a protozoal infection was suspected, and the nodule formation could indicate the persistence of neurophagic sites (Eydal et al., 2012). Furthermore, neuronophagia was commonly observed among the cases studied (18/35), with no predominance for an activation grade. There was a strong tendency however, to occur in samples with inflammation, as a feature of many viral infections (Cantile & Youssef, 2016). Additionally, reactive microglia also develop into rod cells, a bipolar ramified configuration. Again, these are most commonly seen in neurotropic viral diseases, like Borna disease in horses (Cantile & Youssef, 2016), but are also reported in human cases of subacute sclerosing panencephalitis, Alzheimer's disease, and Wilson's Disease (Wierzba-Bobrowicz et al., 2002), and in horses experimentally infected with *T. evansi* (Lemos et al., 2008). Although their function is not completely understood, they are observed when there is diffuse brain injury (Fumagalli et al., 2015; Taylor et al., 2014), going along with the extensive and severe lesions observed in tissues from horses 1 and 22 in this study.

Although Iba1 immunostaining does not differentiate resident from migratory microglia, it has shown an adequate performance that allowed the correct recognition of the heterogenous activation stages of equine microglial cells, and that could be translated into 3 main grades. The statistical model established for microglial cells responding to inflammatory and degenerative lesions can also help to associate certain aetiologies to unresolved cases of encephalitis and encephalopathies.

5.1.2.3 Immunohistochemical demonstration of infectious agents

Immunohistochemistry was also applied for the detection of nine different pathogens that cause encephalitis – RABV, BoDV-1, EHV-1, alphaviruses, flaviviruses, *Listeria* sp., *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis neurona*. In summary, by IHC in 7/35 cases viral or protozoal antigens could be detected. In horse 2 RABV infection was confirmed, double stranded RNA (dsRNA) was found in horses 1, 3, and 17, alphavirus antigen was detected in horses 26 and 31, and antigen of *S. neurona* merozoites was present in horses 1, 9, 19, and 20 (see also 5.3 for cases pathogen-specific discussion).

For this purpose, steps to optimize IHC protocols was carefully considered (Delcambre et al., 2016, 2017; Ramos-Vara & Miller, 2014). Deparaffinization and endogenous peroxidase blocking were done as carried out in the routine laboratory of the Institute of Veterinary Pathology, JLU Giessen as they are similarly performed by many other researchers, who

obtained comparable satisfactory results concerning tissue preparation and tissue background restriction (Delcambre et al., 2016; Ramos-Vara et al., 2008; Ramos-Vara & Miller, 2014; van Alstine et al., 2002). In some cases frequent detachment of spinal cord tissue (horses 16, 19, and 21) was evident, which might have been due to the coagulation core produced in cases of irregular fixation, where only alcohol penetrates the tissue and results in loss of antigenicity (Boenisch, 2005; Werner et al., 2000). Other deleterious effects (e.g. false negative results) due to prolonged storage are known to differ among antigens and can be reversed with optimal antigen retrieval (AR) procedures (Boenisch, 2005; Ramos-Vara & Miller, 2014). To overcome aldehyde cross-linking that binds to proteins during formalin fixation, *heat induced epitope retrieval* (HIER) methods showed the best results in this study with the monoclonal antibodies directed against alphavirus, flavivirus, as well as for the polyclonal anti-*S. neurona* antibody. The use of citrate buffer pH 4.0 at 95°C was able to unmask the epitope and maintained the tissue architecture. Similarly, Delcambre et al. (2016) found in low pH citrate-based solutions the best option to obtain superior staining. Meanwhile, enzyme digestion with proteinase K demonstrated satisfactory results for protozoa *T. gondii* and *N. caninum* identification and dsRNA when combined to light heating incubation at 37°C. Compared to the harsh elevated temperature from HIER that could destroy more sensitive epitopes, enzymatic digestion was conducted in a temperature high enough to activate and increase proteinase K activity without interfering with integrity of the respective epitopes (Leong & Gilham, 1989; Ramos-Vara & Miller, 2014). Antibodies against antigens from RABV, BoDV-1, EHV-1, and *Listeria* sp. did not require any antigenic retrieval and protocols were applied according to the established protocols at the Institute.

As of pathogen-specific antibody reaction, contradictory results were found when comparing the samples previously tested for alphaviruses in Brazil. In this study, nine cases were suggestive to be infected by alphaviruses - horses 24 to 27 (Campos et al., 2013) and horses 29 to 33 (Sousa et al., 2015) - but IHC was positive with only 2/9 brains (horses 26, 31), while a third sample was only positive by RT-PCR (horse 32) and later Mayaro virus (MAYV) was confirmed through amplicon sequencing. This might be explained by the detection spectrum of the monoclonal antibody used was limited to EEEV, WEEV, VEEV, Semliki virus, and Chikungunya virus (CHIKV) and will therefore not react with other species, even when closely related, like MAYV to CHIKV. Madariaga virus (MADV) is close enough to EEEV as it was detected from horse 31 by alphavirus IHC. Meanwhile, for flavivirus group detection with an antibody raised against purified Saint Louis encephalitis virus (SLEV) envelope protein there was positive immunostaining only for the WNV-infected control. Lack of

immunostaining for horse 4, which was positive in the pan-flavivirus semi-nested PCR, could be due to the reactivity spectrum of the antibody (not able to react to Rocio virus), due to missing caudal CNS tissues (more appropriate for investigation), or also due to characteristics of flaviviral infection in horses that coincide with a short viremic phase, cleared by the onset of clinical signs and then only detected with molecular exams (Angenvoort et al., 2013; OIE, 2019h; Patel et al., 2013; Scaramozzino et al., 2001).

CNS immunoreactivity to EHV-1 is also reduced/absent when caudal areas are missing, when there is a prolonged clinical disease duration, and when the virus undergoes the latent cycle (Schultheiss et al., 1997; Smith et al., 1998). These factors were illustrated by the IHC reaction pattern in the positive control used in the study, which was positive only in liver sections, while the CNS remained negative, as well as cases of horses 24, 26, and 27, found to be EHV-1 positive by PCR. Success in immunostaining, on the other hand, is more common with foetal tissues and placenta (Slater, 2014; Studdert et al., 2003).

Difficulty to detect the respective agent is also recognized when parasites are investigated. The mature *S. neurona* structure, the sarcocyst, tends to be irregularly stained or not stained at all by IHC, similarly observed in the cattle tissue here used as positive control. This irregular or non-reactivity is related to the parasitophorous capsule, which remains antigenically unrecognized. Other parasitic structures on the other hand, like the sporozoites (detected in horses 19, 20), merozoites (detected in horse 20), and schizonts demonstrate high antigenicity and can therefore be easily detected by IHC (Dubey et al., 2014, 2015).

5.1.2.4 Immunohistochemical demonstration of dsRNA

The monoclonal J2 antibody to detect double-stranded RNA (dsRNA) was used in this study for the identification of possible viral infections. The major cause of dsRNA presence in cells is a viral infection and its source varies among viruses (Kumar & Carmichael, 1998). For RNA viruses, dsRNA is a by-product from genome replication, while for DNA viruses, it can be an erroneous product due to self-annealing of convergent bidirectional transcription (reviewed by Weber et al., 2006). The common effect of this viral product, is to induce the innate immunity, consequently activating the production of type I interferons (IFN1) (Kumar & Carmichael, 1998; Son et al., 2015; Weber et al., 2006).

In accordance with other publications (Richardson et al., 2010; Weber et al., 2006) and partially with findings by Son et al. (2015), the results obtained in this study demonstrated that positive-stranded RNA viruses, as WNV (positive control for the flavivirus IHC) reacted positively. Likewise, DNA viruses, such as EHV-1 and *Suid herpesvirus 1* (SuHV-1) revealed positive immunostaining, although with marked differences in staining intensity. Positive

results were obtained with the dsRNA IHC in the horse samples, refer to horse 1, suggestive of *Sarcocystis* sp. infection, horse 3 suggestive of flavivirus infection, and horse 17, suggestive of *Clostridium tetani* toxicoinfection. Detection of dsRNA produced by flaviviruses was already demonstrated by Uchida et al. (2015) and reviewed by Son et al. (2015). But for horses 1 and 17, the source for dsRNA formation due to a viral infection was virtually discarded based on the negative results obtained with exhaustive IHC and molecular assays in the study. Although uncertain, an explanation for horse 1 could be supported by the extensive lesions produced by *Sarcocystis* sp., which could have induced cellular stressors like pro-inflammatory cytokines, to be produced like in viral infections (Der et al., 1997; Walochnik & Duchêne, 2016). For horse 17, dsRNA production has yet to be explained as its CNS samples consistently demonstrated negative results for the infectious agents investigated in this study. In the three cases positive for EHV-1 PCR (horses 24, 26, 27), dsRNA immunostaining was expected but a possible immune reaction to the virus by the host could have led to its suppression (Pellet & Roizman, 2013). Latency on the other hand, would be a strategy from EHV-1 to not be detected by the host immune system (Pellet & Roizman, 2013).

The counteracting approaches of RNA viruses to the host immune system are to hide or mask pathogen-associated molecular patterns (like dsRNA) or hit the immune response (Zinzula & Tramontano, 2013). This would explain the lack of immunostaining for dsRNA in the nine horses with presumptive diagnosis of alphavirus infection, later confirmed in three of them (horses 26, 31, and 32), flavivirus (horses 3, 4, 5, 6), and other 10/35 cases of suggestive infectious aetiology. Alphaviruses have variable strategies to overcome the immune response of their hosts; in the case of VEEV and EEEV, the capsid (C) encoding fragment of the viral genome shuts off host protein synthesis (Griffin, 2013). While flaviviruses like DENV and WNV, can delay their detection in the host by hiding dsRNA in compartments synthesized with modified components from the host cell (Uchida et al., 2015; Zinzula & Tramontano, 2013). Also, by the time an animal demonstrates clinical signs, viral protein encoding might be already under IFN-induced antiviral reaction, limiting flaviviral protein detection and therefore, also dsRNA synthesis, which could have happened in case of horse 4 (Pierson & Diamond, 2013).

Additionally, for the other 6/35 horses that were negative for dsRNA and depicted degenerative lesions, these results are closely related to other authors research, who could not induce dsRNA staining in detectable levels by stressed cells undergoing apoptosis (Son et al., 2015). Meanwhile, RABV and BoDV, both negative-stranded RNA (nsRNA) viruses, and brain fragments of a horse without neurological disease were also dsRNA-negative in the IHC. For Son et al. (2015), however, dsRNA is produced by most animal viruses in sufficient amounts

to be detected by immunofluorescence, including those with nsRNA. The authors still suggest that failure in detection might be due to production of smaller amounts of dsRNA in negative-strand RNA virus infections, the use of the less sensitive J2 Mab, the choice of less optimum times for post infection sampling (like during low viraemic phases), or technical issues.

This is the first time that the antibody was tested with brain tissue of naturally infected animals, some still with an unknown etiologic diagnosis. Although interesting results were observed when employing the dsRNA antibody to the 35 samples, detailed sensitivity and specificity research have still to be carried out with a larger equine sample size. Also, disease-specific investigations should be performed to determine if there is variability in reaction within the same animal species, or if it also varies for different hosts.

5.1.3 Optimized molecular assays

5.1.3.1 RNA isolation from FFPE material

Through the comparison of three extraction protocols, a methodology was established that was able to reverse most of the cross-linking subjected to FFPE material, along with increase in extraction yield which allowed a larger RNA input with satisfactory purity rates concerning contaminants (Boos et al., 2019). The best achievement (protocol 3) consisted of simple features added to the deparaffinization step, followed by a moderately harsh lysis, with increased digestion time and volume of the reagents. Moreover, it is important to emphasize that the commercial extraction kits are adequate tools when fresh FFPE material is available and only simple modifications to the protocols are needed to improve their yield for archive samples. When dealing with FFPE material of long storage, the protocol 3 also contributed to satisfactory results in usefulness of the isolated RNA. For a thorough discussion regarding the methods tested, see the publication Boos et al. (2019).

5.1.3.2 Pan-RT-PCRs

Pan-RT-PCRs were optimized after the improved RNA extraction (3.7.1) demonstrated that was possible to amplify up to 170 bp products from housekeeping gene with most of the Brazilian archived samples. Assays targeting high-conserved regions from the genome of pathogens and with short amplicons adjusted mostly for semi-nested reactions with subsequent Sanger sequencing provided a suitable alternative to genus and species-specific assays. With this approach RABV (horse 2), the flavivirus Rocio virus (ROCV, horse 4), and the alphaviruses Madariaga virus (MADV) and Mayaro virus (MAYV) were detected (see 5.3.1 for details). Protozoa were also detected with this approach (horses 1, 2, 9) so that some horses were possibly carrying protozoa pathogenic to other animal species (horses 3 and 5, see 5.3.2.1).

Besides, adaptations from a qPCR assay to detect EHV-1 genes for glycoprotein B were also successful (horses 24, 26, 27) (Leutenegger et al., 2008).

Although other studies have demonstrated reliable results with universal PCRs for arboviruses i.e. (Grywna et al., 2010; Kuno, 1998), the primary focus of most molecular investigations in FFPE tissue was to compare its performance against fresh tissues, usually in assays targeting amplicons too long for stored material (Bhudevi & Weinstock, 2003; Nam et al., 2014). Meanwhile, the use of universal nested or semi-nested PCRs for pathogen detection are less frequently described (Bronzoni et al., 2004; Pfeffer et al., 1997; Scaramozzino et al., 2001). This might be due to challenges regarding genetic diversity and restricted length of conserved regions (Patel et al., 2013), added to high susceptibility to contamination by handling the samples repeatedly (Keer, 2008). However, when using FFFPE tissue there was the definitive need for assays with smaller amplicons, since none of the samples could amplify the 210 bp product from a previously established pan-alphavirus nested PCR (Grywna et al., 2010), the 250 bp from a pan-flavivirus qRT-PCR (Kuno, 1998; Scaramozzino et al., 2001), nor the 382 bp from a pan-coccidia semi-nested PCR (Miller et al., 2009).

Also, the implementation of an ITS1 locus amplification of coccidia was considered for this study to discriminate the parasites at the species level. However, to accomplish such level of specificity might be impossible when using short amplicons in a single plex assay, once these nucleotide differences are found across ~1100nt (Dubey et al., 2015).

The likely effects of contamination were strictly considered in this study, with amplifications only considered successful if concurrently negative controls and non-template controls (NTC) remained negative throughout all steps. In overall, successful amplifications were achieved with dilutions of positive controls originated from cell cultures, fresh tissues, and FFPE material. This demonstrates the exceptional sensitivity of the RT-PCR protocols, providing tools to re-analyse unresolved cases. Furthermore, a base cycling protocol was adopted consisting of 15 min at 95°C, followed by 35 to 40 cycles of denaturation for 30s at 94°C, annealing temperatures ranging according to the primers, elongation for 90s at 72°C, and final elongation for 10 min at 72°C. These cycle conditions were effective in PCR product visualization of all reactions carried out with positive controls, along the consistent negative results for negative controls and NTCs. This similar establishment of protocols facilitate samples/thermo cyclers adjustments and rearrangements of protocols for further use in high-throughput assays, like multiplex PCRs, whole genome sequencing (WGS), and targeted genome sequencing (Avraham et al., 2016; Brinkmann et al., 2017; Karamitros & Magiorkinis, 2018). The practical use of virus family-based and protozoa-based amplifications for cases

suspected of an infectious non-suppurative encephalitis by pathogens that are usually in suboptimal detection levels was also addressed by the described adjustments.

5.1.4 Metagenomic screening as an ancillary tool for investigations

The metagenome was obtained from the CNS of 10/35 (28%) horses from the cohort in cooperation with Arnt Ebinger, Institute of Diagnostic Virology and Dr. Dirk Höper from the Laboratory of NGS and Microarray Diagnostics, Friedrich-Loeffler-Institute, Greifswald. They have applied an untargeted metagenomic next generation sequencing (NGS) modified for low-quality RNA samples (Niller et al., 2020) to detect known, unexpected, or newly emerging pathogens using FFPE material. With midbrain sections from horse 31, MADV infection was confirmed (discussed in 5.3.1.2); for horse 22 a possible infection by *Parascaris equorum* was suggested, while five other animals also presented high number of reads for the parasite (discussed in 5.3.1); horse 7 was considered a carrier of the human-pathogenic bacteria *Elizabethkingia anopheles* (discussed in 5.3.2). For the other three animals (horses 6, 11, 16) an etiology was not possible due to the high amounts of environmental contaminants.

So far, there are only other two studies of untargeted equine metagenomic screening: one investigated plasma, nasal swab, and faeces in a deep sequencing assay to characterize the eukaryotic viral sequences of ill horses with a suspected viral infection (Li et al., 2015), and the other investigated plasma, CSF, and respiratory swab of horses with unexplained neurological and respiratory signs (Altan et al., 2019). These studies are especially important, as they try to provide new candidates for epidemiological studies. However, so little is still known about what can normally be found in the equine genome so that it is not known at what extend horses carry newly discovered pathogens (especially pathogens for other species). Besides, the presence of an infectious agent in a metagenomic screening does not mean *disease*. In this study, an organism was considered as underlying the pathology if it assembled to the samples nucleic acid, if it produced contigs in the "further analysis", if it could be demonstrated by other means in the study – histopathological analysis, IHC, and/or PCR – and if clinical-epidemiological reports from the cases were suggestive of such an infection. These harsh parameters avoided false assumptions for an etiology and were stringent with contaminations (Smuts et al., 2014).

5.2 Epidemiology of the model cohort

Samples used in this study were obtained from an equilibrated population of male and female animals, predominatly from mixed breeds, mostly older than four-years-old, and that died during fall. For the few animals that had an utility specified, seven were used for riding and one for pulling cart. Although no correlation tests were carried out in this study regarding

these characteristics, a higher number of males was infected/carried protozoa (8/9) compared to females (1/9). Similar evidence of male predisposition to protozoal encephalomyelitis was described by Boy et al. (1990), yet, for these authors, it seems that there was an environmental influence towards racing horses, which are male in majority. Certain breeds, like Standardbreds, Thoroughbreds, and Quarter Horses are also commonly observed to get infections like EPM (Reed et al., 2016), while for other pathogens there are not enough evidences for breed predisposition (Radostits et al., 2007). In this study, this lack of correlation was also observed, while a higher number of samples from mixed bred animals could be a regional influence, as well as a possible selection towards working horses and the socio-economic status from owners. Age on the other hand, is in most surveys a determinant to the devleopment of certain conditions. This study focused on primary sources of non-purulent neurological disease in horses, which might have selected samples predominantly of adult and aging animals. This could explain the viral and parasitic findings in this study, reported as a signal of extended exposure time, lack of appropriate sanitary management and/or defficient vaccination programs (Ehizibolo et al., 2012; Tyler et al., 1993). Moreover, the seasonality of neurological diseases in this study shifted towards fall. This finding matches the reports from arbovirus infections with the cases confirmed/suggested here for 10 horses. These samples were retrieved from areas known to have had outbreaks of diseases like eastern equine encephalitis (north and northeast), and where serological studies have demonstrated the circulation of viruses from *Togaviridae*, *Flaviviridae*, and *Orthobunyaviridae* in horses (Midwest) (Campos et al., 2013; Pauvolid-Corrêa et al., 2011, 2017; Silva et al., 2015; Sousa et al., 2015). For other groups of animals, like EPM cases and suggestive of toxicoinfections, death was also mostly reported in fall. Although EPM risk factors involve the presence of the definitive host (opossum) in the property premises, both EPM and food-borne toxicoinfections are more likely a subject of management failure than seasonality (MacKay, 2014; MacKay et al., 2000; Radostits et al., 2007; Saville et al., 2000).

