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# Virological and molecular biological characterization of Equid Herpesvirus 1 (EHV-1) isolates from Germany

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**Nicht was er mit seiner Arbeit erwirbt,**
**ist der eigentliche Lohn des Menschen,**
**sondern was er durch sie wird.**
(John Ruskin)

Meiner Familie

# Contents

# List of Tables

# List of Figures

# Abbreviations

| | |
|---|---|
| A | adenine |
| BAC | bacterial artificial chromosome |
| bp | base pair |
| BHV-1 | Bovine Herpesvirus 1 |
| C | cytosine |
| CHV-1 | Canine Herpesvirus 1 |
| cm | centimetre |
| CMC | Carboxy-Methylcellulose |
| CNS | Central nervous system |
| CPE | cytopathic effect |
| CTLP | cytotoxic T-lymphocyte precursor |
| D | Aspartic acid/Asp |
| EAV | Equine Arteritis Virus |
| ED | equine dermal |
| EDM | Eagle‘s minimum essential medium, Dulbecco‘s modification |
| EDTA | Ethylendiamintetraacetat |
| EHM | Equine herpes myeloencephalopathy |
| EHV-1 | Equid Herpesvirus 1 |
| EHV-4 | Equid Herpesvirus 4 |
| EHV-9 | Equid Herpesvirus 9 |
| EICP | EHV-1 infected cell protein |
| ELISA | enzyme-linked immunosorbent assay |
| FCS | Foetal calf serum |
| FU Berlin | Freie Universität Berlin (Free University Berlin) |
| g | force (Relative centrifugal force/RCF) |
| G | guanine |
| gD | glycoprotein D |
| gC | glycoprotein C |
| HPLC | high-performance liquid chromatography |
| HSV-1 | Herpes Simplexvirus 1 |
| ICP | infected cell protein |
| IE | immediate early |
| IFA | immunofluorescence assay |
| IR | internal repeat |
| FCS | foetal calf serum |
| FITC | fluoresceinthiocyanat |
| LAT | latency associated transcript |
| MEM | Minimum Essential Medium |
| ml | millilitre |
| MLN | mandibular lymph node |
| MOI | multiplicity of infection |

| | |
|---|---|
| N | Asparagine/Asn |
| NCBI | National Center for Biotechnology Information |
| n.d. | not done |
| nm | nanometre |
| nPCR | nested polymerase chain reaction |
| NPS | nasopharyngeal secretion |
| NSAID | non steroidal anti inflammatory drug |
| NT | neutralization test |
| nt | nucleotide |
| ORF | open reading frame |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| REA | restriction enzyme analysis |
| RK | rabbit kidney |
| RPM | rounds per minute |
| S | Serine/Ser |
| SMLN | submandibular lymph node |
| SNP | single nucleotide polymorphism |
| T | thymine |
| TAE | Tris-Acetat-EDTA |
| TR | terminal repeat |
| UL | Unique long |
| V | volt |
| Y | Tyrosine/Tyr |

# 1 Introduction

The focus of this study is on EHV-1 (Equid Herpesvirus 1) induced abortions in Germany. Nine different types of herpes viruses have been reported in equines (Davison et al., 2009). The pathogen EHV-1 is found worldwide and can cause different clinical outcomes like respiratory diseases, abortions, neonatal death and neurological disorders in horses. EHV-4 is formerly known as the rhinopneumonitis virus (Studdert et al., 1981; Thein and Huselstein, 2000) and rarely causes abortion (Benetka et al., 2002). EHV infection in wild animals can also lead to signs like respiratory diseases (Blunden et al., 1998), as well as neurological signs (Fukushi et al., 1997; Kennedy et al., 1996; Taniguchi et al., 2000a; Borchers et al., 2006a); abortions are also observed (Montali et al., 1985; Wolff et al., 1986). EHV isolates from the zebra, antelope and onager have been classified as being close to EHV-1 or EHV-9 (Borchers et al., 2006a; Ghanem et al., 2008).
In recent years, Germany has experienced an increase in number of cases of EHV-1-induced equine abortions. CNS (central nervous system) cases are reported less often (Petzoldt et al., 1982; Stierstorfer et al., 2002).
The results of previous studies on EHV-1 isolates have suggested that there are distinct EHV-1 pathogenic strains. Many authors have therefore tried to differ by restriction enzyme analysis between EHV-1 abortigenic and CNS strains (Allen et al., 1985; Studdert et al., 1992). However, this could not be achieved. Therefore, Nugent et al. (2006) investigated two EHV-1 strains (abortigenic and CNS) by PCR and sequencing. They found a point mutation that seemed to be characteristic of CNS strains. The occurrence of EHV-1, which causes neurological signs, is significantly associated with this single nucleotide polymorphism in the viral polymerase gene (open reading frame/ORF 30). The nucleotide exchange from adenine (A) to guanine (G) at position 2254 has been found to lead to the replacement of the amino acid asparagine (N 752) with aspartic acid (D 752) (Goodman et al., 2007; Van de Walle et al., 2009). The simultaneous occurrence of both variants ($G_{2254}$ and $A_{2254}$) in a single horse has rarely been reported (Allen et al., 2008; Pusterla et al., 2009b), and the effects of this simultaneous occurrence on disease symptoms and severity are not known.
An additional exchange at nucleotide position (nt) 2258 (A to C) in viral polymerase has been reported (Nugent et al., 2006; Smith et al., 2009). This exchange was found to result in the exchange of the amino acid tyrosine (Y 753) with serine (S 753). It is not known whether the exchange at nt position 2258 influences the abortigenic or neuropathogenic potential of EHV-1 strains.