5.3 Detection of pathogens in the CNS and suggestive etiology for unconfirmed cases

With the methods provided, in 20/35 cases (57%), the infectious agent detected could be related/suggested to the disease displayed by the cases. For other 15/35 (43%), an unknown infectious etiology was suggested, or degenerative lesions could have mimicked an infection. Concurrently, 4/35 (19%) were also considered pathogen carriers of *Babesia* spp. (horses 3, 5, 6) and *E. anopheles* (horse 7), detected by PCR and NGS, respectively.

5.3.1 Viral encephalitis

5.3.1.1 Rabies co-infection with *Sarcocystis* sp.

In this study, samples from horses 1 and 2 retrieved from the archives in Mato Grosso state, were sent for further investigation after IF for rabies was only positive for horse 2. Both animals died after a 48h clinical course of hind limbs paralysis and weakness (appendix 4). In this study, confirmed RABV proteins in the IHC and amplification of RABV RNA was possible only for horse 2. However, both samples displayed parasitic-like structures in histology in the spinal cord, also stained by IHC with anti-*S. neurona* antibody and coccidia DNA was amplified by semi-nested PCR.

Both RABV and EPM might be clinically indistinguishable, and definitive diagnosis is achieved by *post-mortem* examination (Radostits et al., 2007). To the best of our knowledge, there are no reports of rabies/protozoa co-infection. Differences were only observed by histology, where lesions from horse 1 were extensively widespread throughout the spinal cord also involving the meninges, accompanied by large malacic areas filled with larger quantities of parasites when compared to horse 2. Also, the inflammatory infiltrate in the spinal cord of horse 2 was practically only lymphocytic, suggesting an older lesion, while horse 1 had a mixed cellular population. A more specific finding during RABV infection would be the formation of Negri bodies within the cytoplasm of neurons, but these were not observed by histology in horses 1 and 2, and are an overall challenge to be identified in horses (Long, 2015).

However, viral aggregates were strongly immunostained within the cytoplasm of almost all neurons and glial cells from horse 2. Concurrently, the successful PCR amplification of a segment from the L-gene and sequencing of a RABV isolate comparable to others previously reported in this region demonstrate the usefulness of the technique for FFPE material and for this sample archived since 2008. The Brazilian isolate (Accession JN897322) obtained from horse 2, was first isolated from cattle in Jaboticabal city, in Säo Paulo state (Carnieli et al., 2012). The authors traced the virus phylogeny back to RABV transmitted by vampire bats *Desmodus rotundus*. The strain belongs to a larger group of hematophagous bat-related viruses of the lineage II, which is also known to circulate on the northern Amazon region (Condori-Condori et al., 2013; Lavergne et al., 2016). This finding strengthens the awareness to the aerial and rural rabies cycles. However, rabies is recognized to occur in every areas in Brazil so that any restriction or preference in the investigation of a specific cycle is not recommended (Queiroz et al., 2012; Rocha et al., 2015).

5.3.1.2 Alphavirus encephalitis

In this study 9/35 cases were confirmed and/or were suggestive to have had an alphaviral infection. These were adult female (horses 24, 26, 27, 29, 30) and male (25, 32, 33) (no information on horse 31) horses that died during fall, overrepresented by the Marajoara breed (which is only found in the Marajó Island, north Brazil). Signs of circling, ataxia, and recumbency were also frequently described among these cases. Histologically, predominance of lymphoplasmacytic infiltrates in all samples, that could also contain eosinophils and macrophages, varying in severity from mild to severe, with forebrain areas more commonly affected, followed by midbrain sections were found. Also, most of the cases had moderate astrogliosis, except for the forebrain region of horse 29 with severe astrocytic activation. Microglial activation was in general moderate among the cases. It was also possible to detect alphaviral epitopes in the IHC of two cases (horses 27 and 31), alphavirus non-structural protein 4 gene amplified from the PCR of two cases (horse 31 and 32) and NGS from horse 31 confirmed MADV in this sample.

Studies conducted in Brazil with fresh brain material from these cases also found alphaviruses by semi-nested RT-PCR (horses 24-27, Campos et al., 2013) and IHC (horses 29-33, Sousa et al., 2015) as the pathogen underlying the cause of death. The clinical and histological findings fit with other cases of alphavirus infection in horses (Long, 2015a; Oberste et al., 1998; Silva et al., 2011). Clinical signs of circling and ataxia are most frequently reported, consistent with the multifocal lesions predominantly in forebrain and midbrain (Oberste et al., 1998; Oliveira et al., 2014; Silva et al., 2015; Silva et al., 2011). Histologically, grey matter of the forebrain and midbrain were the most severely affected areas as described elsewhere (Chénier et al., 2010; Oliveira et al., 2014; Radostits et al., 2007). Additionally, horses 24, 27, 29, 30, 31 and 33 depict scattered eosinophils amidst the predominant lymphocytic inflammatory infiltrate which has not been described in the literature from cases outside Brazil (Chénier et al., 2010; Oberste et al., 1998) but neutrophils have already been detected admixed with lymphocytes in reports of fulminant cases of EEEV in horses from Canada (Chénier et al., 2010), VEEV in horses from Colombia (Roberts et al., 1970), and MADV in a child from Panama (Luciani et al., 2015). A shift to a predominant lymphocytic infiltrate occurs with disease progression to chronic phase (Long & Gibbs, 2014). Moreover, inflammatory infiltrates were accompanied by neuronal necrosis, satellitosis and neuronophagia in the studied cases. Cantile and Youssef (2016) also observed that in more severe cases. Necrotizing vasculitis, as seen in the brain of horses 27, 29, 30, and 32, and parenchymal vacuolization, only observed in the spinal cord of horse 32 has also been described by Sousa et al. (2015). While by IHC of the

brain of horse 27, there was staining limited to the neural soma, and within glial cells of horse 31, in accordance to other findings (Pennick et al., 2012). Moreover, the negative results by RT-PCR for horse 27 might be explained by contaminants carry over presumably due to sampling and fixation procedures (260/230 values of 1.36 and 1.63), even though the material was able to amplify up to 170bp amplicon products of housekeeping gene GAPDH.

As for horse 32, the expected amplification product of 90bp was successfully obtained and its sequencing analysis demonstrated high identities with Mayaro virus (MAYV) from the Semliki Forest Virus group. This virus is closely related to CHIKV and has emerged in Latin America in the last few years as a threat for causing acute, self-limited febrile period, and persistent arthralgia in humans (Hotez & Murray, 2017; Taylor et al., 2005; Theilacker et al., 2013). It is now considered endemic in the north, mid-west, and southeast Brazil, with neutralizing antibodies circulating in horses from the Pantanal region, near horse 32 living location (Pauvolid-Corrêaet al., 2015). The most similar strain to horse 32 sample (KP842813.1) was detected in an outbreak in Peru in 2011, and corresponds to genotype D (Auguste et al., 2015). This is the most widespread genotype through South America and the Caribbean, estimated to have non-human primates like marmosets (*Callithrix argentata*) and wild birds as hosts (Hoch et al., 1981) and *Aedes* spp., *Culex* spp., and *Haemagogus* spp. mosquitos as vectors (Hoch et al., 1981; Long et al., 2011; Serra et al., 2016). The circumstances for a neurological onset are yet incompletely understood. Pathogenesis of MAYV and other arthritic alphaviruses is related to viral invasion into macrophages and viral replication in them, followed by cytokines activation leading to the febrile and regional inflammatory processes (Cavalheiro et al., 2016). Yet, no macrophages nor activated microglial cells were observed in brain samples from horse 32. These findings could represent similarities with Sindbis virus and Semliki Forest virus, which demonstrated neurovirulence in neonatal mice associated to neuronal damage linked to E2 and to Nsp3 regions of the viral genome. E2 protein is believed to be involved in tropism for neurons and passage across the blood-brain-barrier (BBB), while the role for Nsp2 remains undiscovered (Atkins & Sheahan, 2016).

Other findings concerning alphaviruses, involved the screening of Sindbis virus by NGS in brain samples from horses 16, 22, and 35. However, a pathological involvement of this agent could not be confirmed by other means in this study nor linked to the clinical history in these cases.

5.3.1.3 EHV-1 co-infection with alphavirus

EHV-1 infection was suggested in three cases (horses 24, 26, 27) from the study. A fragment from glycoprotein B (ORF33), responsible for viral entry into host cells (Nugent et al., 2006; Telford et al., 1992), was amplified with PCR in the three cases, but no immunostaining was observed. Furthermore, an active alphaviral infection was also suggested for the clinical signs and lesions observed in the cases, as discussed previously in the subtopic 5.3.1.2 and also suggested in an earlier investigation with those samples, that used fresh CNS material (Campos et al., 2013).

Nevertheless, if an investigation were based only on the clinical signs observed, it would be difficult to distinguish equine herpesviral myeloencephalopathy (EHM) from alphavirus encephalitis. An active EHM case normally induces febrile state/fever previous to clinical signs onset, and the disease tends to affect more frequently the caudal segments of the spinal cord and sacral plexus, and thus affects more severely the hind limbs (Dunowska, 2014; McFadden et al., 2016; Slater, 2014). An encephalitic alphavirus frequently induces signs related to brainstem, cerebral cortex, and cerebellum, relatable to the cases in this study (Long & Gibbs, 2014). The gross lesions, on the other hand, could suggest a vasculopathy induced by EHV-1, characterized by the meningeal and medullar haemorrhages, described in the necropsy reports from horses 24, 26, and 27 (Slater, 2014). Although no consistent gross lesions are found in equine alphavirus infections, the meninges are usually congested, especially described for EEEV infection (Long & Gibbs, 2014). Histologically, both viruses produce lesions that could predispose an infection in favour to the other: EHV-1 affects directly vascular endothelium cells producing necrosis, thrombosis, and ischemia, while alphaviruses suppress the host innate immune response (MacDonald & Johnston, 2000; MacLachlan & Dubovi, 2011; Radostits et al., 2007). EHV-1 neurotropic strains have been associated with a higher amplitude and longer duration of viremia, as well as with enhanced neuropathogenicity which could affect or even facilitate a secondary infection (Allen et al., 2008; Marenzoni et al., 2013). However, a genotypic characterization towards a neuropathogenic (D752) or non-neuropathogenic (N752) strain in the cases described in this study was not possible due to the lack of ORF30 amplification, although other studies revealed neuropathogenicity from either genotype (Pusterla & Hussey, 2014).

This is the first study to describe EHV-1 glycoprotein B amplification from the archived brains of Brazilian horses, and the first description of a probable co-infection with an alphavirus. Other reports on herpesvirus as naturally co-infecting agent refer mostly to neonatal loss and abortion (Ellis et al., 1976; Marenzoni et al., 2013; Smith et al., 2010). In these,

neuropathogenic strains of EHV-1 were involved in co-infections with bacterial agents such as pulmonary *Klebsiella pneumoniae*, septicaemic *Actinobacillus equuli* (Marenzoni et al., 2013), and undifferentiated EHV-1 strains co-infection with *Rhodococcus equi* (Perez-Ecija et al., 2016). All these reports have in common with the findings here described, that the herpesvirus was isolated from the same organ as the second infectious agent, and the clinical signs from one infection might mimic or are similarly expressed on both conditions.

5.3.1.4 Flavivirus encephalitis

Samples from the CNS of a 7-year old mixed breed male horse (horse 4) from the north of Mato Grosso state (MT, Carlinda city, southwest Brazil) yielded positive amplification for the non-structural protein 5 (NS5) of the flavivirus ROCV by semi-nested RT-PCR. Other findings regarding clinical history, epidemiology, and histological lesions strongly support the infection. Other three horses (horses 3, 5, and 6) also showed compelling signs of a flaviviral infection, indicated by the clinical history and signs, residence in a risk area (living in flooding areas and near wildlife increases the risk) and the season of infection; these factors have also been considered by other authors (Delcambre & Long, 2014; Prow et al., 2013). However, active disease in these three animals could not be proven with the techniques in this study and might suggest a subclinical or persistent infection, or inadequate sampling.

Flaviviruses circulate among Brazilian equine herds and have been especially detected in the Pantanal from Mato Grosso do Sul state, neighbour to MT (Melandri et al., 2012; Ometto et al., 2013; Pauvolid-Corrêa et al., 2014, 2011; Pauvolid-corrêa et al., 2010; Silva et al., 2013). Neurological disease in horses has been associated so far to SLEV (Rosa et al., 2013) and WNV (Silva et al., 2019) both in the Brazilian southeast region. Natural disease by ROCV on the other hand, was so far only detected in humans from an outbreak of encephalitis in south-eastern São Paulo state in Brazil in late 1970's (de Souza Lopes et al., 1978; Medeiros et al., 2007). However, re-emergence and spread of ROCV through the country is feared as it has been detected in serological studies of human patients with dengue-like symptoms (Saivish et al., 2020). Although no clinical disease in horses has been reported, ROCV induced monotypic serum reaction (IgG) in 46/753 horses (6.1%) surveyed from southeast and southwest Brazil (Silva et al., 2014). Death from horses in this study was reported to have occurred in late summer (horses 3, 4, 5, 6), also described for human cases of ROCV and other arboviruses (Iversson, 1977; Long & Gibbs, 2014). Duration of illness could be only assessed from horse 4, with 72h of clinical onset until death, while in ROCV fatal infections, death occurred between 3 to 64 days (Rosemberg, 1980).

Discussion

Clinically, signs from all four suspected cases were highly unspecific, but match with findings of behavioural changes, gait abnormalities, and cranial nerves alterations reported in human cases of ROCV (Rosemberg, 1980). Histological alterations in the cerebral cortex of horses 3, 4, 5, and 6 revealed predominant lymphoplasmacytic inflammatory infiltrates that ranged from mild to severe. All four cases had neuronal necrosis, satellitosis and neuronophagia, with mild diffuse microglial activation, but severe astrogliosis only in the cerebral cortex of horse 4. Meningitis was also observed in horses 3 and 5, like in cases of mice experimentally infected with ROCV (de Barros et al., 2011). Although the histologic lesions observed in human and equine flavivirus infections by ROCV, WNV, and SLEV are similar to those found in horses 3, 4, 5, 6 in this study, lesions tend to occur more frequently in ventral/caudal areas of the CNS – thalamus, basal forebrain, brainstem, dentate nucleus of cerebellum, and spinal cord. Therefore, lack of complete sets of CNS might have hindered an etiologic confirmation by IHC (Autorino et al., 2002; Cantile et al., 2001; Cantile & Youssef, 2016; Porter et al., 2003; Toplu et al., 2015). Furthermore, antibody detection in flavivirus cases is limited and considered by the OIE as partially suitable for confirmation of clinical cases (OIE, 2019h). Still, horse 3 showed positive immunostaining for dsRNA within the neuropil, which could suggest an infection by a positive strand RNA virus (Weber et al., 2006). The semi-nested RT-PCR was able to amplify a small sequence of the flaviviral NS5 from the cerebral cortex and cerebellar peduncle from horse 4, but not from horses 3, 5, and 6. ROCV persistent infection in hamsters was demonstrated by Henriques et al. (2012) to be similarly difficult to detect by qRT-PCR using the proper brain areas for investigation and could be therefore, below the semi-nested RT-PCR detection. Moreover, no flavivirus (nor other arbovirus) was assembled in the metagenome from horse 4. This could indicate a limitation in the technique to identify pathogens in low concentration and it might translate that the 200bp cut off in the NGS is still too long for quality compromised samples. Yet, the absence of a complete CNS set for investigation in horse 4, especially from brainstem and thoracolumbar spinal cord, pose a serious limitation to the effectiveness of the tests carried out in the study.

ROCV cannot be ruled out as a potential neuro-infectious agent for horses and should be considered therefore in encephalitis cases. Horses are known to be extremely sensitive to flaviviruses, especially when exposed to risk factors that facilitate an infection. Additionally, most of the epidemiological and biological aspects of ROCV infection are largely unknown, and disease in species other than humans are only known from experimental studies (de Barros et al., 2011; Henriques et al., 2012). Horses were demonstrated to be suitable sentinels for WNV and other neuro-infectious viruses, and further investigations based i.e. on virus replication in

equine cells could help understand ROCV pathogenicity.

5.3.2 Protozoal encephalitis

5.3.2.1 Equine protozoan myeloencephalitis and horses as carriers of Babesia spp.

For 9/35 (26%) cases investigated, protozoa were identified either through parasite identification and antigen detection by IHC using the anti-*S. neurona* antibody (horses 1, 2, 9, 19, 20), and through coccidia DNA amplification (horses 1, 2, 3, 5, 6, 9). Horse 18 is included in this section as a suggestive case. Horses 3, 5, and 6 were considered carriers of *Babesia* sp., identified with the semi-nested PCR, and the horse 2 was already described in the subsection 5.3.1.1 Rabies virus. Furthermore, metagenomics analysis was able to screen *Sarcocystidae* pathogens from other cases (horses 11, 16, 22, 35), but no evidence of clinical disease could be determined for them.

In general, infections by *S. neurona* have been sporadically reported throughout Brazil in the last 30 years. Horses 1 and 2 refer from the Brazilian Midwest region, while horses 9, 18, 19, and 20 originated from the Rio Grande do Sul (RS) state, the southernmost region in the country and the place where the first *S. neurona* infection was reported in Brazil in 1986 (Faria et al., 2017; Lins et al., 2008; Barros et al., 1986). In this region, serological results of 36% of slaughterhouse horses indicated exposure to the protozoan (Portella et al., 2017). Also, the natural reservoirs of EPM, the opossums, are found endemically, not only in the south, but widespread in the country (Cáceres et al., 2016).

The animals considered clinically infected (horses 1, 2, 9, 18, 19, 20) with protozoa in this study were adult mixed breed males that died mostly during fall (5/6) or summer (1/6). Clinical signs were unspecific and suggested multifocal lesion locations depending on the parasite location in the CNS (Dubey et al., 2015; Radostits et al., 2007). Progressive weakness (horses 1, 2) and ataxia (horses 9, 18, 20) involving especially hind limbs are also frequently described (Dubey et al., 2015). Gross lesions, when observed, were confined to the CNS, characterized by a mixture of acute and chronic patterns, as red areas of the spinal cord amidst with yellow areas in the white matter (horses 1, 9), and diffusely cherry-red brain (horse 19). These findings are similar to those described in other studies regarding protozoal infections, where lesions are more frequently observed in the brainstem and spinal cord as focal, segmental spinal haemorrhagic pattern (Mayhew et al., 1978; Witonsky et al., 2014). Hence, the most dramatic microscopic lesions were also observed in the spinal cord (horses 1 and 9). Other studies propose that the asymmetry in hind limbs muscle atrophy is highly suggestive of EPM, but in this study none of the cases described such lesions in the necropsy reports (Dubey et al., 2015; Radostits et al., 2007; Witonsky et al., 2014).