The aim of this thesis is to identify the non-neuropathogenic genotype ($A_{2254}$) in EHV-1 abortigenic strains. In this study, EHV-1 field isolates from abortion cases in Germany (1987-2009), and selected EHV-1-related and EHV-9 strains from wild equines and cattle, were investigated. Furthermore, blood samples were obtained from the animals involved in two abortion outbreaks on two different stud farms in Germany at intervals of several weeks for a serological survey.

# 2 Review of literatures

## 2.1 Taxonomy of Equid Herpesviruses

The family of *Herpesviridae* is divided into three subfamilies ($\alpha$-, $\beta$-and $\gamma$-*Herpesvirinae*) based on their biological and antigenic properties, and their genome configuration (Davison et al., 2009). Nine equine herpes viruses have been identified. Six of them belong to the subfamily of $\alpha$-*Herpesvirinae* (EHV-1, -3, -4 and -9; Asinine Herpesvirus 1 and 3). Three belong to the subfamily of $\gamma$-*Herpesvirinae* (EHV-2, -5; Asinine Herpesvirus 2).
The investigated viruses Equid Herpesvirus 1 (EHV-1), Equid Herpesvirus 4 (EHV-4) and Equid Herpesvirus 9 (EHV-9) belong to the subfamily $\alpha$-*Herpesvirinae* and to the genus Varicellovirus (see Tab. 1).

Table 1: The classification of equine herpes viruses by Davison et al., 2009

| Taxon | Name | Acronym | Common name |
|---|---|---|---|
| Order | *Herpesvirales* | | |
| Family | *Herpesviridae* | | |
| Subfamily | $\alpha$-*Herpesvirinae* | | |
| Genus | Varicellovirus | | |
| Species in the genus | Equid Herpesvirus 1 | EHV-1 | Equine abortion virus |
| | Equid Herpesvirus 3 | EHV-3 | Equine coital exanthema virus |
| | Equid Herpesvirus 4 | EHV-4 | Equine rhinopneumonitis virus |
| | Equid Herpesvirus 8 | EHV-8 | Asinine Herpesvirus 3 |
| | Equid Herpesvirus 9 | EHV-9 | Gazelle Herpesvirus |
| Tentative species | | | |
| In the genus | Equid Herpesvirus 6 | EHV-6 | Asinine Herpesvirus 1 |
| Subfamily | $\gamma$-Herpesvirinae | | |
| Genus | Percavirus | | |
| Species in the genus | Equid Herpesvirus 2 | EHV-2 | Equine Herpesvirus 2 |
| | Equid Herpesvirus 5 | EHV-5 | Equine Herpesvirus 5 |
| Unassigned species in | | | |
| the subfamily | Equid Herpesvirus 7 | EHV-7 | Asinine Herpesvirus 2 |

## 2.2 Equid Herpesvirus 1, 4 and 9

### 2.2.1 Morphological structure

A virion is composed of core, capsid, tegument and envelope. The viral genome consists of a linear double-stranded DNA of 124 to 235 kilobase pairs located in the inner body (core). The core is surrounded by an icosahedral capsid with a diameter from 120 to 300 nm (nanometre) consisting of 162 capsomeres. The capsid is surrounded by the tegument, an amorphous protein which occupies the space between capsid and envelope. The envelope contains membrane glycoproteins. They are important for virus replication and include several proteins which elicit an immune response.

### 2.2.2 Genome structure

Until 1981, EHV-1 was divided into respiratory and abortigenic strains. By studying the genomes with restriction enzymes it was found, however, that the abortigenic and respiratory strains are related but distinct viruses. Therefore, the nomenclature changed to EHV-1 and EHV-4 (Studdert et al., 1981; Studdert, 1983).
The sequencing of the entire viral genomes of EHV-1 and EHV-4 brought about a breakthrough in the differentiation of pathotypes.

**EHV-1** Telford et al. (1992) determined the DNA sequence of the EHV-1 strain Ab4 (GenBank Accession No.M86664), which can cause abortion and neurological disease. The EHV-1 DNA has been characterized with a base composition of 56.7 % G + C (Darlington and Randall, 1963; Soehner at al., 1965; Telford et al., 1992). The double-stranded DNA, 150.223 bp in size, encodes 80 open reading frames. The open reading frames encode proteins with specific functions (Telford et al., 1992).
The DNA consists of two components: L and S (Whalley et al., 1981). The S component is divided in a unique sequence ($U_S$) and the large inverted repeat ($IR_S$ and $TR_S$). The L component consists of a unique sequence ($U_L$) and a small inverted repeat ($IR_L$ and $TR_L$) (Chowdhury et al., 1990). Bovine foetal isolates were identified as EHV-1, though there are some changes in the viral DNA explained by the virus adaption to the new host system (Chowdhury et al., 1988).

**EHV-4** The sequenced EHV-4 strain NS80567 was 145.597 bp in size. A long unique region (112.398 bp) was flanked by a short inverted repeat (27 bp). The short inverted repeat was associated with a short unique region (12.789 bp) that was flanked by an inverted repeat (10.178 bp). EHV-4 encodes 76 different open reading frames, but three are duplicated in the inverted repeat. Thus, 79 open reading frames resulted. The base composition of EHV-4 was 50.5% G + C (Telford et al., 1998).

**EHV-9** The size of the sequenced EHV-9 strain (NC011644) was 148.371 bp. EHV-9 contained 80 open reading frames and has a G + C base composition of 56%. The identity between the nucleotide sequences of the ORFs in EHV-9 and EHV-1 were 86 to 95% (Fukushi et al., 2012).
In various studies, the gB region of EHV-1 and EHV-9 were compared and homologies found (Fukushi et al., 1997; Ibrahim et al., 2007). The gB region of EHV-9 showed a 97% homology to that of EHV-1 and 92% to that of EHV-4 (Fukushi et al., 1997). Ibrahim et al. found 2007 that the EHV-1 gB sequence showed 98% homology to that of onager and zebra, 97.8 % homoglogy to that of gazelle (Ibrahim et al., 2007). These figures indicate that the wild equid EHV-9 isolates are quite similar to EHV-1.

### 2.2.3 The DNA polymerase (open reading frame 30)

In the ORF (open reading frame) 30 at position 2254 a single nucleotide change was found to be associated with the outcome of neurological signs (Nugent et al., 2006). The ORF 30 of EHV-1, EHV-4 and EHV-9 were characterized as DNA polymerase, which contribute significantly to the replication. The beginning and the end of the ORF 30 varies between EHV-1, EHV-4 and EHV-9

(see Tab. 2). Between the ORF 30 of EHV-1 and EHV-4 a 88.3% identity was found (Telford et al., 1992). The ORF 30 sequence of EHV-9 was similar to that of HSV-1 (www.ncbi.nlm.nih.gov). Moreover, there is a great similarity between the ORF 30 sequence of EHV-1 and EHV-9, the ORF 30 sequence of the giraffe herpesvirus (EHV-9) was found identical 99.5% to EHV-9 and 94.6% to the ORF 30 sequence of EHV-1 (Kasem et al., 2008).

Table 2: Comparison of the ORF 30 localisation between EHV-1, -4 and -9

| Virus | Start (bp) | Stop (bp) | Functions of proteins | Source |
|---|---|---|---|---|
| EHV-1 ORF 30 | 51.501 | 55.184 | DNA polymerase | www.ncbi.nlm.nih.gov |
| EHV-4 ORF 30 | 51.263 | 54.924 | DNA polymerase | Telford et al., 1998 |
| EHV-9 ORF 30 | 51.097 | 54.759 | DNA polymerase | www.ncbi.nlm.nih.gov |

It was demonstrated for HSV-1 (herpes simplex virus) that the putative DNA polymerase catalytic subunit stimulates the synthesis of the DNA and increases its processivity (Purifoy et al., 1977; Gallo et al., 1989). In a further study on the function of EHV-1 ORF 30 undertaken by Loregian et al. (2006), they defined the ORF 30 to encode the putative DNA polymerase catalytic subunit (pORF 30) and to provide functions close to those of their HSV-1 counterparts.

### 2.2.4 Latency

The ability to cause latent infection is a common feature of herpes viruses and a survival strategy. Latency is defined as a reversible non-productive infection of cells through a replication-competent virus (Garcia-Blanco and Cullen, 1991). Most mammalian Alphaherpesviruses are neurotropic and latency is usually observed in sensory ganglia, where they evade the immune system (Roizman and Sears, 1987).

LATs (latency-associated transcripts) are antisense oriented transcripts which accumulate in host cells during latent infections. The LATs are encoded by immediate early genes (Fraser et al., 1992).

EHV-1 establishes latency in the trigeminal ganglia and submandibular lymph nodes (SMLN) (Welch et al., 1992; Slater et al., 1994; Pusterla et al., 2010b). Horses with a positive detection of latent EHV-1 in SMLNs were generally younger than horses with latent EHV-1 in the trigeminal ganglia, but the statistical significance is lacking (Pusterla et al., 2010b).

EHV-4 is localized in the state of latency in the trigeminal ganglia (Borchers et al., 1997).

Zebras were assumed to be the natural reservoir (Borchers et al., 2005b; Schrenzel et al., 2008) and the latent carrier of EHV-9, confirmed by the evidence of viral DNA in the trigeminal ganglia of a zebra without showing clinical signs (Borchers et al., 2008).