Histologically, the intensity of the lesions ranged from mild to severe, with parenchymal vacuolization and vast malacic areas observed as lesions intensified, sometimes accompanied by haemorrhagic foci. Spinal cord sections were more frequently affected, usually accompanied by a mononuclear inflammation admixed with eosinophils. While eosinophils are present in most parasitic infections of the CNS, in most allergic processes, and in some intoxications, Mott cells and giant cells are often seen during chronic inflammation (Boos et al., 2012; Furr, 2015b). Their presence has been described mainly in cases of protozoal encephalitis associated with *Trypanosoma evansi* infection in horses (Rodrigues et al., 2009; Rodrigues et al., 2005), but parasitic morphology in the IHC and Sanger sequencing from PCR products from horses 1, 2, and 9 indicated an infection by a *Sarcocystis* sp. Concurrently, the severe granulomatous meningomyelitis observed in horse 9 is a classical lesion pattern associated to protozoan infections, typically with correspondent lesions such as neuronal necrosis, and activated amoeboid microglia cells (Gitter cells) (Gerhold et al., 2014; Hodo et al., 2016; MacKay et al., 2000). Although satellitosis and/or neuronophagia are most reported in viral infections, they might also occur in a protozoal lesioned tissue due to parasitic intracellular replication, as observed within the neuronal soma of horse 20 by IHC. Furthermore, the neuronal lesion triggers microglial nodules (Summers et al., 1995). In this study, protozoa were only observed by IHC (horses 1, 2, 9, 19, 20), and for horse 1 an unidentified larvae egg was also found but no clinical correlation was found so far. Protozoal feeding and motile forms, as the sporozoites and merozoites of *S. neurona* are found in tissue sections in approximately one-third to one-half of cases (Boy et al., 1990; Witonsky et al., 2014). Parasites might be observed either free in tissues (horses 1, 2, 9) or within neurons (horse 20), leukocytes (horses 1 and 2), vascular endothelium (horses 19 and 20), and glial cells (Reed & Andrews, 2004; Witonsky et al., 2014). Meanwhile, the only surely identified parasite structure in the CNS of horses are mature schizonts (Dubey et al., 2015). Other interesting finding by IHC was positivity for dsRNA antibody in spinal cord sections from horse 1. Although uncertain, an explanation could be the extensive lesions produced by *Sarcocystis* sp., which could have induced cellular stressors like pro-inflammatory cytokines, similar to a viral infection (Der et al., 1997; Walochnik & Duchêne, 2016).

For horses 3, 5, and 6, PCR results indicated that *Babesia* spp. were present, even though the semi-nested PCR optimized in this study was aligned with coccidia sequences. The Apicomplexa phylum possibly share similarities that also enabled the detection of these parasites, displaying high identities with the sequences retrieved from these cases (Li, 2003). In the metagenomic screening from horse 6, reads from *Babesia* sp. XXB-HangZhou (accession

KT895089.1), along with *B. microti*, *B. divergens*, and *B.* sp. *spanish dog,* can suggest this animal as carrier of the cited parasites to other animal species (Gaffar et al., 2003).

Amplification of coccidia DNA was not possible by the semi-nested reaction with samples from horses 18, 19, 20, which might occur in samples with low pathogen load (Frickmann et al., 2013). Also, the universal coccidia PCR, kindly performed by the Institute of Parasitology, JLU Giessen, aiming for the amplification of a 382bp amplicon was unsuccessful for all samples tested. In this case, the extended fixation time of the samples which led to an unbreakable crosslinking of DNA with proteins, frequently observed in FFPE tissues, might have hindered a positive outcome (Bonin et al., 2003; Gerhold et al., 2014; van Beers et al., 2006).

5.3.1 Verminotic meningoencephalomyelitis by *Parascaris equorum*

Findings concerning horse 22 suggested a parasitic neuroinfection, most probably induced by the *Ascaridae Parascaris equorum*. This diagnosis was suggested based on the clinical history, severity of lesions comparable to other worms knowingly migrating through the CNS of horses and other animals, and the successful assembly of sequences from *P. equorum* with NGS. Also, consistent negative results for all other infectious agents tested in this study supports this finding.

The history from this 10-year-old male American Quarter Horse lasted around one month, starting as swelling of the left side of the face, passing through a myriad of vestibular and cerebellar signs, leading to health decline till external decubitus. Even though a possible hematozoan infection was considered, tests (non-specified which) results were negative, there was no reference to whether the animal received anti-helminth treatment, nor if there were lesions in other organs that could correspond. Gross lesions in the CNS referred to as yellow areas in the brainstem white matter could fit with a nematode infection. In general, tracts of nematode migration throughout the brain of horses leave brown discoloration and haemorrhagic foci when larvae hit a vessel, or tortuous tracks are excavated, leading to traumatic injuries later observed as microcavitation (vacuolization) and malacia (Bak et al., 2017; Cantile & Youssef, 2016; Mittelman et al., 2017). Also, the “mass-like” formation at the cerebellar base, was later confirmed in the histological exam as a choroid plexus papilloma (CPP). There are only a few reports of CPP in horses, but the clinical signs described fit to the ones in horse 22 (ataxia and/or incoordination, cranial nerve alterations) (Pirie et al., 1998; Sardon et al., 2008; Haghdoost & Zakarian, 1985).

Meanwhile the inflammatory histologic lesions in horse 22 were more severe than those described by other studies with *Setaria digitata* and *Parelaphostrongylus tenuis* migration

through the CNS of horses, which consisted mostly of granulomatous and eosinophilic meningoencephalitis (Cantile & Youssef, 2016; Frauenfelder et al., 1980). Lesions in the CNS of horse 22 approximate in severity of those produced by *Halicephalobus gingivalis* – a severe predominant mixed inflammatory infiltration of brain and meninges, with variable amounts of eosinophils, numerous macrophages, and eventually giant cells, added to extensive areas of parenchyma vacuolization and spheroids formation (Cantile & Youssef, 2016; Hermosilla et al., 2011; Onyiche et al., 2018). *Baylisascaris procyonis*, the common roundworm of raccoons, infects humans and other primates that serve as intermediate hosts (Langelier et al., 2016; Wise et al., 2005) and can induce severe and frequently fatal CNS disease with similar histologic lesions (Gozalo et al., 2008; Langelier et al., 2016; Wise et al., 2005). *Parascaris equorum* is closely related to *B. procyonis*, and although there are no records of cerebral larva *migrans* due to *P. equorum* infection, its route of infection and the dramatic proportion of lesions observed on horse 22 could then be explained. Tissue migration of ascarids is far more aggressive and continuous than other parasitic infections that cause *larva migrans*, such that migration tracks through the CNS and other tissues. Ascarid larvae are only accidentally observed as they tend to move, rather than to remain in the CNS, like it occurs with *P. tenuis*, (Cantile & Youssef, 2016). This might explain why no parasitic structures were observed, neither in histology or special staining. Another factor (which might be exclusive of *B. procyonis*) is that the larvae continue to grow as they migrate, exacerbating mechanical damage to tissues. Finally, the severe inflammatory reactions similarly observed in horse 22 and cases of *B. procyonis*, occur due to the release of excretory-secretory proteins by migrating larvae, as well as the release of toxic eosinophils proteins by the host (Moertel et al., 2001; Wise et al., 2005). Likewise, larvae migration from other ascarids as *Ascaris suum* and *P. equorum* itself are reported to occur between the small intestine, liver, and lungs (Cantile & Youssef, 2016; Clayton & Duncan, 1979). Inatomi et al. (1999) described this behaviour known as visceral larva migrans (VLM) as being the probable mechanism utilized by *A. suum* to cause encephalopathy in a healthy adult man in Japan.

Furthermore, the severe microglia activation observed in spinal cord and third ventricle of horse 22 was accompanied by Gitter cells, which the Iba1 IHC cannot distinguish between microglia from macrophages recruited from the periphery. An invasion of parasite-laden macrophages was also considered as a source of parasitic genetic material available in the CNS. However, the compelling clinical history and lesions described in horse 22 strongly support the direct parasitic migration hypothesis.

Reports on *P. equorum* in faeces, albeit in lower frequency than other worms, have been described in the African continent, central Europe, Latin America (including Brazil), India, and in Saudi Arabia (Ehizibolo et al., 2012; Güiris et al., 2010; Laugier et al., 2012; Lem et al., 2012; Matto et al., 2015; Morsy et al., 2016; Rehbein et al., 2013; Teixeira et al., 2014). In these regions, horses are highly exposed to parasitic infections, either by feeding in infected pasture/silvopasture/rural area, poor body condition or by no anthelminthic medication history, and poor management practices (Francisco et al., 2009; Jajere et al., 2016; Sokół et al., 2015). In cases where an erratic migration through the CNS might occur, few treatments have been proven successful (Furr, 2015a; Tanabe et al., 2007), and therefore, it is essential for veterinarians and public health workers to be aware of possible new infections and complications. *P. equorum* sequence assembly was detected for other 5/10 samples (horses 7, 11, 16, 32, 35) sent for NGS in this study. In these cases, samples showed high counts for environmental contaminants, what possibly hampered a correct assignment of reads. Also, the histology in these cases were not indicative of a parasitic infection (Cantile & Youssef, 2016) However, there is a knowledge gap regarding the "normal" metagenome of horses and what would be the possible relations of these animals with parasites like *P. equorum*.

5.3.2 Suggestive etiology for unconfirmed cases

For 15/35 (43%) horses, an infectious etiology could not be demonstrated with the tests carried out in this study, similar to other Brazilian research with horses (Cunha et al., 2016).

Undetected pathogens causing non-suppurative encephalitis

In the clinical history from horse 7, RABV infection was suggested, but promptly refuted after a thorough histological analysis and negative results for IHC, for pan-lyssavirus PCR, and no assemblies for the virus with NGS. Furthermore, poor sampling could have hindered the identification of other pathogens since clinical signs suggested that lesions would be in brainstem, cerebellum, and eventually cerebral cortex, but only parts of spinal cord, cerebral cortex and trigeminal ganglion were available. In this regard, infections led by agents with midbrain tropism like *Listeria monocytogenes*, *Burkholderia pseudomallei*, *Neisseria meningitidis,* and bunyaviruses, that were all listed in the metagenomic screening but with low number and unassembled reads, could not be properly assessed (Cantile & Youssef, 2016; Currie et al., 2000; Tauro et al., 2015). Furthermore, the histological lesions consisted of moderate multifocal lymphocytic ganglio-meningoencephalomyelitis, with moderate astrocytic activation associated to malacic areas, severe microglia activation in the forebrain presenting numerous Gitter cells, and moderate microglia activation in the spinal cord. There was also

diffuse neuronal necrosis, neuronophagia and satellitosis but no formation of microglia nodules. Giemsa, PAS, and Gram staining did not detect possible parasites and bacteria within the lesions. An interesting metagenomic finding, on the other hand, revealed suitable assemblies with the bacteria *Elizabethkingia anophelis*, which recently has been associated to been carried by horses and possibly involved in a zoonotic cycle (Johnson et al., 2018). It is still unknown whether *E. anophelis* causes disease in horses, but there are reports of human infection, especially of hospitalized patients with severe pre-existing diseases, and includes the onset of neurological disease (Castro et al., 2017; Frank et al., 2013; Nielsen et al., 2018).

For other 6/10 cases suggestive to have an infectious cause, toxic infections like the ones caused by *Clostridium botulinum* and *C. tetani* toxins should be considered. Mostly due to signs of ataxia, flaccid paralysis evolving to sternal recumbency, and apnoea by the time of death, a botulism outbreak could have involved four horses from the same property (horses 10 to 13) in Porto Alegre, south Brazil. Exposure to *C. botulinum* neurotoxin (BoNT) cannot be ruled out for these four animals as the issue usually occurs when badly stored silage or feed offer the bacteria proper anaerobic growing conditions (Ostrowski et al., 2012; Riet-Correa et al., 2001). Although no specific macroscopic nor microscopic findings are observed with botulism, the diagnosis could be supported by the history of feeding issues, the neurological signs, GI stasis and diarrhoea, and by ruling out other diseases with similar clinical presentation, namely EPM, rabies, equine encephalomyelitis, as well as listeriosis (Furr, 2015b; Radostits et al., 2007). This suggestion is further supported by the fact that more animals from the same properties died within the same period under similar circumstances. Furthermore, although *C. botulinum* was detected in the metagenomic analysis, the bacteria is also an environmental contaminant and the assessment of its toxins would be more appropriate. This could be achieved with proteomics to determine if there was neurotoxic protein expression (Kaiser et al., 2018; Terilli et al., 2011), or the amplification of neurotoxin genes through specific qPCRs (Johnson et al., 2014; Johnson et al., 2012).

For horses 14 and 17, in the etiologic diagnosis, *Clostridium tetani* should be considered by the clinical presentation of muscular stiffness (horses 14 and 17) for about one week and the presence of ulcerated skin wound in the left hind limb (horse 14), and slow feeding (horse 17). A final diagnosis for tetanus is however, difficult to achieve when there is a poor clinical history available, unknown vaccination history, added to the fact that there are no gross nor histological findings that are confirmatory (Furr, 2015b; Radostits et al., 2007).

For horse 23, 34, and 35, lack of complete history and/or necropsy reports and availability of only one CNS region for investigation strongly hindered the identification of a

pathogen. Furthermore, high levels of sample contamination from horse 35 further hindered a proper metagenomic screening, making unfeasible to discriminate a real cause of infection from environmental contamination. Yet, the results obtained from the histology and negative results for commonly agents that circulate in horses from Brazil could match the reports of re-emerging vector diseases and the circulation of new viruses in the country (de Souza et al., 2015; Lima-Camara, 2016; Pauvolid-Corrêa et al., 2017; Pauvolid-Corrêa et al., 2015). The glial reaction pattern and inflammatory infiltration was mostly lymphoplasmacytic, which could suggest a viral infection. Besides, samples were collected from areas where arboviruses are known to circulate and to infect horses (alphavirus and flavivirus infections).

Undetected infectious agents from degenerative lesions

For 3/35 cases (8% - horses 8, 15, and 28) that presented degenerative lesions in the CNS an infectious etiology could not be determined. This was similar to cases 16 and 21 (6% - horses 16 and 21) that presented local ganglial inflammation accompanied by degenerative lesions in the CNS.

Although a complete clinical history was lacking for horse 28 case, it was accompanied by histological description of hepatic and renal lesions that could not rule out an infectious etiology and/or hepatic encephalopathy. A viral etiology should be carefully considered, especially after reports on the circulation of equine pegivirus (EPgV) in Pará state, the same origin of horse 28 (de Souza et al., 2015). The genus Pegivirus belongs to the *Flaviviridae* family, with EPgV and Theiler`s disease-associated virus (TDAV) recognized to cause acute onsets and persistent infections of hepatitis in horses from North America, France, and United Kingdom (Kapoor et al., 2013; Lyons et al., 2014). Besides, persistent infection by other flaviviruses, like in ROCV experimentally infected hamsters demonstrated to produce similar cerebral lesions – pseudo laminar neuronal medio-cortical necrosis and mild to moderate gliosis; hepatic lesions – frequent necrotizing hepatocytes and portal spaces filled with mononuclear infiltrate, and kidney lesions such as mild interstitial lymphoplasmacytic infiltrate (Rosemberg, 1980).

While there was no complete clinical history referred from horse 16, a 16-year old female, samples from this animal were sent for RABV detection in Brazil. The clinical signs by the time of death might have been suggestive of this infection. Necropsy findings of urine retention and a possible intestinal hypomotility might suggest a degenerative disease along the spinal cord, possibly involving the nerve roots and the brainstem (Cantile & Youssef, 2016; Radostits et al., 2007). Also, space-occupying masses could display similar alterations, added to ganglionitis as paraneoplastic syndrome (Radostits et al., 2007). The histological

examination of the CNS revealed more severe lesions in the striatum, and were consistent with hypertensive lesions like sclerotic blood vessels, hyaline globules deposition around small blood vessels (positive to PAS and negative to Congo red for amyloid deposition) and discrete parenchymal degeneration throughout the CNS (Price & Kasner, 2014; Summers et al., 1995). Apart from that, the lymphocytic ganglioneuritis could not be associated to any of the infectious agents tested in this study, with special reference to rabies virus (Bassuino et al., 2016; O'Toole et al., 1993). With the material available for investigation, a hypertensive encephalopathy was the most likely diagnosis. It is suggested that this condition occurs due to acute failure of cerebrovascular autoregulation resulting in inappropriate dilation of small arteries and arterioles and disruption of the BBB (MacKenzie et al., 1976). Apparently, the δ-isoenzyme protein kinase C regulates intracerebral blood vessel permeability and its selective inhibition could provide appropriate reduction on related neurological signs (Price & Kasner, 2014; Qi et al., 2008).

Post-traumatic myelomalacia/myelopathy - Acute myelomalacia

A traumatic lesion was thought to be the cause for the spontaneous recumbency, masticatory movements, caudal insensibility, and pain in the atlas region of horse 15. At necropsy, the pregnant 7-year-old mare presented a focal extensive area of dark redness with an amorphous substance overlaying the meninges at spinal cord T5-T12. A swab from the lesion site was collected and showed no significant bacterial growth. The first differential diagnosis hypothesized were of spinal myelitis due to parasitic invasion or EHV-1 (Cantile & Youssef, 2016). These tend to cause spontaneous traumatic lesions in the spinal cord, but both produce additional inflammatory infiltration in the affected tissue, not observed in horse 15 (Blazejewski et al., 2015; Slater, 2014). Furthermore, spinal cord was negative for all tests carried in the present study. However, it should be mentioned that brain tissue was not available in this case. According to Radostits et al. (2009), myelomalacia occurs rarely as an entity separate from encephalomalacia. A similar report of haemorrhagic myelomalacia describes the development of lesions after dorsal recumbency during a surgery under general anaesthesia (Lerche et al., 1993). For horse 15, however, there were no reports on any procedures that would induce such injury. In this case, another pre-existing ischemic condition, probably of traumatic origin, like a compressive or stenotic vertebral instability, could have been worsened with the advanced pregnancy. Comparable cases were described in canine progressive haemorrhagic myelomalacia, demonstrated by ischemic necrosis of the spinal cord parenchyma (Castel et al., 2017; Zilio & Arias, 2013).