Following acute infection, the virus can go into latency and can remain for lifetime in the host. The detection of latent EHV-1 varies in different studies from 54 to 88% (Allen et al., 2008; Carvalho et al., 2000) as explained subsequently. Allen et al. (2008) detected latent EHV-1 DNA in 54% (71/132) SMLN (submandibular lymph nodes) of abattoir horses, which were collected during post mortem examination. Only SMLN but not ganglia were investigated. The detection of transcripts of EHV-1 glycoprotein B by reverse transcription PCR achieved the differentiation

of active from latent EHV-1 infections (Allen et al., 2008). In another study (Carvalho et al., 2000) from different samples (blood, neuronal tissue, nasal swab, serum, visceral tissues including lung, spleen, liver and retropharyngeal lymph nodes) of 116 abattoir horses, EHV-1 DNA was found in 88% (267 samples from 116 abattoir horses). The authors developed a highly sensitive PCR but weather it detects „...true latent EHV-1 or a low level of persisent EHV-1 infection can not be fully determined"(Carvalho et al., 2000).
A trigger for the viral reactivation out of latency can be stress, overcrowding, treatment with corticosteroids (Pusterla et al., 2010a; Pusterla et al., 2010b) or vaccination. This reactivation can result in abortion or CNS disorders.

### 2.2.5 Epidemiology

EHV-1 is a highly contagious pathogen. Infections with EHV-1 are common in horses worldwide and occur mainly enzootically.
An estimate of the prevalence of EHV-1 suggested 60% of the horse population is latently EHV-1 infected (Lunn et al., 2009). Most of the horses become infected very early as foals or yearlings (van Maanen et al., 2002).
Abortions occur seasonally during November to May (Borchers et al., 2006b). EHV-1-induced abortions occur mostly between the 8th and 10th months of pregnancy. Abortions induced by EHV-1 are never found before the 5th month of pregnancy. A higher EHV-1 susceptibility of the uterine endothelial cells in late pregnancy is proposed as the cause of abortions in the last stages of pregnancy (Smith et al., 1996).
EHV-1, the causal agent of virus-induced abortion can rarely also induce neurological signs. But Wilson (1997) commented that special strains of EHV-1 can possibly trigger neurological diseases. In the reported rare occurrence of neurological cases a seasonal appearance was observed similar to the seasonal occurrence of abortions (Wilson, 1997). Neurological signs are observed mainly in mares after an abortion (Henninger et al., 2007). In recent years the neurological EHV-1 (EHM/Equine herpes myeloencephalopathie) has become an emerging disease in the United States of America (Lunn et al., 2009).
The latently infected horses are virus carriers and the reactivation of latent virus represents a potential danger to other horses. Through the high prevalence of EHV-1 an infection of young horses is very likely. Latently-infected pregnant mares could abort as a consequence of the reactivation through stress or other factors. After abortions, the foetus and all foetal parts can contain the infectious virus, and are highly contagious (Allen and Bryans, 1986). The infectious virus is excreted via the nasal secretions (Pusterla et al., 2009b), therefore, virus transmission by direct contact or via droplets after coughing or snorting is possible. The climatic conditions and virus concentration in the air (pasture, barn) should be taken into account as risk factors for the virus transmission (van Maanen et al., 2000). It is not only the aborted foetus and the placenta that are detected as being highly contagious, the infectious virus has also been found in the semen of stallions (Hebia-Fellah et al., 2009). In the semen of two of three naturally infected stallions EHV-1 was observed for close to three weeks, but the spermatozoa were not found directly associated with the shedding EHV-1 (Walter et al., 2012). The risk of EHV-1 transmission via semen from stallion to mare is still unclear.
In contrast, the EHV-4 infection occurs mostly with respiratory signs. EHV-4 infections show no

clear seasonal incidence. The infection begins in the nasopharynx and remains restricted to the upper airways. In the first 12 days the virus is excreted through the secretions (Benetka et al., 2002). Sporadic abortions caused by EHV-4 are very rare (Allen and Bryans, 1986; Benetka et al., 2002). With regard to the occurrence of neurological diseases caused by EHV-4 only a few reports are available (Allen and Bryans, 1986; Benetka et al., 2002).
EHV-1-related infections in wild equids could result in neurological signs or abortion (Wohlsein et al., 2011). Montali et al. reported in 1985 an EHV-1-induced abortion in an onager. A zebra in the adjacent pen developed a short time later neurological signs. It was demonstrably caused by the same EHV-1 strain (Montali et al., 1985).
Equine Herpesvirus 9 (EHV-9) is closely related to EHV-1 (Kasem et al., 2008; Schrenzel et al., 2008) and serologically cross-reactive with EHV-1 (Fukushi et al., 1997). EHV-9 was formerly known as Gazelle Herpesvirus 1 (GHV-1) (Fukushi et al., 1997). In contrast to fatally-infected mice and rats, in experiments with EHV-9-infected horses only mild clinical symptoms were seen (Taniguchi et al., 2000a; Yanai et al., 1998). Burchell's zebras were proposed to be the natural host of EHV-9 (Borchers et al., 2005b).
EHV-1 strains have the ability to infect cattle and under natural conditions cause illnesses (Crandell et al., 1988).