Post-traumatic myelomalacia/myelopathy

The onset of neurological signs observed on horse 21 was only described after a fall, resulting in fracture between C5-C6 vertebrae. This might be an indication that the animal was already presenting signs of hindlimb weakness and ataxia. Furthermore, after verifying lymphocytic trigeminal ganglioneuritis, the laboratory of origin performed IHC against rabies which yielded negative result, similar as in this study. In the report of histological alterations observed in Brazil, there was the description of lymphocytic inflammatory infiltration in other ganglia, namely paravertebral and celiac. Nevertheless, infectious agents commonly involved in CNS disease in horses were excluded from the diagnosis due to negativity for the tests carried out in the study. Degenerative diseases that could have led to the traumatic event were carefully considered, and included investigations concerning polyneuritis equi and equine degenerative myeloencephalopathy (Hahn, 2008; Radostits et al., 2007). However, fractures of cranial cervical vertebrae are fairly common in horses (Tyler et al., 1993), they can lead to the destruction of primary sensory neurons in the dorsal roots and ganglia, leading to orthograde fibre degeneration in the spinal dorsal funiculus (Summers et al., 1995). This would explain the sparse malacic areas associated to macrophages, mild astrocytosis, and necrotic neurons observed in the spinal cord fragments of this animal.

Spinal cord subluxation

Subluxation affecting the spinal cord from horse 8 at C5-C6 led the animal to recumbency for four days right after a penicillin injection. After careful investigation to rule out an infectious agent that could possibly underline the clinical history and lesions, a degenerative condition was assumed. Additionally, the animal was cachectic, and parasitized by at least three different helminths, *S. vulgaris* within the mesenteric arteries, and *P. equorum* and *A. perfoliate* within the intestinal lumen.

While the penicillin shot might have led to the fall of the animal, the trauma occurred during the accident might have produced the microscopic lesions observed in the cervical spinal cord and in its intumescence. In adults, traumatic injury by falling sideways onto the neck base or pulling back whilst tied up commonly affect the C3-C6 arch, and the articular processes or articular process joints of the C5-C7 (Hepburn, 2015). According to Cantile and Youssef (2016), in more severe cases there is axonal loss with astrogliosis, demonstrated in this case with Luxol fast-blue stain.

5.4 Outlook

With the increase in cases of emergent, re-emergent zoonotic and neglected diseases not only in developing regions, but also in economically stable countries, there is the need of detecting pathogens with methodologies accessible to all levels of infrastructure. In this light, a significant contribution was already achieved in this study, with the optimization of tissue-based assays using FFPE material, continuously on demand in several sectors of veterinary and human health.

The next step to complement and increase the success of pathogen detection, would include the development of a multiplex system for infectious and zoonotic diseases that is easy-to-handle and accessible for less equipped laboratories and basic bioinformatics skills. Investigations would particularly beneficiate from a multiplex sequencing assay using the target enrichment technology. Genus-specific degenerate oligonucleotides will increase the sensitivity of pathogen detection and decrease host background detection especially for FFPE material. For this methodology, reagents can be prepared in a basic laboratory setting and sequencing can be performed with a portable sequencer, like the MinIon (Oxford Science Park, Oxford, UK). Moreover, this methodology can be supported by the study presented here, as it provides several morphologic, antigenic, and molecular detection of pathogens.

The optimized methodology for equine samples as model system will then also enable zoonotic pathogen assessment in humans and could also be transferred to important livestock species, like cattle, small ruminants, and pigs, which share susceptibility to several infectious and zoonotic agents. It is then expected that during an outbreak e.g., basic laboratories located near affected areas will be able to provide an etiology using the method quickly and precisely.

6. Summary

Worldwide reports on clinical disease and serological surveys from horses with encephalitis and encephalopathies reveal the increase in emergent, re-emergent, and neglected diseases, mostly due to an infectious agent. Diseases caused by arboviruses or by henipaviruses are known zoonoses, while equine herpesvirus 1 (EHV-1) or protozoal encephalitis are detrimental to animal's performance. However, while most of diagnostics tools are adjusted to fresh tissues, formalin-fixed paraffin-embedded (FFPE) tissues are vastly used in pathology. Therefore, this study used FFPE tissue from the central nervous system (CNS) of 35 horses from Brazil suffering from non-suppurative encephalitis or encephalopathies as study model. The main aim was to provide adequate diagnostic tools adopted to FFPE tissues for morphologic, antigenic, and molecular detection as well as metagenomic screening of relevant pathogens. Samples available for the study consisted of a mixed population of male and female animals, mostly > 4 years-old, predominantly of mixed breeds. Most of the clinical signs (21/35) pointed to a multifocal distribution of lesions in the CNS, and most cases (20/35) occurred in fall. Histologically, 28/35 cases had inflammation in at least one CNS region or ganglion, and 7/35 had degenerative lesions. Score systems established to assess the degree of inflammatory cell infiltration and glial reaction patterns, revealed the predominance of mild lymphocytic inflammation, and 3 statistically distinguishable grades of astrocytic and microglial activation throughout the cohort. In 20/35 cases, an infectious agent could be related to disease - rabies virus/coccidia coinfection (1/35), alphavirus (6/35), flavivirus (4/35), alphavirus/EHV-1 coinfection (3/35), coccidia (5/35) detection, and helminth larvae migration (1/35). For other 15/35 cases, an unknown infectious etiology was suggested, or degenerative lesions could have mimicked an infection. Concurrently, 4/35 cases were also considered pathogen carriers of *Babesia* sp. or *Elizabethkingia anopheles*. The long-term stored FFPE materials used in this study provided valuable epidemiological information and identification of pathogens important for animal and human health. Missing CNS regions, environmental contamination, and protein cross-links bound during fixation might have hindered pathogen identification. In this regard, optimization of techniques such as multiplex sequencing assays using target enrichment could further improve the sensitivity of pathogen detection and decrease host/environment background. Moreover, the methods optimized in this study enable zoonotic pathogen assessment in humans and can also be transferred to important livestock species, like ruminants and pigs, which share susceptibility to several infectious and zoonotic agents. It is expected that during an outbreak e.g., basic laboratories located near affected areas will be able to provide an etiology using the methods quickly and precisely, with the routine procedures of tissue fixation.

7. Zusammenfassung

Weltweite Berichte über klinische Krankheiten und serologische Untersuchungen von Pferden mit Enzephalitis und Enzephalopathien zeigen den Anstieg der auftretenden, wiederauftauchenden und vernachlässigten Krankheiten, meist aufgrund eines infektiösen Erregers. Krankheiten, die durch Arboviren oder durch Henipaviren verursacht werden, sind bekannte Zoonosen, während das Pferdeherpesvirus 1 (EHV-1) oder die Protozoen Enzephalitis die Leistung des Tieres beeinträchtigen. Während jedoch die meisten Diagnosewerkzeuge an frisches Gewebe angepasst sind, wird Formalin-fixiertes Paraffin-eingebettetes (FFPE)-Gewebe in den Pathologieeinrichtungen in großem Umfang eingesetzt. Daher verwendete diese Studie FFPE-Gewebe aus dem Zentralnervensystem (ZNS) von 35 Pferden aus Brasilien, die an nicht-suppurativen Enzephalitis oder Enzephalopathien leiden, als Studienmodell. Das Hauptziel bestand darin, geeignete diagnostische Instrumente für FFPE-Gewebe für den morphologischen, Antigen- und molekularen Nachweis sowie metagenomisches Screening relevanter Krankheitserreger bereitzustellen. Die für die Studie verfügbaren Proben bestanden aus einer gemischten Population von männlichen und weiblichen Tieren, die meisten > 4 Jahre alt, überwiegend von gemischten Rassen. Die meisten klinischen Anzeichen (21/35) deuteten auf eine multifokale Verteilung von Läsionen im ZNS hin, und die meisten Fälle (20/35) traten im Herbst auf. Histologisch hatten 28/35 Fälle Entzündungen in mindestens einer ZNS-Region oder einem Ganglion, und 7/35 hatten degenerative Läsionen. Score-Systeme zur Beurteilung des Grades der entzündlichen Zellinfiltration (DICI) und gliale Reaktionsmuster zeigten die Dominanz einer geringgradigen lymphatischen Entzündung und 3 statistisch unterscheidbare Grade der Astrozyten- und Mikrogliaaktivierung in der gesamten Kohorte. In 20/35 Fällen konnten Infektionserreger nachgewiesen werden: Tollwutvirus/Kokzidien-Koinfektion (1/35), Alphavirus (6/35), Flavivirus (4/35), Alphavirus/EHV-1-Koinfektion (3/35), Kokzidien (5/35) und Helminth *larvae migrans* (1/35). In anderen 15/35 Fällen wurde eine unbekannte infektiöse Ätiologie vorgeschlagen, oder degenerative Läsionen hätten eine Infektion imitieren können. Gleichzeitig wurden 4/35 Fälle auch als Pathogenträger von *Babesia* sp. oder *Elizabethkingia anopheles* betrachtet. Die in dieser Studie verwendeten langzeitgelagerten FFPE-Materialien lieferten wertvolle epidemiologische Informationen und die Identifizierung von Krankheitserregern, die für die Gesundheit von Menschen und Tier wichtig sind. Fehlende ZNS-Regionen, Umweltkontaminanten und Protein-cross links, die während der Fixierung gebunden sind, könnten die Identifizierung von Krankheitserregern verhindert haben. In diesem Zusammenhang könnte die Optimierung von Techniken wie Multiplex-Sequenzierungsassays mittels *Target enrichment* die Empfindlichkeit des Pathogennachweises weiter verbessern und

gleichzeitig den Host-/Umgebungshintergrund verringern. Darüber hinaus ermöglichen die in dieser Studie optimierten Methoden die Beurteilung zoonotischer Krankheitserreger beim Menschen und können auch auf wichtige Tierarten wie Rinder, kleine Wiederkäuer und Schweine übertragen werden, die Anfälligkeit für mehrere infektiöse und zoonotische Erreger teilen. Es wird erwartet, dass z. B. bei einem Ausbruch grundlegende Laboratorien in der Nähe betroffener Gebiete in der Lage sein werden, schnell und präzise eine Ätiologie mit den vorgeschlagenen Methoden bereitzustellen, und zwar nach den Routineverfahren der Gewebefixierung, die von den meisten Pathologieinstituten durchgeführt werden.

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9. Appendix

9.1 Literature review

Appendix 1: Alphaviruses with risk of causing neurologic disease in horses

Continued

Antigenic complex/ single species	Species	Antigenic variety/strain	Primary vector	Amplifying hosts/reservoir	Distribution	Disease in horses (in other species)
Eastern Equine Encephalitis	*Eastern equine encephalitis virus*	I	*Culiseta melanura, Aedes taeniorhynchus*	Passerine birds	North America, Caribbean	Encephalitis
Madariaga virus	*Madariaga virus*	II, III, IV	*Culex* spp.	Birds	South and Central America	Encephalitis
Venezuelan Equine Encephalitis	*Venezuelan equine encephalitis virus*	TRD (IAB*)	*Culex* spp.	Birds, rodents, horses	Americas	Encephalitis
		P676 (IC*)	*Ae. taeniorhynchus*	Birds, rodents, horses	South and Central America	Encephalitis
		3880 (ID*)	*Ae. albopictus*, *Cx. aikenii*	Rodents	South and Central America	Unknown
		Mena II (IE*)	*Cx. taeniopus*	Rodents	Central America, Mexico	Encephalitis
		Everglades FE3-7C (II*)	*Cx. cedeci*	Rodents (cotton rats – *Sigmodon* spp.)	Florida (USA)	Unknown
	Mosso das Pedras virus (IF*)	78V3531	*Culex* spp.	Unknown	Brazil	Unknown
	Mucambo virus (III*)	A, C, D	*Culex portesi*, *Aedes* spp.	Rodents (*Oryzomys capito)*	Brazil, Trinidad	Febrile illness
	Pixuna virus (IV*)	BeAr35645	*Trichoprosopon digitatum, Cx.* spp., *Anopheles nimbus*	Rodents (*Proechimys guyannensis*)	Brazil, Argentina	Febrile illness
	Cabassou virus (V*)	CaAr508	*Culex* spp.	Not reported	French Guiana	Unknown
Western Equine Encephalitis	*Western equine encephalitis virus*	WEEV	*Cx. tarsalis, Ae. albopictus*	Passerine birds	Americas	Encephalitis
	Highlands J virus	B-230	*Cx. tarsalis*	Passerine birds	Eastern NA	Encephalitis
	Fort Morgan virus	FMv CM4-146	*Oeciacus vicarius*	Cliff swallows, house sparrows	Western NA	Unknown (encephalitis in birds)
		Buggy Creek 81V8122	*Cx. tarsalis Oe. vicarius*	Cliff swallows	Oklahoma (USA)	Unknown

Antigenic complex/ single species	Species	Antigenic variety/strain	Primary vector	Amplifying hosts/reservoir	Distribution	Disease in horses (in other species)
	Sindbis virus	Babanki virus	*Culex* spp., *Anopheles*. spp., *Ae*. spp.	Birds	Madagascar, Cameron, Senegal	Encephalitis**
		Karelian fever virus	*Culex.* spp.	Passerine birds	Russia	Unknown
		Kyzylagach	*Cx. modestus*	Herons	Transcaucasia	Unknown
		Ockelbo virus	*Culex spp., An. maculipennis*	Passerine birds, Anseriformes	Europe	Encephalitis** (encephalitis in pigeons)
		Sindbis-like	*Culex* spp.	Birds	Europe, Africa, Australia, Asia	Unknown
Tonate virus (III*)	*Tonate virus*	B, CaAn410d	*Cx. portesi*, *Oe. vicarius*	House sparrow, cliff swallow	French Guiana, North America	Unknown
Rio Negro virus (VI*)	*Rio Negro virus*	Ag80-663	*Culex* spp., *Ps. cingulata*	Rodents	Brazil, Argentina	Unknown
Barmah Forest virus	*Barmah Forest virus*	BH2193	*Cx. annulirostris, Ae. vigilax*	Dog, brushtail possums, horse	Australia	Unknown neurologic, but polyarthritis reported
Semliki Forest virus	*Semliki Forest virus*	SFV4 Me Tri virus	*Ae. africans, Ae.* spp.	Unknown	Africa	Encephalitis
	Ross River virus	T48, 213970, NB5092	*Ae. vigilax, Cx. annulirostris,*	Dog, brushtail possums, horse	Australia	Unknown neurologic, but polyarthritis reported
	Getah virus	Sagiyama virus, Getah virus M1	*Ae. vexans*, *Cx. tritaeniorhynchus*	Pigs, horses	Japan, Australasia	Unknown neurologic, but febrile illness and oedema of limbs reported
	O'nyong-nyong virus	Igbo Ora virus Strain Gulu Strain SG650	*An. funestus*, *An. gambiae*	Unknown	Africa	Unknown
	Mayaro virus	Genotype D and L (Caribbean, SA), N (Peru)	*Haemagogus* spp., *Ae. aegypti*	Non-human primates, birds	South America	Unknown (pot. neurologic, fever, myalgia, arthralgia in humans)
	Chikungunya virus	37997	*Ae. aegypti, Ae. albopictus*	Non-human primates	Africa, Asia, Latin America	Unknown (encephalitis, fever, myalgia, arthralgia in humans)
	Una virus	BeAr13136	*Psorophora ferox*	Non-human primates	Latin America	Unknown (pathogenic for mice)

Antigenic complex/ single species	Species	Antigenic variety/strain	Primary vector	Amplifying hosts/reservoir	Distribution	Disease in horses (in other species)
Middelburg virus	*Middelburg virus*	Ar-749, MIDV-857, ArB-8422, ArTB-5290	*Ae. vittatus*, *Amblyomma variegatum*	Birds	Africa	Sporadic encephalitis

Viruses classified according to the International Committee on Taxonomy of Viruses (ICTV). BFV: Barmah Forest virus. MIDV: Middelburg virus. NA: North America. Pot. neurologic: potentially neuropathogenic. TRD: Trinidad Donkey. WEEV: Western equine encephalomyelitis virus. * old classification for the VEE group. ** Sindbis-positive cases from horses with encephalitis investigated by van Niekerk et al. (2015) were closely related to Ockelbo strain and Babanki strain.

References: Eastern and Western equine encephalitis virus (Bruno et al., 1961; Ferreira et al., 1994; Heinemann, 2006; Iversson et al., 1993; Jennings et al., 1966; Mitchell et al., 1987; Vasconcelos et al., 1991), Madariaga virus (Arrigo et al., 2010; Weaver et al., 1999), Venezuelan equine encephalitis virus (Bigler et al., 1974; Blosser & Burkett-Cadena, 2017; Hoyos-López et al., 2016; Pisano et al., 2013; Vasconcelos et al., 1991), MIDV and Sindbis virus (van Niekerk et al., 2015), SFV (Robin et al., 1974).

Appendix 2: Flaviviruses with risk of causing neurological disease in horses

Transmission, group	Virus species	Virus name, isolate or strain[a]	Primary vector	Amplifying hosts	Distribution	Disease in horses (in other species)
Mosquito-borne, Japanese encephalitis virus group	*Saint Louis encephalitis virus* (SLEV)	Kem217	*Culex quinquefasciatus, Cx. declarator, Cx. coronator*	Birds (Formicariidae)	Americas	Encephalitis (idem in humans)
	West Nile virus (WNV)	WNV Lineage 1 (clade 1 and 1b [Kunjin virus])	*Culex* spp.	Passerine birds (crows, sparrows, robins)	Africa, Middle East, Europe, NA, CA, SA, Australia	Encephalomyelitis (idem humans, birds, sheep, camelid)
		WNV Lineage 2	*Culex* spp.	Wild birds	Africa, Europe	Encephalitis (idem humans, birds)
	Murray Valley encephalitis virus (MVEV)	18629	*Cx. annulirostris, Ae. normanensis*	Rabbits, grey kangaroos	Australia, Papua New Guinea	Polioencephalomyelitis (idem humans)
	Japanese encephalitis virus (JEV)	JAOARS982, M28, Nakayama, SA(V), SA-14	*Cx. tritaeniorhynchus*	Pigs	Asia, Oceania	Encephalitis (idem humans, swine [stillbirth])
Mosquito-borne, Ntaya virus group	*Ilheus virus*	Ilheus virus (ILHV)	*Ae. scapularis*	Birds, small rodents	Latin America	Unknown (encephalitis in humans)
		Rocio virus (ROCV)	*Psorophora ferox, Aedes scapularis*	Birds	Brazil	Unknown (encephalitis in humans and lab rodents)
Tick-borne, mammalian host	*Powassan virus* (POWV)	POWV Lineage 1 (enzootic)	*Ixodes cookei, Ix. marxi, Ix. persulcatus.*	Lagomorphs, skunk, dogs, birds, small mammals	North America, Russia	Meningoencephalitis (idem humans, dogs)
		Lineage 2 (deer tick virus)	*Ix. scapularis*	White-footed mouse	Canada, USA	Unknown (encephalitis in humans)
	Louping ill virus (LIV)	LI-31 (prototype)*	*Ix. ricinus,* also transm. by goat and sheep milk	Sheep, grouse, small rodents	Iberian Peninsula, United Kingdom	Encephalitis (idem sheep, dog)
	Tick-borne encephalitis virus (TBEV)	European subtype (TBEV-Eur)	*Ix. ricinus*	Small rodents	Europe, Turkey, Russia	Meningoencephalitis (idem lambs, kids, dogs, humans)
		Siberian subtype (TBEV-Sib)	*Ix. persulcatus*	Small rodents	Russia, northern Finland	Unknown (encephalitis in humans)
		Far Eastern subtype (TBEV-FE)	*Ix. persulcatus, Ix. ovatus*	Small rodents	Far-eastern Asia, Japan, Russia	Unknown (encephalitis in humans)

[a] Viruses classified according to the International Committee on Taxonomy of Viruses (ICTV). * Other viruses within the species are the subtypes British, Irish, Spanish, Turkish sheep encephalitis virus, and Greek goat encephalitis virus. References: SLEV reviewed by Heinen et al. (2015), Hoyos-López et al. (2016), and Vasconcelos et al. (1991). WNV by Bakonyi et al. (2013) and Hoyos-López et al. (2016). MVEV by Mackenzie et al. (2017). JEV by (OIE, 2019d). ROCV (de Barros et al., 2011; Iversson, 1980). POWV by Fatmi et al. (2017) and Krow-Lucal et al. (2018). Louping ill by Hubálek et al. (2014). TBEV by Kaiser (2016) and Bogovic & Strle (Bogovic, 2015).