### 2.2.6 Clinical signs

**Respiratory disease** For EHV-1 respiratory infection, a short incubation time of one to three days is noticeable (Gibson et al., 1992). In rare cases mild or subclinical upper respiratory infections occurs. Clinical signs can last for two to seven days (Allen and Bryans, 1986). In older horses, the respiratory infections may pass off asymptomatically (Allen et al., 2004).
The incubation period for EHV-4 varies from three to 10 days. The disease manifests itself as a sudden onset of fever, anorexia, lymph node swelling and catarrh (Allen and Bryans, 1986) and can be complicated by secondary bacterial infection, chronic cough in terms of a mucopurulent rhinitis or pharyngitis. In general, the animals recover relatively quickly. In most horses the EHV-4 infection occurs in the first two years (Crabb and Studdert, 1995). In older animals, the rhinopneumonitis is mostly asymptomatic or only performance weaknesses are observed. Complications due to secondary bacterial infections exacerbate the disease process. Through reinfection, mild respiratory signs can occur.

**Abortion** The incubation period for abortion varies between nine days to four months (Allen, 1998). EHV-1 infection can result in abortion of non-viable or stillborn foals (Murray et al., 1998). The mares are often infected silently, but sometimes oedema of the legs or anorexia are noticeable (van Maanen, 2002).
EHV-4-induced abortions are very rare (Allen and Bryans, 1986; Crabb and Studdert, 1995).

**Neurological disorder** The incubation time for EHV-1-induced neurological signs varies between six to 10 days (Henninger et al., 2007). Fever is the first sign of infection (Gilkerson and Barrett, 2008; Pronost et al., 2012). The neurological signs vary from mild ataxia to severe paralysis (Henninger et al., 2007). The clinical signs range from ataxia, swaying, weakness and disorientation followed by paralysis of fore and/or hind limbs (Thein, 1996; Stierstorfer et al.,

2002; Allen et al., 2004) with the horses becoming recumbent (Crowhurst et al., 1981). Urinary incontinence caused by a bladder paralysis and anal dysfunction can also occur (Goehring and van Oldendruitenborgh-Oosterbann, 2001). The prognosis for recumbent horses is bleak and complications could lead to the death of infected horses (Studdert et al., 2003).
In the rare cases of neurological diseases induced through EHV-4, movement disorders and paralysis are noticeable (Benetka et al., 2002).

**Disease in stallions** In EHV-1-infected stallions, scrotal oedema, loss of libido and fever were reported (Tearle et al., 1996). In a neurological EHV-1 outbreak on a thoroughbred stud farm, stallions also suffered neurological signs like ataxia (Greenwood and Simson, 1980). Nothing is known yet about infection with EHV-4 in stallions.

**Equid Herpesvirus 1 in cattle** There are only a few investigations about EHV-1 infections in cattle (Chowdhury et al., 1988; Crandell et al., 1988; Pagamjav et al., 2007). In two cases, an infection with EHV-1 in bovine resulted in abortion (Smith, 1976; Crandell et al., 1988).

**Disease in gazelle, blackbuck, onager and zebra** Neurological signs like depression, convulsion and weakness were observed in gazelles and blackbuck (Kennedy et al., 1996; Yanai et al., 1998).
EHV-1 infection can result in abortions in the following species: zebras and onager (Montali et al., 1985; Wolff et al., 1986).
In a zebra stallion with a systemic EHV-1-like infection, two different kinds of illnesses were observed: first a rhinitis and pulmonary oedema, and additionally an orchitis and epididymitis (Blunden et al., 1998).

**Equid Herpesvirus 9** EHV-9 was observed to induce neurological signs like encephalitis in Thomson‘s gazelles (Fukushi et al., 1997) and giraffe (Kasem et al., 2008).

### 2.2.7 Treatment

In the case of respiratory infections a treatment is uncommon. Fever can be treated with antipyretics and to avoid bacterial secondary infections, prophylactic antibiotics could be given (van Maanen, 2002).
Abortions happen suddenly. The mares should be separated and the barn must be cleaned. Furthermore, the placenta and/or foetus should be kept for virological and pathological examination or be disposed of safely.
For neurological EHV-1 disease, no specific treatment exists. Nutrition and the reduction of the inflammation in the CNS via NSAIDs (nonsteroidal anti-inflammatory drug) are essential (Pusterla et al., 2009a). In the case of bladder dysfunction, bladder control and catheterization are indicated. If faeces are no longer deducted, the faeces must be removed (Friday et al., 2000). Recumbent horses should be placed in standing position by using slings (Pusterla at al., 2009a), if that not succeed the sternal position is recommended with regular rearrangement of the position. Treatment with corticosteroids could prevent harm caused by immune-complexes, but their immune-suppressive impact is a contradiction since they can prolong the viraemia (Allen and

Bryans, 1986). NSAIDs may be considered as an alternative.
Vaccines are available but not fully protective, therefore an antiviral chemotherapy is needed. Antiviral medication reduce viral replication and prevent viral replication in in-contact horses. Acyclovir inhibits the viral DNA synthesis by blocking the viral polymerase without influencing the cellular DNA polymerase (Henninger et al., 2007), but in the stage of latency the virus is unaffected (Field et al., 2006). A limiting factor in the treatment with acyclovir is the bioavailability of the drug (Garré et al., 2007). However, there are several reports on the efficacy of acyclovir in EHV-1 infections (Murray et al., 1998; Friday et al., 2000; Henninger et al., 2007).

### 2.2.8 Prophylaxis and Management

For the decrease of the EHV-1 infection risk, a reduction of stress by overcrowding and assignment of the boxes is thought to be helpful (Pusterla et al., 2009a). There should be a separation of the foaling and stable areas. Furthermore, rules of good hygiene can be drawn up.
Vaccination is a valid prophylaxis method and reduces the risk of EHV-1 abortions (Ostlund, 1993). Vaccinated horses were experimentally infected with a neuropathogenic EHV-1 strain and a significant reduction of viral shedding was observed (Goodman et al., 2006). This supports the relevance of vaccination.
There are modified live-virus vaccines (Prevaccinol EHV-1, Intervet) and inactivated vaccines (Duvaxyn EHV-1/-4, Fort Dodge; Resequin EHV-1/-4, Intervet; Resequin EHV-1/-4 plus, Intervet). In Germany, only the inactivated vaccine Duvaxyn EHV-1/-4 (Fort Dodge) is licensed to prevent respiratory disease and abortion (Pusterla et al., 2009a). The other inactivated vaccines (Resequin EHV-1/-4, Intervet; Resequin EHV-1/-4 plus, Intervet) only have a protection claim against respiratory disease (Patel and Heldens, 2005).
Due to the increasing number of cases of neurological diseases in the United States a study of the effectiveness and a comparison between the modified live-virus vaccine (Rhinomune$^{TM}$, Pfizer) and the inactivated combination vaccine (Fluvac Innovator 6 combination vaccine, Fort Dodge) was done. In case of infection, horses vaccinated with the modified live-virus (Rhinomune$^{TM}$, Pfizer) showed a shorter period of fever, and moreover a better protection against the neurological EHV-1 form was observed (Goodman et al., 2006).
In another study a recently developed modified live-virus (MLV) vaccine (not on the market) based on an abortigenic strain (NY03) was researched. Horses were vaccinated with the MLV vaccine and then experimentally infected with a neuropathogenic EHV-1 strain. An immune response and a reduction in clinical signs and viraemia were observed (Van de Walle et al., 2010). The vaccine Duvaxyn also reduced the duration of virus excretion as well the clinical signs. However, there is no 100% protection against the disease or virus shedding (Heldens et al., 2001).

### 2.2.9 Pathogenesis

**Respiratory disease** EHV-1 and -4 are both transmitted via nasal infection and replicate in the epithelial cells of the upper respiratory tract. Progress of the infection results in inflammation of the nasopharyngeal mucosa and degeneration of epithelial cells in the respiratory tract. The infectious viruses invade the local lymph nodes and ganglia. In the cells, necrosis and intranuclear inclusion bodies are observed (van Maanen, 2002). In most of the EHV-4 infections, the infection

is limited to the respiratory tract. EHV-4 infections rarely cause abortion or neurological disease. A latency can develop and in this case the latently-infected horses appear clinically healthy (Benetka et al., 2002). The trigeminal ganglia could be demonstrated to be a location for latent EHV-1 and EHV-4 (Borchers et al., 1999). EHV-4 infections do not usually develop cell-associated viraemia (Edington et al., 1986; Slater et al., 2006). Only one case of cell-associated EHV-4 viraemia has been published (Matsumura et al., 1992).