Appendix 3: Orthobunyaviruses with risk of causing encephalitis in horses

Virus species	Name of the virus	Vector	Amplifying hosts	Distribution	Disease in horses (in other spp.)
Bunyamwera orthobunyavirus	Bunyamwera virus (BUNV)	*Aedes* spp. *Psorophora* spp.	Unknown	Americas	Encephalitis and abortion (idem in children and immunocompromised patients, abortion and teratogenic in ruminants)
Cache Valley orthobunyavirus	Cache Valley virus (CVV)	*Culiseta inornate*, *Culex tarsalis*, *Aedes* spp.	Wild ruminants (i.e. white-tailed deer), horse	Americas	Unknown (meningoencephalitis in humans, teratogenic in ruminants)
Maguari orthobunyavirus	Maguari virus (MAGV)	*Aedes* spp., *Culex* spp., *Anopheles* spp.,	Birds are suggested*	Central and South America	Encephalitis (idem in humans)
California encephalitis orthobunyavirus	California encephalitis virus	*Ae. dorsalis*, *Ae. melanimon*	Rabbits, squirrels	Americas	Unknown (encephalitis in children and puppies, abortion and teratogenic in ewes)
Jamestown Canyon orthobunyavirus	Jamestown Canyon virus	*Aedes* spp., *Anopheles* spp., *Culex* spp., *Culiseta* spp.	White-tailed deer	Americas	Unknown (neuroinvasive in humans)
Fort Sherman orthobunyavirus	Fort Sherman virus	*Culicidae* spp.	Unknown	South America	Unknown (febrile disease in humans)
La Crosse orthobunyavirus	La Crosse virus (LACV)	*Aedes triseriatus*	Rodents (*Tamias striatus*, *Sciurus carolinensis*, *S. niger*) and lagomorphs	North America	Unknown (encephalitis in children and puppies, abortion and teratogenic to ewes)
Snowshoe hare orthobunyavirus	Snowshoe hare virus (SSHV)	*Aedes* spp., *Cs. inornate*, *Cs. impatiens*	Snowshoe hare (*L. americanus*, *Myodes rutilus*, *Dicrostonyx torquatus*)	North America, northern Europe, Asian Russia	Encephalitis, fever (idem in children)
Main Drain orthobunyavirus	Main Drain virus	*Culicoides variipennis*	Lagomorphs	North America	Encephalomyelitis (abortion, teratogenic for ewes)
Shuni orthobunyavirus	Kaikalur virus	*Culicoides* spp., *Cx. theileri*	Ruminants, horses	Africa (Nigeria, South Africa)	Encephalitis (idem in cattle and abortion, stillbirth, malformation)

Viruses classified according to the International Committee on Taxonomy of Viruses (ICTV), revised by Hughes et al. (2020). References: BUNV (Figueiredo, 1999), MAGV* (Pauvolid-Corrêa et al., 2017; Tauro et al., 2015). Cache Valley virus, La Crosse virus, and Snowshoe hare virus revised by Hubálek et al. (2014), Main Drain virus (Emmons et al., 1983), Shuni virus (van Eeden et al., 2012) and revised by Hubálek et al. (2014). Main Drain virus (Emmons et al., 1983).

9.2 Samples and epidemiology

Appendix 4: Epidemiological data of horses from Brazil

Continued

ID	Sex	Age	Breed	Clinical history and signs	Lesion location	Samples available	Death	Relevant gross lesions
Horse 1	Male	6 years	Not available	In the last 20 days, 4/6 animals got sick with the same signs of posterior paralysis and weakness. 48h of clinical evolution	Caudal to T2	Spinal cord	Summer, Dec. 2008	Lumbar spinal cord with intense redness, yellowish areas in the white matter
Horse 2	Male	8 years	Appaloosa	In the last 20 days, 4/6 animals got sick with the same signs of posterior paralysis and weakness. 48h of clinical evolution	Caudal to T2	Spinal cord	Summer, Dec. 2008	Lumbar spinal cord with intense redness, yellowish areas in the white matter
Horse 3	Male	3 years	Not available	Depression, blindness, ataxia, incoordination, circling, tremors, muscular spasms	Cerebral cortex, brainstem (vestibular nucleus), cerebellum	Cerebral cortex	Summer, Feb. 2009	Not available
Horse 4	Male	7 years	Mixed	Behaviour alterations and sialorrhea. Clinical course of 72h until death	Cerebrum-limbic, trigeminal nerve (5^{th})	Cerebral cortex, cerebellar peduncle	Summer, Feb. 2009	Not available
Horse 5	Male	8 years	Not available	Apathic, evolution to staggering gait, circling, falling, lateral recumbency, pedalling, mandibular stiffness, hyper salivation. Suggestive of rabies	Cerebral cortex, brainstem (vestibular nucleus, trigeminal nerve [5^{th}])	Cerebral cortex, cerebellum	Summer, Feb. 2009	Not observed
Horse 6	Female	10 years	Mixed	Depression, tremors, dysmetria, flaccid paralysis of limbs	Cerebral cortex, cerebellum, motor nerve	Cerebral cortex, cerebellum	Summer, Feb. 2009	Not available

ID	Sex	Age	Breed	Clinical history and signs	Lesion location	Samples available	Death	Relevant gross lesions
Horse 7	Female	4 years	American Quarter Horse	Vestibular syndrome (right-side facial paralysis, tongue paralysis), blindness, circling to the right, incoordination, dysmetria	Cerebral cortex, brainstem (vestibular nucleus), cerebellum	Cerebral cortex, trigeminal nerve, spinal cord	Fall, Mar. 2009	Not observed
Horse 8	Male	2.5 years	Mixed	Recumbency for 4 days. He felt after penicillin injection.	Suggestive of spinal cord lesion	Cerebral cortex, cerebellum, spinal cord	Fall, Mar. 2013	Cachectic animal. Luxation between C5-C6. Intestines: *A. perfoliata*, *P. equorum.* Mesenteric artery: *S. vulgaris*
Horse 9	Male	N.a.	Mixed	Impairment of hind limbs mobility	Caudal to T2	Spinal cord (lumbar with lesion), nerve	Fall, Mar. 2013	Dorsal cervical medullar channel: focal haemorrhage
Horse 10	Female	6 years	Mixed	Fed with bakery waste and native pasture. Ataxia of hind limbs, evolution to tetraparesis, sternal recumbency. Tried to get up, kept appetite. Some animals presented pedalling movements. Clinical course of 6 days	Cerebellum, UMN/LMN	Cerebral cortex, cerebellum, spinal cord,	Fall, Apr. 2013	Intestine: yellow liquefied content

ID	Sex	Age	Breed	Clinical history and signs	Lesion location	Samples available	Death	Relevant gross lesions
Horse 11	Female	1.5 year	Mixed	Fed with bakery waste and native pasture. Ataxia of hind limbs, evolution to tetraparesis, sternal recumbency. Tried to get up, kept appetite. Some animals presented pedalling movements. Clinical course of 6 days, until euthanasia	Cerebellum, UMN/LMN	Cerebral cortex, cerebellum, spinal cord	Fall, Apr. 2013	CNS: N.o. Eye: moderate bilateral palpebral oedema. Small intestine: yellow liquefied content. Large intestine: parched content
Horse 12	Female	6 years	Mixed	Fed with bakery waste and native pasture. Ataxia of hind limbs, evolution to tetraparesis, sternal recumbency. Tried to get up, kept appetite. Some animals presented pedalling movements. Pregnant, clinical course of 4 days	Cerebellum, UMN/LMN	Cerebral cortex, spinal cord	Fall, Apr. 2013	CNS: N.o. Eye: severe bilateral palpebral oedema. Intestines: liquefied content
Horse 13	Female	7 years	Mixed	Fed with bakery waste and native pasture. Ataxia of hind limbs, evolution to tetraparesis, sternal recumbency. Tried to get up, kept appetite. Some animals presented pedalling movements. Clinical course of 2 days until euthanasia	Cerebellum, UMN/LMN	Cerebral cortex, pons, cerebellum, spinal cord	Fall, Apr. 2013	CNS: N.o. Eye: severe bilateral palpebral oedema. Intestines: liquefied content. Mesentery: severe petechial haemorrhage.
Horse 14	Male	2 years	Crioulo	Muscular stiffness (8 days), was falling for nothing. Suggestive of tetanus	Cerebellum, decreased synaptic resistance	Midbrain, spinal cord,	Fall, Apr. 2013	CNS: N.o. Skin: superficial lesion on left hind limb

ID	Sex	Age	Breed	Clinical history and signs	Lesion location	Samples available	Death	Relevant gross lesions
Horse 15	Female	7 years	American Quarter Horse	From one day to another, the animal was in recumbency, masticatory movements, caudal insensibility, pain when the atlas was touched. Advanced pregnancy. Swab of spinal cord necrotic material negative in the bacteriology	ANS, LMN (sacrococcygeal spinal cord)	Spinal cord (several fragments)	Fall, May 2013	Spinal cord T5-T12: focal extensive area of dark redness, white amorphous substance over meninges. Lung: diaphragmatic lobes with multiple white dots 0.2 cm
Horse 16	Female	16 years	Not available	Not available. Suggestive of rabies, rabies negative immunofluorescence	Inconclusive	Striatum, obex, spinal cord, trigeminal ganglion (only place with inflammation)	Spring, Oct. 2013	Bladder: full, multifocal haemorrhagic areas in the mucosa. Small intestine: red areas in the mucosa, mucous content. Kidneys: diffusely dark red
Horse 17	Female	4 years	Mixed	Chicken faeces in the feed trough. Weakness, thoracic limbs stiffness, tremors. Suggestive of botulism, rabies.	Cerebellum, caudal to T2	Spinal cord (cervical, lumbar, sacral)	Fall, Mar. 2014	Not observed
Horse 18	Male	6 years	Mixed	One month with pelvic limbs ataxia	Cerebellum	Mesencephalon, spinal cord	Fall, Apr. 2014	Brain: oedema. Intestine: *A. perfoliata.* Mesenteric artery: *S. vulgaris*

ID	Sex	Age	Breed	Clinical history and signs	Lesion location	Samples available	Death	Relevant gross lesions
Horse 19	Male	6 years	Crioulo	Stiffness for one week, no apparent lesions, felt down and did not get up for 1 day before death	Decreased synaptic resistance	Cerebral cortex, spinal cord	Fall, May 2014	Encephalon: diffuse cherry-red colour, meningeal vessels hyperaemic
Horse 20	Male	7 years	Mixed	2 weeks with instability in the hind limbs, no trauma reported, sternal recumbency for one week. Worsening of signs after exercise; receiving treatment for EPM by the time of death	Cerebellum, UMN/LMN	Cerebral cortex (frontal, parietal), medulla oblongata, spinal cord	Spring, Sep. 2014	Not available
Horse 21	Male	6 years	Crioulo	10 days before death, he felt trying to mate, after that, blindness, head rotation and loss of balance	Fracture C5-C6, brainstem (vestibular nucleus), cerebellum	Spinal cord, trigeminal nerve	Spring, Oct. 2014	Fracture ventral region of C5-C6, with focal haemorrhage
Horse 22	Male	10 years	American Quarter Horse	1 month with left side infra orbital swelling; head rotation, circling to the right, accumulated food on the left, right-side face deviation (no ptosis), left eye in ventral position, absent left facial reflex and diminished on the right, intense ataxia. Treatment*. 3 weeks later febrile peaks. External decubitus 28 days after first signs, difficulty to stand/support. Euthanasia	Brainstem (vestibular nucleus, trigeminal nerve [5th], oculomotor nerve [3rd], hypoglossal nerve [12th]), cerebellum	Hippocampus, 4th ventricle (small fragment of cerebellum), choroid plexus, spinal cord	Early winter, June 2011	Telencephalon: meningeal vessels stuffed. Brainstem: focal yellowish areas, soften especially in the white matter. Cerebellar base/4th ventricle: reddish exuberant tissue, spongy consistence.

ID	Sex	Age	Breed	Clinical history and signs	Lesion location	Samples available	Death	Relevant gross lesions
Horse 23	N.a.	N.a.	Not available	Not available	Inconclusive	Spinal cord	2007	Not available
Horse 24	Female	6 months	Marajoara	Severe depression, difficulty to keep in station, prolonged periods standing in the same place or in lateral recumbency, limbs abducted or crossed, when moved walked in circles, head down, slightly right-rotated, closed eyelids, tongue paralysis, muscular tremors, anorexia, dehydration	Cerebral cortex, brainstem (vestibular nucleus, trigeminal nerve [5th], hypoglossal nerve [12th]), cerebellum	Cerebral cortex, obex, cerebellum, spinal cord	Fall	Haemorrhage of leptomeninges and spinal cord
Horse 25	Male	1.5 year	Marajoara	Anorexia, hypermetria, difficulty to walk, walking in circles, skin of the head with excoriation	Brainstem (vestibular nucleus, motor nucleus), cerebellum	Olfactory bulb, cerebral cortex, hippocampus, corpus callosum, cerebellum, choroid plexus, spinal cord	Fall	Haemorrhage of leptomeninges and spinal cord
Horse 26	Female	5 years	Marajoara	Sternal recumbency, head turned to flanks and supported on the ground, tongue paralysis, respiratory difficulty	Brainstem (vestibular nucleus - hypoglossal nerve [12th])	Hippocampus, thalamus, mesencephalon, cerebellum	Fall	Haemorrhage of leptomeninges and spinal cord
Horse 27	Female	4 years	Marajoara	Found dead. Walked in circles for 2 days, felt one day before death	Brainstem (vestibular nucleus)	Cerebral cortex, hippocampus, pons, spinal cord	Fall	Haemorrhage of leptomeninges and spinal cord

ID	Sex	Age	Breed	Clinical history and signs	Lesion location	Samples available	Death	Relevant gross lesions
Horse 28	N.a.	N.a.	Not available	Not available**	Inconclusive	Cerebral cortex, hippocampus, cerebellum, spinal cord	Fall	Not available
Horse 29	Female	Adult	Mixed	No support on thoracic limbs, 1 day after had torticollis, walking in circles, sensibility in the whole body	C6-T2, brainstem (vestibular nucle-us), peripheral sensory neurons	Cerebrum, telencephalon	Fall, 2005	Not observed
Horse 30	Female	Adult	Mixed	Apparent blindness, lack of balance, decreased proprioception, hypotonic tongue, decreased palpebral reflex and of threat	Cerebral cortex, brainstem (motor nucleus, vestibular nucleus: hypo-glossal and facial)	Pons, mesencephalon	2006	Not available
Horse 31	N.a.	N.a.	Not available	Dead during an EEE outbreak	Inconclusive	Mesencephalon	2009	Not available
Horse 32	Male	6 months	Mangalarga Marchador	Lateral recumbency, decree-sed reflex on hind limbs, paresis of limbs, head rests on the floor when in sternal recumbency, dehydrated	Brainstem, C1-C7	Cerebral cortex, thalamus, spinal cord	Fall, 2011	Not observed
Horse 33	Male	5 years	Mixed	Ataxia and deficient proprioception for 15 days	Cerebellum and brainstem	Parietal cortex, thalamus	Fall, 2012	Not observed
Horse 34	N.a.	N.a.	Not available	4/50 animals of the herd had “neurological signs”	Inconclusive	Cerebral cortex	2013	Not available
Horse 35	N.a.	N.a.	Not available	4/50 animals of the herd had “neurological signs”	Inconclusive	Cerebral cortex	2013	Not available

Ca: calcium. CNS: central nervous system. EEE: eastern equine encephalomyelitis. EHV-1: equine herpesvirus type 1. EPM: equine protozoal meningoencephalitis. IF: immunofluorescence. IHC: immunohistochemistry. N.a.: Not available. N.o.: none observed. Mg: magnesium. P: phosphorus. PCR: polymerase chain reaction. * treatment with dexamethasone, Ceftiofur, enrofloxacin, flunixin meglumine; febrile peaks treated with dipyrone. ** Partial histological report available: liver – periportal hepatitis, kidney – interstitial mononuclear inflammatory infiltrate.

9.3 Pathological studies

Appendix 5: Histological analysis

Continued

ID	Material received	DICI [a]	Cellular morphology				Neuro necros.	Satell./ neur.	Vasc.[b]	Mening.	Mening. degree [c]	Vacuoli	Malacia
			lymph	macro	plasma	eosino							
1	S. cord	3.44	+++	+	<+	+	+	+	1	+	1	-	+
2	S. cord	3.14	+++	-	<+	-	+	-	1,2	-	0	-	+
3	C. cortex	4.6	+++	-	<+	-	+	+	1,2	+	2	+	-
4	Cereb.,	0	-	-	-	-	-	-	-	-	0	-	-
	c. cortex	3.8	+++	-	<+	-	+	+	1,3,5	-	0	+	+
5	Cereb.,	1.57	+++	-	<+	-	-	-	5	+	1	-	-
	c. cortex	1.94	+++	-	-	-	+	+	4	+	1	-	-
6	Cereb.,	0	-	-	-	-	-	-	4,5	-	0	-	-
	c. cortex	4.5	+++	-	-	-	+	+	5	-	0	-	-
7	S. cord,	2.09	+++	-	<+	-	+	+	4,5	-	0	+	-
	c. cortex,	2.77	+++	-	<+	-	+	-	-	+	2	+	-
	trig. g.	*	+++	-	-	-	+	-	-	-	0	-	-
8	S. cord,	0	-	++	-	-	-	-	3,4,5	-	0	+	+
	cereb.,	0	-	-	-	-	-	-	-	-	0	+	-
	c. cortex	0	-	-	-	-	-	-	5	-	0	+	-
9	S. cord,	5.85	++(+)	++	+	+	+	+	1,2	+	3	-	+
	nerve	*	+++	-	-	-	+	+	-	-	-	-	-
10	S. cord,	0	-	-	<+	-	+	-	-	-	0	-	-
	cereb.,	0	-	-	-	-	-	-	3	-	0	+	-
	c. cortex	0.76	+++	-	-	-	+	-	1,3,5	-	0	-	-
11	Pons,	0	-	-	-	-	-	-	-	-	0	-	-
	cereb.,	1.65	+++	-	-	-	-	-	-	-	0	+	-
	c. cortex	1,02	+++	-	-	-	+	+	4	-	0	+	-

ID	Material received	DICI [a]	Cellular morphology				Neuro necros.	Satell./ neur.	Vasc.[b]	Mening.	Mening. degree [c]	Vacuoli	Malacia
			lymph	macro	plasma	eosino							
12	S. cord,	0	-	-	-	-	-	-	-	-	0	-	-
	c. cortex	0	-	-	-	-	-	-	5	-	0	+	-
13	S. cord,	0	-	-	-	-	-	-	-	-	0	-	-
	cereb.,	0	-	-	-	-	-	-	-	-	0	+	-
	c. cortex	0	-	-	-	-	-	-	5	-	0	+	-
14	S. cord,	0	-	-	-	-	-	-	-	-	0	+	-
	obex	0	-	-	-	-	-	-	2,5	-	0	+	+
15	S. cord	0	-	-	-	-	+	-	1,2	-	0	+	-
16	S. cord,	0	-	-	-	-	-	-	-	-	0	+	-
	trig. g.,	*	+++	-	-	-	-	-	-	-	0	-	-
	obex,	0	-	-	-	-	-	-	-	-	0	+	-
	striatum	0	-	-	-	-	-	-	4,5	-	0	+	-
17	S. cord,	0	-	-	-	-	-	-	1,2,5	-	0	+	-
	c. cortex	0	-	-	-	-	+	+	4	-	0	+	-
18	S. cord,	0.75	+++	+	<+	-	+	+	4	-	0	+	-
	mesen.	3.6	+++	++	<+	<+	-	+	5	-	0	-	-
19	S. cord,	1.2	+++	-	-	-	-	-	-	-	0	-	-
	c. cortex	2	+++	-	<+	-	+	-	3,5	+	1	-	-
20	S. cord,	1.28	+++	+	-	-	+	+	1,4	-	0	-	-
	cortex f,	2.38	+++	-	-	-	+	-	-	+	1	-	-
	cortex p,	2.38	+++	-	-	-	-	-	4	-	0	-	-
	bulb	2.38	+++	-	<+	-	-	-	-	-	0	-	-
21	S. cord,	0	-	-	-	-	+	-	-	-	0	-	+
	trig. g.	*	++	+	<+	-	-	+	-	-	0	-	-
22	S. cord,	4.21	+++	+	+	+	+	+	1,2,4,5	+	3	+	-
	c. cortex,	4	+++	-	-	<+	+	+	1	-	-	+	-
	cereb.,	2.52	+++	-	-	-	-	-	2	+	1	-	-
	c. plex.	0	-	-	-	-	-	-	-	-	-	-	-

ID	Material received	DICI [a]	Cellular morphology				Neuro necros.	Satell./ neur.	Vasc.[b]	Mening.	Mening. degree [c]	Vacuoli	Malacia
			lymph	macro	plasma	eosino							
23	S. cord	1.45	+++	<+	<+	-	-	-	4	-	0	-	-
24	S. cord,	0.93	+++	-	<+	-	+	+	-	-	0	+	-
	cereb.,	3.93	+++	-	<+	-	-	-	-	-	0	+	-
	c. cortex,	4.98	+++	-	<+	-	+	-	-	-	0	-	-
	obex,	2.3	+++	<+	<+	<+	+	+	-	-	0	-	-
25	Cereb.,	3	+++	-	-	-	+	+	4,5	+	2	+	-
	c. plex.,	*	+++	-	-	-	-	-	-	-	0	-	-
	c. cortex,	1.85	+++	<+	<+	-	+	-	4	+	2	+	-
	hippoc.,	1.85	+++	<+	<+	-	+	+	2,4	+	2	-	-
	c. call.,	2.48	+++	<+	<+	-	+	+	4	-	0	-	-
	s. cord,	0	-	-	-	-	-	-	-	-	0	-	-
	bulb	2.48	+++	-	-	-	-	-	4	+	1	-	-
26	Hippoc.,	0.54	++	-	<+	-	-	-	4	+	1	+	-
	cereb.,	0	-	-	-	-	-	-	-	-	0	+	-
	mesen.	2.21	++	-	<+	-	-	-	-	-	0	-	-
27	Hippoc.,	4.2	+++	-	<+	<+	-	-	-	+	1	-	-
	pons,	0	+++	<+	-	<+	-	-	-	+	1	-	-
	s. cord,	1.32	+++	-	<+	-	-	-	-	-	0	-	-
	c. cortex	4.2	+++	<+	<+	-	-	-	-	+	1	-	-
28	S. cord,	0	-	-	-	-	-	-	4	-	0	+	-
	c. cortex,	0	-	-	-	-	-	-	4	-	0	-	-
	hippoc.,	0	-	-	-	-	-	-	-	-	0	+	-
	cereb.	0	-	-	-	-	-	-	-	-	0	+	-
29	Forebrain	2.31	+++	<+	+	<+	+	+	1,4	-	0	-	-
	midbrain	5.7	+++	-	+	-	-	+	1	-	0	-	-
30	Pons,	1.94	+++	<+	-	<+	+	+	4	-	0	+	-
	mesen.	1.94	+++	-	-	-	-	+	4	-	0	-	-
31	Mesen.	4.47	+++	-	<+	<+	-	+	4,5	-	0	-	-

ID	Material received	DICI [a]	Cellular morphology				Neuro necros.	Satell./ neur.	Vasc.[b]	Mening.	Mening. degree [c]	Vacuoli	Malacia
			lymph	macro	plasma	eosino							
32	S. cord,	0.32	+++	-	<+	-	+	+	1	-	0	+	-
	c. cortex,	1.53	+++	-	-	-	+	+	-	-	0	-	-
	thalamus	1.53	+++	-	<+	-	-	-	1	-	0	-	-
33	cortex p.	2.11	+++	-	<+	<+	+	+	4,5	-	0	-	-
	thalamus	2.11	+++	-	-	-	+	-	-	-	0	-	-
34	C. cortex	2.23	+++	<+	-	-	-	-	4	+	2	-	-
35	C. cortex	0.31	+++	<+	-	-	-	-	4	+	2	-	-

Cereb.: cerebellum. C. cortex: cerebral cortex. C. plex.: choroid plexus. Cortex f: cortex frontal. Cortex p: cortex parietal. Eosino: eosinophils. ID: identification. Lymph: lymphocytes. Macro: macrophages. Mening: meningitis. Mesen.: mesencephalon. Neuro necros.: neuronal necrosis. Neuronop.: neuronophagia. Plasma: plasma cells. Satell./neur.: satellitosis and/or neuronophagia. S. cord: spinal cord. Telencep.: telencephalon. Vacuoli: vacuolization.

[a] Degree of inflammatory cells infiltration: * - not assessed
0 – no inflammatory cells infiltration
>0 to 2 – mild inflammatory infiltrate
>2 to 4 – moderate inflammatory infiltrate
> 4 – severe inflammatory infiltrate

[b] Vascular alteration: 1 – vasculitis
2 – haemorrhage
3 – congestion
4 – reactive endothelium
5 – oedema

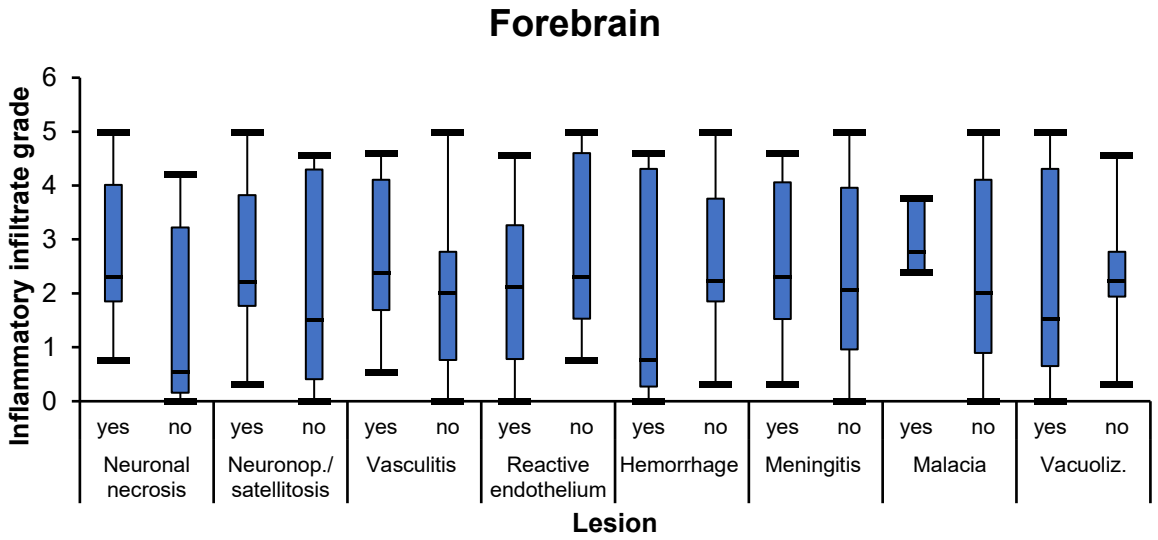
[c] meningitis degree: 0 – no meningitis
1 – mild
2 – moderate
3 – severe

Appendix 6: Mean of inflammatory cells in score for each CNS area

Variable	n	Mean	St. D	Min	Max
Forebrain					
A	20	19.122	16.364	0	48.250
B	20	1.0705	0.5546	0	2.0000
C	20	125.26	83.190	0	372.80
Midbrain					
A	10	48.300	43.229	0	141.00
B	10	1.4438	0.7842	0	2.9375
C	10	138.31	65.204	0	221.50
Cerebellum					
A	8	8.5000	14.590	0	42.500
B	8	0.3750	0.5175	0	1.0000
C	8	42.750	100.91	0	289.60
Spinal cord					
A	16	21.583	26.425	0	83.600
B	16	1.0069	0.8688	0	2.8000
C	16	122.54	136.31	0	494.40

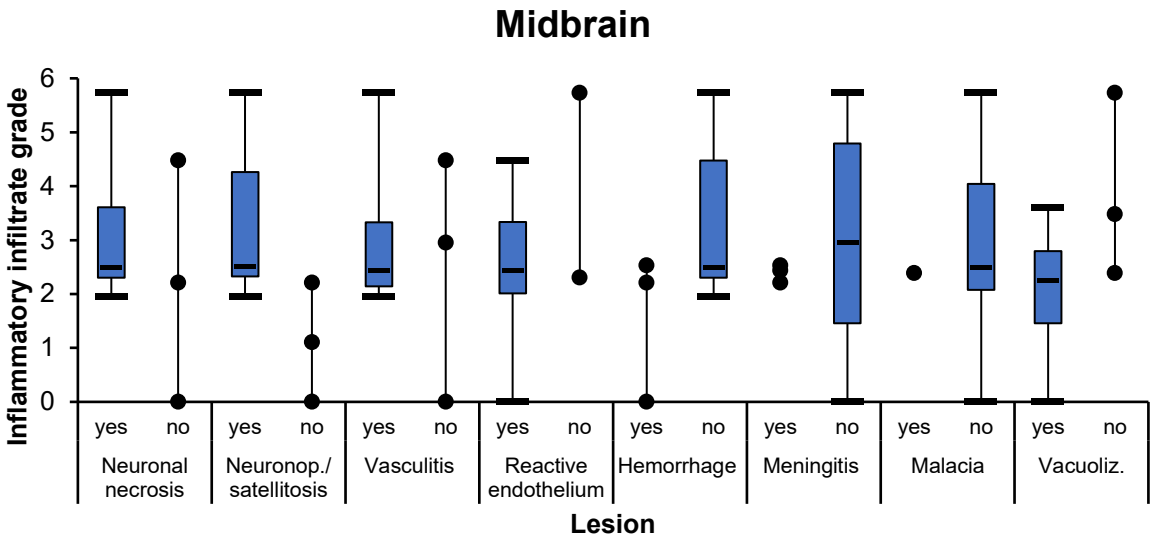
A: mean of inflammatory cells in perivascular infiltrates. B: mean of inflammatory cell layers in perivascular infiltrates. C: mean of inflammatory cells in the parenchyma. n: number of fragments available. St. D.: standard deviation.

Appendix 7: Wilcoxon-Mann-Whitney-test for correlation of inflammation to other lesions in forebrain (n= 20)



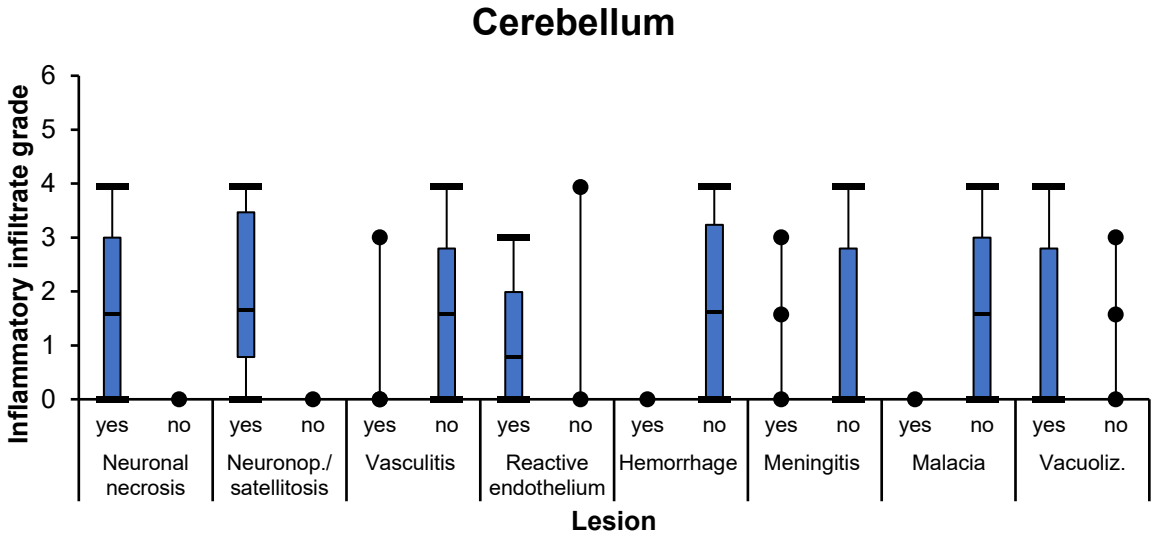
There was no statistically significant correlation ($p > 0.05$) between the severity of inflammation from samples presenting (yes) or not presenting (no) lesions in forebrain fragments. Neuronop.: neuronophagia. Vacuoliz.: vacuolization.

Appendix 9: Wilcoxon-Mann-Whitney-test for correlation of inflammation to other lesions in midbrain (n= 10)



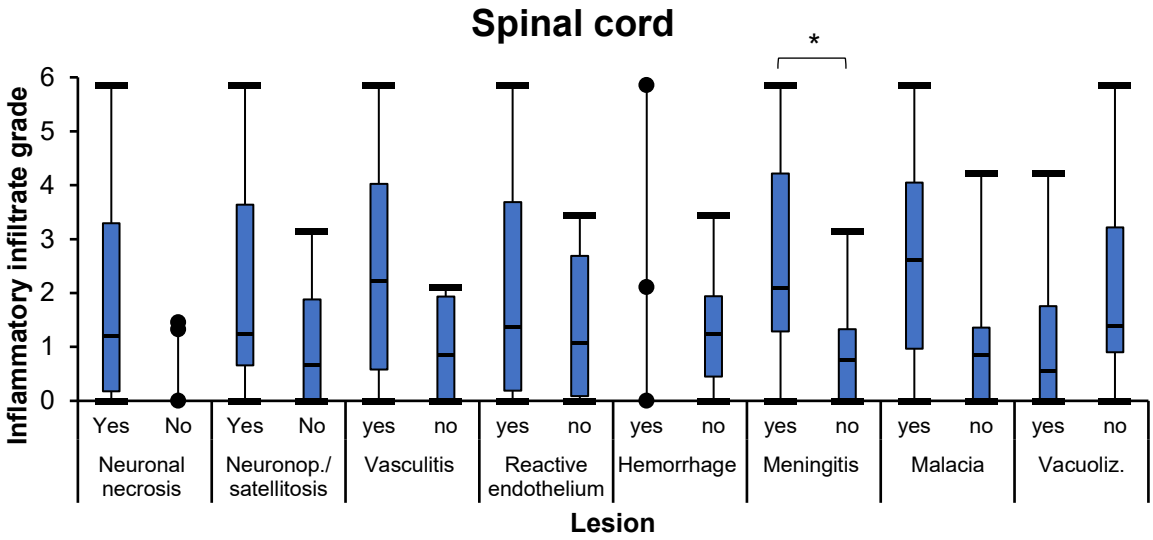
There was no statistically significant correlation ($p > 0.05$) between the severity of inflammation from samples presenting (yes) or not presenting (no) lesions in midbrain fragments. Results plotted in points refer to lesions observed in less than 5 cases. Neuronop.: neuronophagia. Vacuoliz.: vacuolization.

Appendix 8: Wilcoxon-Mann-Whitney-test for correlation of inflammation to other lesions in cerebellum (n= 8)



There was no statistically significant correlation ($p > 0.05$) between the severity of inflammation from samples presenting (yes) or not presenting (no) lesions in cerebellum. Results plotted in points refer to lesions observed in less than 5 cases. Neuronop.: neuronophagia. Vacuoliz.: vacuolization.

Appendix 10: Wilcoxon-Mann-Whitney-test for correlation of inflammation to other lesions in spinal cord (n= 16)



Higher degrees of spinal cord inflammation were statistically correlated to the occurrence of meningitis. There was no statistically significant correlation ($p > 0.05$) between the severity of inflammation from samples presenting (yes) or not presenting (no) other lesions in spinal cord. Results plotted in points refer to lesions observed in less than 5 cases. * $p \leq 0.05$. Neuronop.: neuronophagia. Vacuoliz.: vacuolization.