**Abortion** EHV-1 primary-infected adult horses show either mild respiratory signs or are asymptomatic. EHV-1 infections leads to viraemia (Allen, 1998). The leukocyte-associated viraemia may take up to 14 days and is seen as a prerequisite for an abortion (Lunn et al., 2009) followed by virus replication in blood vessels in the uterus (Patel et al., 1982). Herpes viruses have the ability to go into latency. Abortions can occur in newly EHV-1 infected horses or by reactivation of latent EHV-1. In the first case, EHV-1 invades through the nasal epithelia and quickly infects leukocytes and the endothelial cells of the blood vessels. Then, EHV-1 viraemic spread occurs from regional lymph nodes to mononuclear cells (PBMC/peripheral blood mononuclear cells), resulting in cell-associated viraemia in monocytes and lymphocytes. If latent EHV-1 is reactivated, the virus starts a leucocyte-associated viraemia from the regional lymph node (Allen, 1998).
In aborted foetuses, pulmonary oedema, petechiation in the lungs, splenomegaly, hepatomegaly, hepatic necrosis and sometimes ascites are found. Microscopically intranuclear inclusion bodies are found in the liver, lung, spleen and thymus. Additionally, necrosis in the liver and spleen, and necrotizing bronchiolitis or pneumonitis are detected (Allen et al., 2004).
In the pathogenesis of abortion, several development paths have been discussed. The most likely pathogenesis is the so called endotheliotropism of the virus. The viral replication takes place in the endothelium of blood vessels. Thrombosis of the blood vessels of the endometrium results, followed by ischaemic degeneration of the uterus. A non-infected foetus is expelled (Edington et al., 1991). A comparison between early and late EHV-1 infection showed that the degree of EHV-1 antigen in the uterus is approximately equal to each other, but the vascular damage in the late-infected uterus is more pronounced (Smith et al., 1996). Smith and Borchers (2001) suggested a transplacental spread with an infarction of mikrocotyledones followed by the break-down of the uteroplacental barrier and resulted in the EHV-1 infection of the foetus. Depending on the viral load it comes either to an abortion or the birth of a weak foal which is already carrying the virus (Patel and Heldens, 2005). Different virus strains may have different degrees of endotheliotropism and thus result in different endothelial damage (Smith et al., 2000). The ability of virus crossing the placental barrier depends on the size and expanse of endometrial lesions (Gerst et al. 2003). This possibly explains the differences in the abortigenic potential. The uterine endothelial cells show a higher susceptibility in late pregnancy, possibly caused by cell surface proteins during pregnancy (Smith et al., 1996).
A second hypothesis for the pathogenesis of abortions is that the virus in the latent stage gets to the foetus transplacentally in lymphocytes, thereby the virus circumvents the immune response of the host. A reactivation of the latent virus in the foetus can lead to an abortion (Allen et al., 2004).
The third theory dealth with the effects of antibody-antigen complexes, so-called immune complexes. These are formed on the walls of the endometrial blood vessels and lead to thrombosis.

This leads possibly to the abortion of a non-infected foetus. The pathogenesis was proposed to be similar to that for the EHV-1 neurological disease (Crabb and Studdert, 1995).

**Neurological disease** Neurological signs mostly occur shortly after acute respiratory disease or abortion. In an EHV-1 infection experiment with intracerebral EHV-1-infected baby mice EHV-1 replication in neurons and glial cells was proven (Nowotny et al., 1987). In horses, very rare macroscopic lesions like haemorrhages in the meninges and the brain parenchyma were observed. Vasculitis, congestion, ischemic degeneration and thrombosis were detected in histological investigations (Wilson, 1997). Diffuse multifocal myeloencephalopathy, vasculitis, haemorrhage, thrombosis and ischemic neuronal injury were mentioned in pathological investigations (Edington et al., 1986).
The first virus replication is in the endothelial cells of the upper airways. Via viraemia PBMCs (peripheral blood mononuclear cells) are infected and transport the virus to the CNS vasculature causing infection of the endothelium of the arteries and veins of the CNS (Goehring et al., 2011). EHV-1 antigen has been found in the endothelium of the arteries and veins of the CNS. In connection with an immune-mediated vasculitis with secondary ischemic degeneration of the neuroparenchym, neurological signs could be induced (Borchers et al., 2006b). The number, size and the localization of the lesions resulted in varying degrees and severity of clinical signs (Goehring et al., 2005). The infection spreads very quickly and the sanctions to avoid the disease came mostly later than the infectious rate (Crowhurst et al., 1981). The biphasic increase of the body temperature correlated with the initial infection and the start of viraemia (Gilkerson and Barrett, 2008).
Even with regard to the pathogenesis of EHM, several theories exist. The most favoured theory for the formation of EHM is the cell tropism of the virus. The neurotropism of certain EHV-1 strains leads to a cell-associated viraemia in the endothelial cells of the blood vessels of the CNS (Patel et al., 1982; Patel and Heldens, 2005). The infection of PBMCs was investigated and a different frequency of viral DNA in the PBMC subpopulation was shown. Most frequently CD8+ lymphocytes contained viral DNA, followed by the B-lymphocytes, however, the role of this finding in the pathogenesis is still unclear (Wilsterman et al., 2011).
Intraperitoneally with EHV-1 infected mice showed myelitis and a haematogenous route of infection was suggested. Neuronal spread was also mentioned (Hasebe et al., 2002). In experimentally intranasally infected adult mice, a neurotropic spread in the brain was demonstrated in contrast to an endotheliotropic spread in internal organs. In detail, EHV-1 infected the neuroepithelium in the olfactory area of the mice and used the neurons to spread (Gosztonyi et al., 2009). In horses, a direct infection of neural cells caused by EHV-1 is not known yet.
In contrast to other herpes viruses such as herpes simplex virus (HSV-1) with a primary neurotropism, the neurological form of EHV-1 leads to a vasculitis in the brain and the spinal cord (Edington et al., 1986). A second theory deals with an immune complex-related process. Thrombosis can be caused by forming immune-complexes (Crabb and Studdert, 1995). This can cause immune-mediated vasculitis with secondary degeneration of nerve tissue resulting in neurological signs. A correlation between the antibody titers and protection was not seen. The viraemia therefore occurs in the presence of antibodies. A more pronounced humoral immunity was observed and probably initiated by circulating immune complexes. The vascular lesions and virus propagation