Appendix 11: Spearman's rank correlation for the severity of inflammation in each CNS area and the severity of meningeal inflammatory infiltrate

			n	r_s	P
Forebrain	-	Meningitis degree	20	0.2773	0.2366
Midbrain	-	Meningitis degree	10	0.0069	0.9850
Cerebellum	-	Meningitis degree	8	-0.0439	0.9177
Spinal cord	-	Meningitis degree	16	0.5311	**0.0343**

r_s: spearman's correlation coefficient

9.4 Immunohistochemical studies

Appendix 12: t-Test for dependent variables. Significance between the means of astrocytes stained in lesioned and non-lesioned areas in different CNS regions

		Paired differences						
				95% confidence interval of the difference				
Variable	n	Mean	St. D	Min	Max	t value	df	P value
F L – NL	25	45.9	36.4	5.60	175	6.30	24	**.0000**
M L – NL	14	36.3	32.2	3.40	115	4.22	13	**.0010**
C L – NL	12	38.3	25.2	14.0	102	5.26	11	**.0003**
Sc L – NL	22	33.6	14.3	5.60	68.2	11.01	21	**.0000**

C: cerebellum. df: degree of freedom. F: forebrain. n: number of fragments available for investigation. L: lesioned. M: midbrain. NL: non-lesioned. Sc: spinal cord. St. D: standard deviation.

Appendix 13: Wilcoxon-Mann-Whitney-test for differences between the mean of GFAP positive astrocytes in inflammatory and in degenerative/reactive lesions in different CNS regions

CNS region	Lesion type	n	Mean	Median	Min	Max	St. D	U	P value
Forebrain	Inflammatory	19	67.3	49.6	15.6	195	42.5	59.0	0.8987
	Deg. /reactive	6	57.1	56.1	23.2	83.2	22.9		
Midbrain	Inflammatory	9	70.8	70.4	27.4	115	26.8	38.5	**0.0327**
	Deg. /reactive	5	40.2	37.8	24.8	59.8	13.1		
Cerebellum	Inflammatory	6	62.4	56.3	21.4	101.7	29.5	24.0	0.3367
	Deg. /reactive	6	47.5	41.1	26.0	74.6	18.9		
Spinal cord	Inflammatory	11	44.4	42.2	16.8	76.6	19.1	45.0	0.3084
	Deg. /reactive	11	52.2	47.8	16.8	86.0	20.9		

Deg.: degenerative. Max: maxima. Min: minima. n: number of fragments available for investigation. St. D: standard deviation.

Appendix 14: t-Test for dependent variables. Significance between the means of Iba1 positive microglia in lesioned and non-lesioned areas from different CNS regions

		Paired differences				t		P
				95% confidence interval of the difference				
Variable	n	Mean	St. D	Min	Max	value	df	value
F L - NL	25	14.2	18.3	-13.6	52.6	3.87	24	**0.0007**
M L - NL	14	14.2	16.0	-6.2	46.4	3.31	13	**0.0056**
C L - NL	12	3.3	9.1	-17.6	13.4	1.25	11	0.2365
Sc L - NL	22	20.9	32.4	-6.2	133.4	3.02	21	**0.0065**

C: cerebellum. df: degree of freedom. F: forebrain. L: lesioned. M: midbrain. Max: maxima. Min: minima. n: number of fragments available. NL: non-lesioned. Sc: spinal cord. St. D: standard deviation.

Appendix 15: Wilcoxon-Mann-Whitney-test for differences between the mean of microglial cells stained in inflammatory and in degenerative/reactive lesions in the different regions of the CNS of horses

CNS region	Lesion type	n	Mean	Median	Min	Max	St. D	U	P value
Forebrain	Inflammatory	19	23.9	16.0	0	63.2	20.5	82.0	0.1100
	Deg./reactive	6	12.6	5.0	0	50	19.4		
Midbrain	Inflammatory	9	33.1	32.0	10.2	67.4	20.1	39.5	**0.0230**
	Deg./reactive	5	8.3	9.4	0	17	8.1		
Cerebellum	Inflammatory	6	10.4	11.4	0	17	6.3	27.0	0.1467
	Deg./reactive	6	5.6	5.4	0	11.8	5.2		
Spinal cord	Inflammatory	12	37.6	18.4	0	163.8	47.3	83.0	0.1282
	Deg./reactive	10	14.7	10.3	0	60.2	18.1		

Deg.: degenerative. Max: maxima. Min: minima. n: number of fragments available for investigation. St. D: standard deviation.

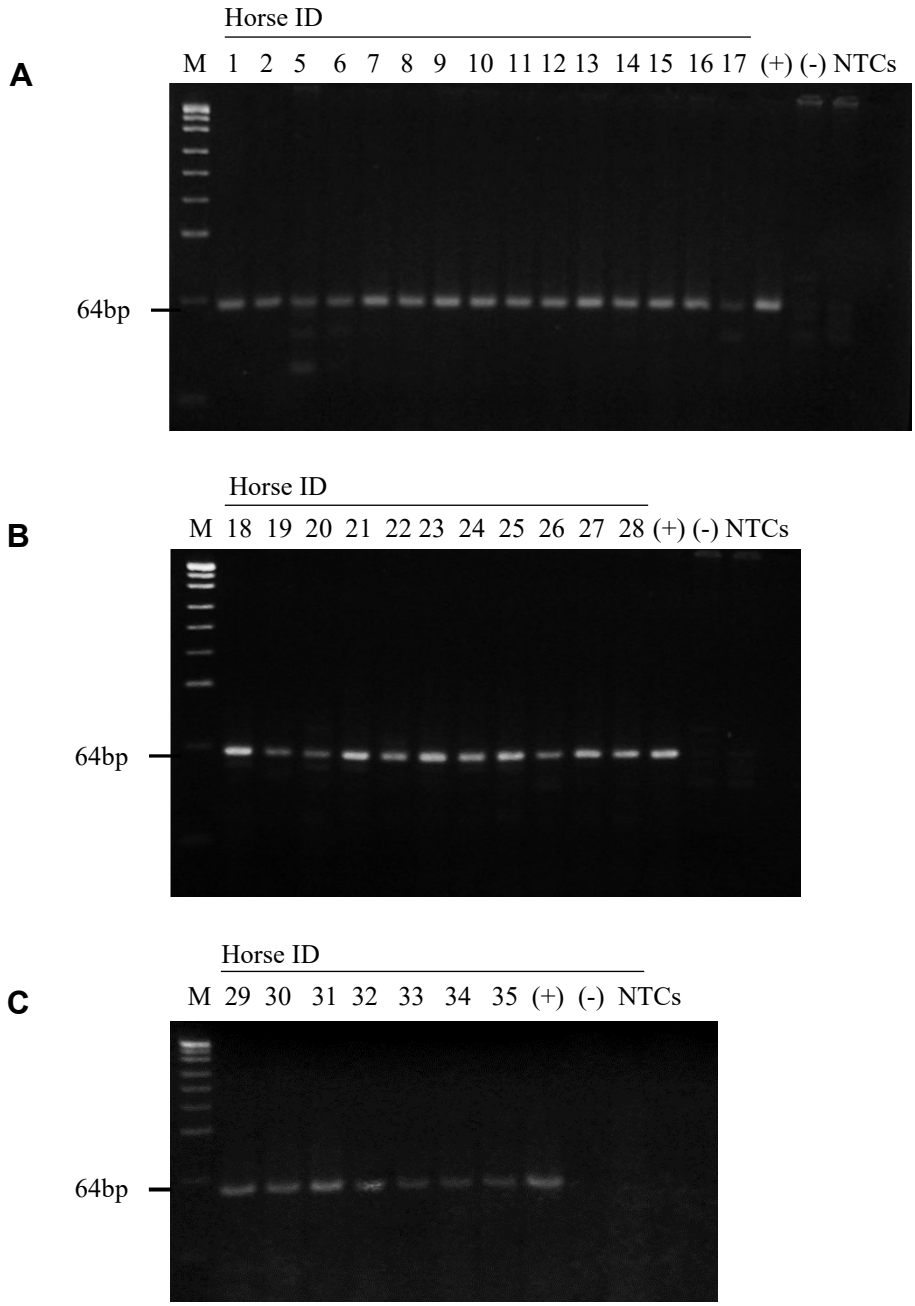
9.5 Molecular analysis

Appendix 16: Yield and purity of RNA and DNA extraction of FFPE material (n=35)

Horse ID	RNA1			RNA2			DNA		
	RNA ng/µl	260/280	260/230	RNA ng/µl	260/280	260/230	DNA ng/µl	260/280	260/230
1	50.4	1.83	1.58	32.6	1.79	1.69	104	1.72	1.36
2	24.6	1.85	1.65	123.8	1.8	1.81	51.2	1.63	0.94
3	30.3	1.8	1.57	700	1.89	1.95	14.8	1.55	2.1
4	19	1.7	1.27	*	*	*	34.2	1.6	1.6
5	57.8	1.84	1.76	32.3	1.69	1.33	28.3	1.67	0.37
6	27.2	1.65	1.39	48.2	1.71	1.64	26.7	1.49	0.83
7	61.9	1.93	1.94	60.6	1.95	1.82	185.5	1.85	1.98
8	284.2	1.89	1.93	*	*	*	29.9	1.69	1.46
9	341.4	1.83	1.74	90.8	1.81	1.75	23.4	1.62	1.09
10	346.6	1.94	2	212.9	1.94	2	61.3	1.75	1.62
11	375.4	1.95	1.81	324	1.95	1.96	114.9	1.79	1.77
12	120.1	1.87	1.75	*	*	*	39.1	1.76	1.65
13	91.4	1.89	1.81	*	*	*	135.5	1.79	1.78
14	232	1.92	1.84	*	*	*	75.9	1.8	1.48
15	38.5	1.77	1.38	*	*	*	44.7	1.76	1.28
16	689.3	1.88	1.9	310.3	1.91	1.85	26.3	1.73	0.81
17	248.1	1.92	1.91	*	*	*	43.9	1.81	1.2
18	329.8	1.9	1.84	235.2	1.95	1.95	52.8	1.83	1.08
19	392.4	1.95	1.94	214.2	1.96	1.97	64.7	1.83	1.09
20	545.5	1.9	1.98	542.7	1.9	1.94	54	1.76	1.37
21	410.9	1.93	1.9	249.4	1.87	1.9	201.3	1.74	1.61
22	786	1.82	1.76	205.6	1.83	1.85	77.6	1.67	1.56
23	238.4	1.82	1.94	141.2	1.84	1.79	26.8	1.7	0.93
24	329.2	1.78	1.75	320.5	1.79	1.87	64.3	1.51	1.1
25	192.7	1.68	1.55	19.1	1.43	1	41.4	1.51	1.5
26	96	1.72	1.71	48.7	1.73	1.61	29	1.46	0.82
27	170.7	1.74	1.63	44.4	1.68	1.36	9.4	1.42	0.59
28	423.5	1.78	1.84	*	*	*	125.8	1.62	1.65
29	97.2	1.7	1.57	45.5	1.61	1.37	19.6	1.67	1.73
30	166.3	1.77	1.69	102.9	1.76	1.57	74.9	1.6	1.36
31	162.3	1.81	1.88	120.4	1.8	1.84	54.9	1.7	1.26
32	338.6	1.79	1.91	296.3	1.78	1.85	53.2	1.72	1.14
33	343.9	1.77	1.85	151.4	1.73	1.68	23.7	1.71	0.77
34	302.7	1.81	1.81	62.2	1.79	1.68	13	1.63	0.7
35	226.1	1.83	1.78	46.1	1.72	1.39	78.5	1.68	1.58

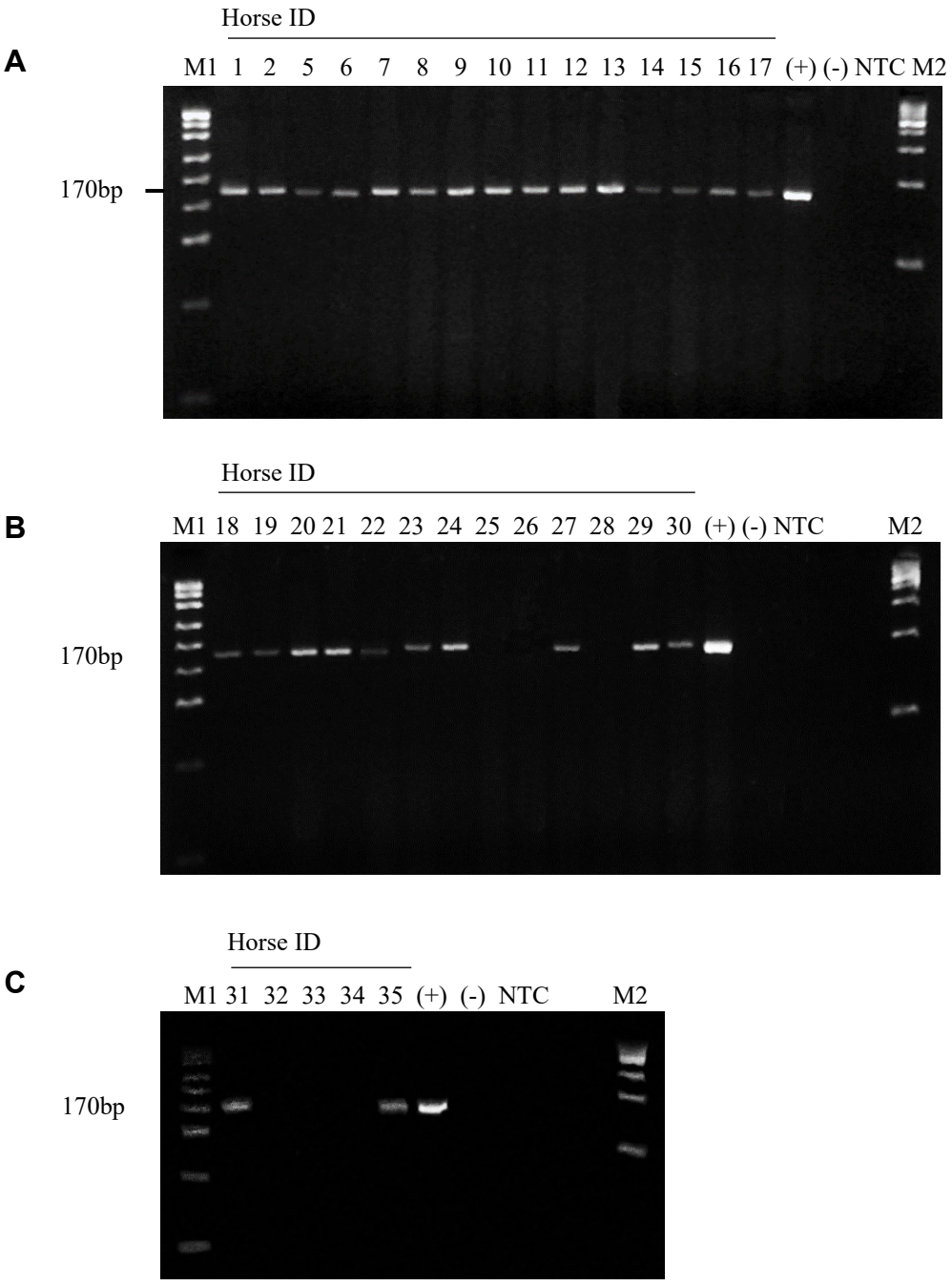
260/280: purity rate of the extracted RNA and DNA in relation to proteins. 260/230: purity rate of RNA and DNA extracted in relation to contaminants. ID: identification. RNA1: first RNA extraction. RNA2: second RNA extraction. * not performed.

Appendix 17: Equid glyceraldehyde phosphate dehydrogenase (GAPDH) amplification of 64bp product in 4% agarose gel in 33/33 horses.



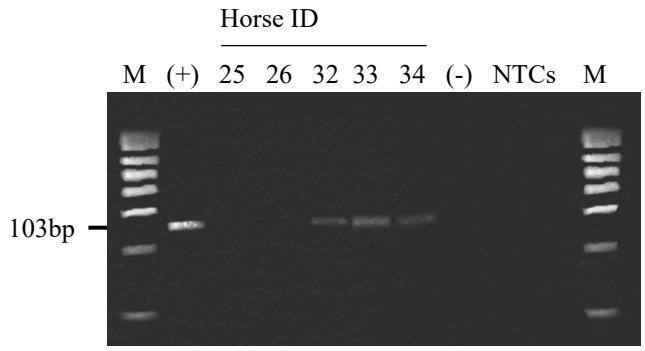
(M) pUC19 marker. (+) positive control. (-) negative control. (NTCs) no-template controls. ID: identification.

Appendix 18: Equid glyceraldehyde phosphate dehydrogenase (GAPDH) amplification of 170bp in 4% agarose gel of 27/33 horses.



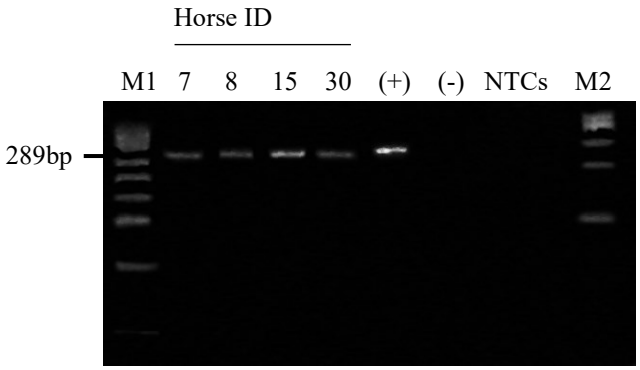
(M1) pUC19 marker. (M2) GR100 marker. (+) positive control. (-) negative control. (NTC) no-template control. ID: identification.

Appendix 19: Equid glyceraldehyde phosphate dehydrogenase (GAPDH) amplification of 103bp in 4% agarose gel.



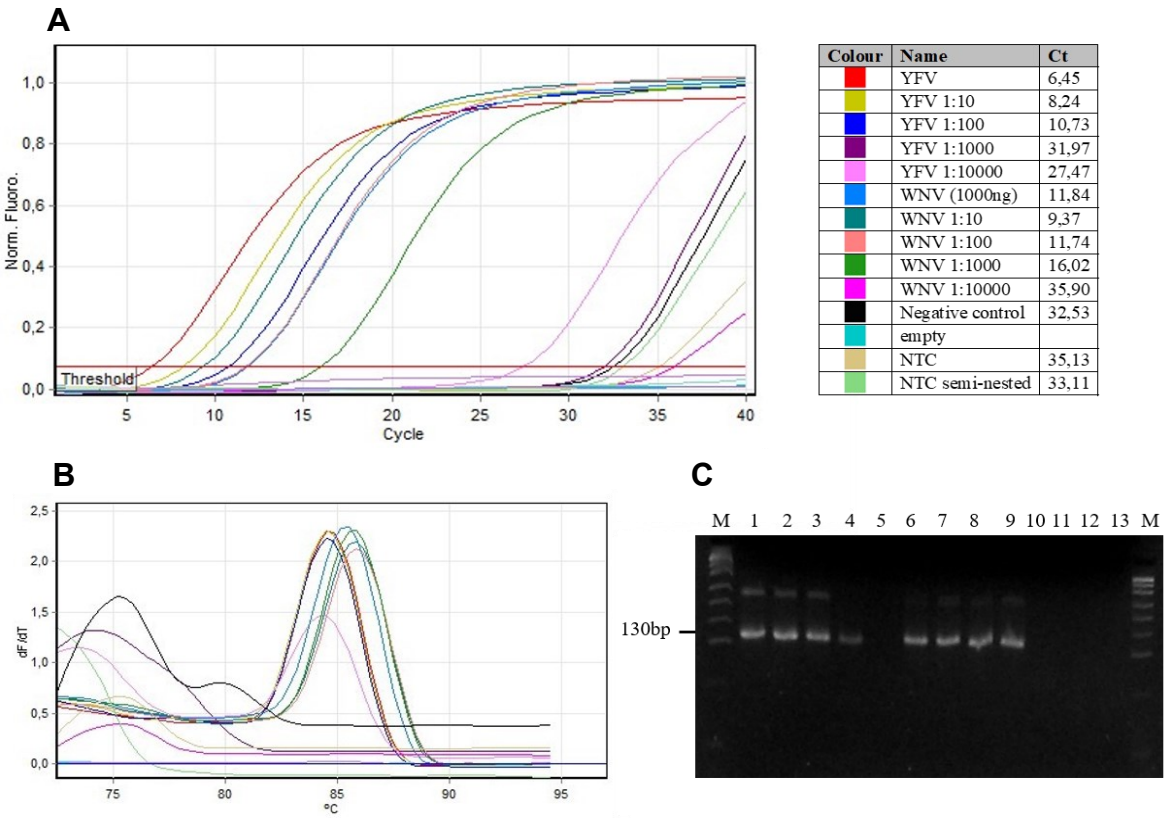
Expected product was amplified by samples of Horse 33 and Horse 34. (M) pUC19 marker. (+) positive control. (-) negative control. (NTCs) no template controls.

Appendix 20: Equid glyceraldehyde phosphate dehydrogenase (GAPDH) amplification of 298bp in 4% agarose gel.



Expected product was amplified by samples of Horse 7, 8, 15, and 30. (M1) pUC19 marker. (+) positive control. (-) negative control. (NTCs) no-template controls. (M2) GR100 marker.

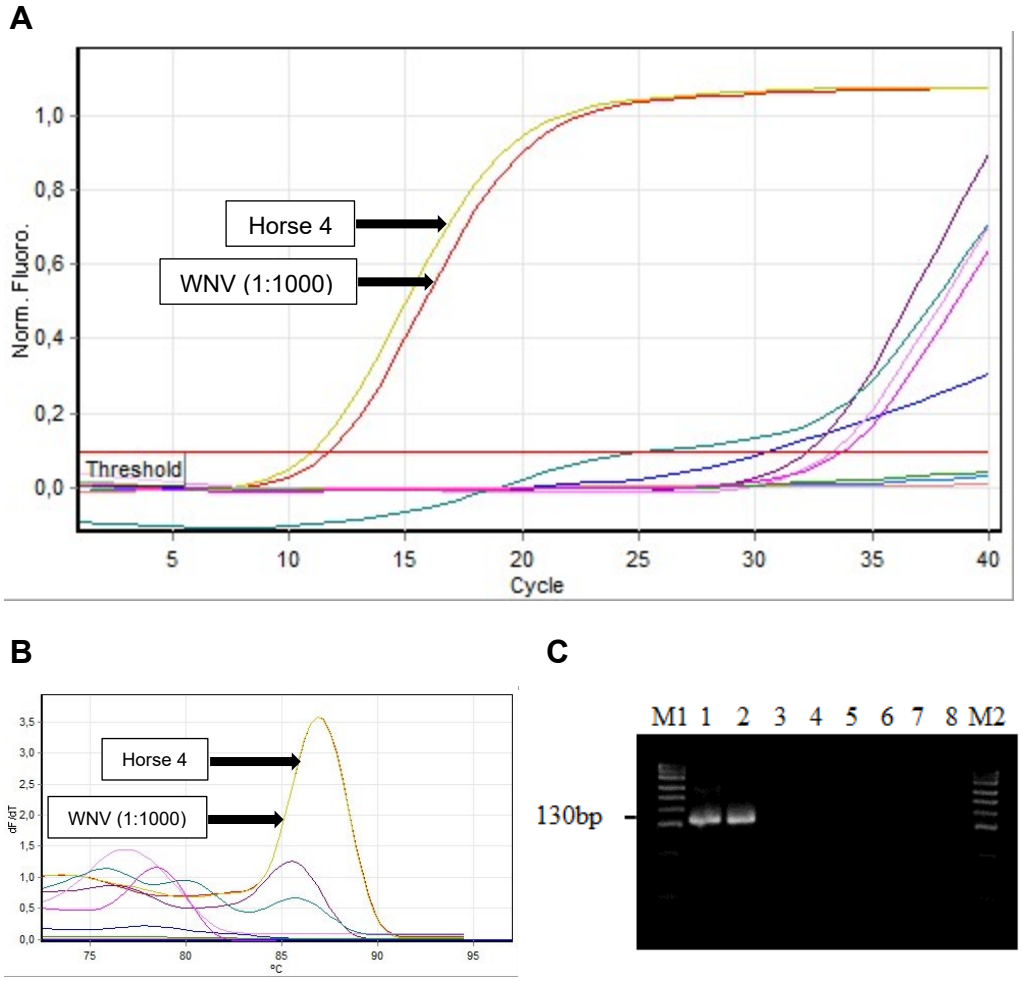
Appendix 21: Pan-Flavivirus non-structural protein 5 gene amplification curve from positive controls with semi-nested SYBR Green RT-qPCR.



Colour	Name	Ct
	YFV	6,45
	YFV 1:10	8,24
	YFV 1:100	10,73
	YFV 1:1000	31,97
	YFV 1:10000	27,47
	WNV (1000ng)	11,84
	WNV 1:10	9,37
	WNV 1:100	11,74
	WNV 1:1000	16,02
	WNV 1:10000	35,90
	Negative control	32,53
	empty	
	NTC	35,13
	NTC semi-nested	33,11

(A) Amplification curves obtained after serial dilution of positive controls. (B) Melting curve analysis. (C) 4% agarose gel. M: marker pUC19.

Appendix 22: Pan-flavivirus non-structural protein 5 gene amplification curve with semi-nested SYBR Green RT-qPCR



(A) Amplification curves obtained with WNV control and samples from Brazil. (B) Melting curve analysis, WNV (red) and Horse 4 (yellow). (C) 4% agarose gel, amplification of 130bp product from WNV (lane 1) and Horse 4 (lane 2). M1 and M2: pUC19 marker.

9.6 Material sources

Agricultural Research Service, United States Department of Agriculture, Beltsville, USA
Polyclonal rabbit anti-*Sarcocystis neurona* (Dr. Jitender Dubey)

AppliChem GmbH, Darmstadt, Germany
Tris ultrapure, 1kg (A1086,1000)

Bachofer
Light plate

Bioline GmbH, Luckenwalde, Germany
BioLine MyTaq ™ HS Mix kit (BIO-25046)

Biozym Scientific GmbH, Hessisch Oldendorf
Biozym Phor Agarose
Multicycler® PTC 200

Carl Roth GmbH + Co. KG, Karlsruhe
Boric acid (6943.3)
EDTA (X986.2)
Imidazol (3899.3)
Proteinase k (7528.4)
Sodium chloride (7029.3)
Sodium hydroxide (6771.1)
Tris (4855.2)

Cleaver Scientific Ltd, Rugby, United Kingdom
MultiSUB Mini (MSMINI7)
MultiSUB Choice (MSCHOICE15)

Dako A/S, Glostrup, Denmark
Polyclonal rabbit anti-GFAP (Z0334)
Polyclonal rabbit anti-*Toxoplasma gondii* (B1013)
Swine anti-rabbit IgG (ZO196)
Rabbit Immunoglobulin Fraction (Solid-Phase Absorbed) (X0936)

Dianova GmbH, Hamburg, Germany
Bovine serum albumin (BSA) (001-000-161)

Eastmann Kodak Inc., Rochester, USA
Kodak 1D
Kodak digital Science, Electrophoresis Documentation and Analysis System 120

Eppendorf AG, Hamburg, Germany
Micro Centrifuge (5415C)

Fermentas Life Sciences, Germany
pUC19 DNA/Mspl (Hpall) Marker 23 (SM0221)

Friedrich-Loeffler-Institut, Wusterhausen, Germany
Monoclonal mouse anti-*Neospora caninum* (Dr. Schares)

Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Vortex laboratory agitator

Institut for Virology, Justus-Liebig University, Giessen, Germany
Monoclonal mice anti-BDV-N (Bo18) (Dr. Herzog)

Institut for Virology, Philipps-University, Marburg, Germany
Polyclonal rabbit anti-BDV-p24

Invitrogen™ Life Technologies, Karlsruhe, Germany
UltraPure™Agarose (16500-500)

Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA
Mouse peroxidase-anti-peroxidase (PAP) (223-005-024)

Leica Biosystems Nussloch GmbH, Nussloch, Germany
Leica slide stainer (ST4040)

Merck GmbH KGaA (Merck Millipore), Darmstadt, Germany
Citric acid
Cresyl violet (1.05235.0025)
Giemsa's Solution (1.09204.0500)
Potassium metabisulfite
Sodium acid
Titrisol (1.09970)

Michael Hardt, Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen, Leipzig, Germany
Polyclonal rabbit anti-EHV-1 antibody

MJ Research Bio-Rad Laboratories GmbH, Munich, Germany
PTC-200 Peltier Thermal Cycler with dual 48 well

My Bio Source, Inc., San Diego, USA
Monoclonal mouse anti-Alphavirus group (MBS832145)

National Center for Biotechnology Information, Bethesda, USA
Basic Local Alignment Search Tool (BLAST®) - available at www.http://blast.ncbi.nlm.nih.gov

Nikon Instruments Europe B.V., Düsseldorf, Germany
Nikon Eclipse 80i (2CE-MGEH-6)
Nikon DS-Fi1
NIS-elements Basic Research 3.2 64bit software (2CE-MPCH-3)

Nippon Genetics Europe GmbH, Germany
Midori-green dye (M602)

PAA Laboratories GmbH, Pasching, Austria
Dulbecco's Modified Eagle Medium (DMEM)
Foetal bovine serum, 500 ml (A15-151)

Goat serum (B11-035)
Horse serum (donor) (B15-023)
Penicillin/streptomycin (10.000 U/10mg in normal saline), 100 ml (P11-010)
Swine serum (B15-030)
Trypsin-EDTA (L11-001)

Qiagen GmbH, Hilden, Germany
QIAamp® DNA FFPE Tissue Kit (6404)
RNeasy® FFPE Kit (73504)
RNeasy® Mini Kit (74106)
QIAGEN® Multiplex PCR Kit (206145)
QIAGEN One Step RT-PCR Kit (210212)
QuantiTect® Reverse Transcription Kit (205311)
Rotor Gene® Q, Rotor-Gene Q 5plex Platform (9001570)

R. Langenbrinck Labor- und Medizintechnik, Emmendingen, Germany
SuperFrost® Plus Objektträger (3-0060)

Sakura Finetek Germany GmbH, Staufen, Germany
Miles Tissue-Tek® Film® Coverslipper (4742)

Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA
Monoclonal mouse anti-bunyavirus group 3591 (sc-58098)
Monoclonal mouse anti-flavivirus 3571 (sc-58128)

Sarstedt AG & Co., Nümbrecht, Germany
Cell culture flask 25cm³ (833.910.002)

Scicons English and Scientific Consulting, Szirák, Hungary
Monoclonal mouse anti-dsRNA (J2)

Sigma-Aldrich Co. LLC., St. Louis, MO, USA
Diamino-benzidin-tetrahydrochlorid dehydrated (DAB)
Proteinase k (8038-16)

Thermo Fischer Scientific Inc., Carlsbad, MA, USA
6x Orange DNA Loading Dye (R0631)
ExoSAP-IT™ PCR Product Cleanup Reagent (78200.200.UL)
Gene Ruler™ 100bp DNA Ladder (SM0242)
NanoDrop 2000 Spectrophotometer and Software
RNase-away® ready-to-use (7005-11)
Sequenza™ Slide Rack with Coverplates™

Tom Hall, Ibis Biosciences, Carlsbad – CA, USA
BioEdit Sequence Alignment Editor BioEdit v7.2.5
available at www.mbio.ncsu.edu

Ulrich Eskens, The Hessen State Laboratory, Giessen, Germany
Polyclonal rabbit anti-rabies virus

Universitat Autònoma de Barcelona, Departament de Sanitat I Anatomia Animals, Barcelona, Spain
Polyclonal rabbit anti-*Listeria* sp. (Dr. Mariano Domingo)

Vector Laboratories Inc., Burlingame, CA, USA
Biotinylated goat anti rabbit IgG (H+L) (BA 1000)
Biotinylated horse anti-mouse IgG (H+L) (BA 2000)
Avidin-biotin complex (ABC) Vectastain® ABC-HRP Kit (peroxidase, standard) (PK-4000)

Wako Chemicals GmbH, Neuss, Germany
Polyclonal rabbit ant-Iba1 (019-19741)

9.7 Buffers and solutions

9.7.1 Histochemical staining

9.7.1.1 Periodic-acid Schiff staining

0.5% Periodic acid
Dilute 0.125g Periodic acid in 25 ml distilled water

Sulfitwater
300 ml distilled water added to 15 ml of 10% Potassium metabisulfite ($K_2S_2O_5$)
15 ml of 1N HCL

9.7.1.2 Giemsa

Giemsa (stock solution)
4g Giemsa stain powder dissolved at 60°C
250 ml glycerol
250 ml methanol

Giemsa (work solution)
4 ml Giemsa stock solution
96 ml acetate buffer distilled water pH 6.8

9.7.1.3 Luxol-fast-blue

Luxol-fast-blue MBS
0.1% Luxol fast blue MBS in 95% ethanol – 1g/1000 ml of ethanol and 5 ml 10% acetic acid (filtered)

Cresyl-violet (stock solution
1% aqueous cresyl-violet

Cresyl-violet (work solution)
0.1% cresyl-violet aqueous solution added to 5 drops of 10% acetic acid in 30 ml, heated at 50°C and filtered

Lithium carbonate solution
0.05% aqueous solution

9.7.1.4 Goldner

Light green
0.1 – 0.2g diluted in 100ml distilled water added to 0.2ml glacial acetic acid

9.7.1.5 Gram

Picric acid-acetone solution
0.2g picric acid
100 ml acetone

9.7.2 Immunohistochemistry

1% BSA/TBS
1g of bovine serum albumin (BSA)
100 ml TBS

3% BSA/TBS
3g of bovine serum albumin (BSA)
100 ml TBS

Citrate buffer pH 4.0
Main solution A: 0.1M citric acid (21.01g $C_6H_8O_7xH_2O$ in 1000 ml distilled water)
Main solution B: 0.1M sodium citrate (29.41g $C_6H_5O_7Na_3xH_2O$ in 1000 ml distilled water)

- 9 ml of the main solution A and 41 ml of the main solution B with 450 ml distilled water
- Set the pH 4.0 with 0.1M hydrochloric acid (HCl)
- Maintain the solutions at 2-8°C

Citrate buffer pH 6.0
Main solution A: 0.1M citric acid (21.01g $C_6H_8O_7xH_2O$ in 1000 ml distilled water)
Main solution B: 0.1M sodium citrate (29.41g $C_6H_5O_7Na_3xH_2O$ in 1000 ml distilled water)

- 9 ml of the main solution A and 41 ml of the main solution B with 450 ml distilled water
- Maintain the solutions at 2-8°C

DAB/Imidazol solution
100 µg 3,3'-Diamino-benzidin-tetrahydrochlorid dehydrated (DAB)
200 ml Imidazol/HCl buffer pH 7.08
Filter, protected from direct light, with paper filter
Mix the solution with magnetic mixer
Add 70µl of 30% hydrogen peroxide immediately before staining

1.5% goat serum/TBS
1.5 ml goat serum
98.5 ml TBS

1.5% goat serum/1% BSA/TBS
1.5 ml goat serum
1 ml bovine serum albumin (BSA)
97.5 ml TBS

20% horse serum/TBS
20 ml horse serum
80 ml TBS

Imidazol/HCL buffer pH 7.1
6.81g Imidazol
Dissolve in a little of distilled water
Fill the graduate container until 1000 ml with distilled water
500 ml of 0,1HCL (Titrisol)
Measure the pH
Maintain the solution at 2-8°C
Good to use for one week

Kardasewitch
200 ml of 25% ammoniac solution
800 ml of 70% ethanol

Papanicolaou solution (Hematoxylin)
Dilute the Papanicolaou hematoxylin with distilled water at a 1:20 proportion and filter

Proteinase K
5 µg Proteinase K
10 ml buffer NaCL-PBS pH 7.4
Adjust volume according to recipient used for slides' immersion

20% swine serum/TBS
20 ml swine serum
80 ml TBS

Tris-buffered saline (TBS) stock solution (10L) pH 7.6
60.57g Tris (tris[hydroxymethyl]aminomethane)
Dilute in 600 ml distilled water
Add 1n HCL (Titrisol) until completes 1000 ml
Measure pH: 7.65
72g NaCl
Add distilled water until complete a 10 l bottle

9.7.3 Cell culture

DMEM for cell passage
500 ml DMEM, high glucose
50 ml foetal calf serum
5 ml penicillin/streptomycin

9.7.4 Molecular biology

Agarose gel 2%
UltraPure™Agarose
TBE buffer 0.5x
Midori Green, 1:10

Appendix

Agarose gel 4%
Biozym Phor Agarose
Microwave preparation instructions:
Choose an Erlenmeyer that would fit 2-4 times the final volume of the gel
Pour 0.5x cooled TBE buffer with a magnet, and let the Agarose scatter while the solution is mixed quickly
Let the Agarose settle to reduce foam formation while cooking
Heat the solution in the microwave for 1min in half power level
Let it rest for 15 min
Heat the solution in the microwave for 2min in half power level
Mix the solution slowly to dissolve the rest of the Agarose
Bring it to a higher temperature for 1min
Mix slowly
Add distilled water to acquire the initial weight
Mix thoroughly
Add ethidium bromide
Before laying the solution in the PCR chamber, let it cool down until 50-60°C. After that, let it solidify at room temperature at the chamber and later keep it at 4°C for 20 min

DEPC water RNase-free
1 ml of Diethylpyrocarbonate (DEPC)
1 litre of bi-distilled water
Mix overnight
Autoclave at 120°C for 1h and 15 min

Proteinase K (20 mg/ml)
20 mg of Proteinase K lyophilized, ≥ 30 mAnson-U/mg,
1 ml of 20mM Tris-HCL pH 7,5

Tris Borate EDTA buffer (TBE) 5x stock solution
54g of Tris ultrapure
27.5g of boric acid,
20 ml of 0.5M EDTA,
Adjust pH to 8.3
Maintain refrigerated at 4°C

TBE 0.5x solution
100 ml of TBE 5x stock solution
900 ml of bi-distilled water

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