might be influenced through immunological mechanisms like cytokines and immune complexes derived from cytotoxic lymphocytes (Allen, 1998).
A new approach was the idea that only certain strains of EHV-1 caused myeloencephalitis and neurological signs (Studdert et al., 1984). The neuropathogenic potential in some EHV-1 strains might be explained by a point mutation in the viral polymerase gene ORF 30 (Allen, 2006; Allen and Breathnach, 2006; Nugent et al., 2006). Different strains of virus (non-neuropathogenic or neuropathogenic) induce different levels of viraemia (Allen and Breathnach, 2006) and thus exhibit a different virulence. Strains with the mutation in the ORF 30 show not only a higher neuropathogenic potential, but also a higher viraemia and greater viral shedding.

**Neuropathogenic genotype in EHV-1** Studdert suspected as early as 1984 that only certain EHV-1 strains have neuropathogenic potential (Studdert et al., 1984). Later the sequence of two EHV-1 strains, one associated with neurological disease (Ab4) and the other not (V592), were investigated (Nugent et al., 2006). They found a variation rate of 0.1% with 50 insertions or deletions of one or more nucleotides and 110 single nucleotide substitutions. It was further noted that the nucleotide position 2254 in the viral polymerase gene ORF 30 was significantly associated with neurological versus non-neurological outbreaks (see Tab. 3). A change from A to G at nucleotide position 2254 results in an exchange of a single amino acid (asparagine to aspartic acid N752 to D752) in the catalytic centre of the DNA polymerase (ORF 30) and has a significant association ($P \langle 0.01$) with neurological signs (Nugent et al., 2006).

Table 3: Overview of the expected occurrence of the neuropathogenic genotype ($G_{2254}$) in the ORF 30

| Pathotype | Genotype | Nukleotide position 2254 | Amino acid position |
|---|---|---|---|
| abortion | EHV-1 | $A_{2254}$ | N 752 |
| respiratory | EHV-1 | $A_{2254}$ | N 752 |
| neurological | EHV-1 | $G_{2254}$ | D 752 |

A decisive proof of this hypothesis was provided in the work of Goodman et al. (2007). By BAC (bacterial artificial chromosome) cloning and *in vitro* mutagenesis, a mutant and a revertant, with and without the relevant mutation in the polymerase gene, were generated. Both virus mutants N752 and D752 showed in cell culture the same growth and replication properties. In experimentally infected mice, the same loss of body mass and the same levels in viraemia and virus load in infected tissues was obtained. Differences were expressed, however, in a stronger tropism of the strain carrying the neuropathogenic mutation for CD4+ T cells *in vitro* and in a much more pronounced viraemia and clinical signs of infection experiments in the horse. The key observation in experimentally infected horses was that only the neuropathogenic strain could induce ataxia, and this correlated with the stronger viraemia. This confirmed that the D752 strain possessed neuropathogenic potential (Goodman et al., 2007).
In a study by Ma et al. (2010), a difference in cellular tropism or replication behaviour could not be observed and this supports the findings of Goodman et al. (2007). But in other studies differences in the cell culture behaviour of both strains were found. In cell culture the neuropathogenic strains showed a higher replication rate and higher viral titers (Allen and Breathnach, 2006). The

number of infected cells was found to be higher for the neuropathogenic strains in comparison to the non-neuropathogenic strains (Vandekerckhove et al., 2010).
In the USA, EHV-1 myeloencephalopathy (EHM) is an emerging problem. To estimate the prevalence of latent infections with EHV-1 neuropathogenic strains, Allen et al. (2008) investigated SMLNs (submandibular lymph nodes). Interestingly, the frequency of detection ranged from 28% by conventional nested PCR to 54% by sequence-capture nested PCR. The neuropathogenic genotype was found in 18% (13/71) latently infected thoroughbred broodmares, 11 of the 13 were additionally infected with the non-neuropathogenic EHV-1 strain in the same sample (Allen et al., 2008). Therefore, these horses were infected with more than one EHV-1 strain.
Pusterla et al. (2010b) investigated SMLN and trigeminal ganglia, and found both genotypes (neuropathogenic and non-neuropathogenic) in the same horse. Other evidences of dual infection with both genotypes were described by Allen et al. (2008) and Pusterla et al. (2009b). No case of simultaneous infection with $G_{2254}$ and $A_{2254}$ EHV-1 genotypes was found in 419 foetal isolates from Kentucky's thoroughbreds (Smith et al., 2009). Interestingly, in each of the mentioned studies different tissues and different PCR techniques were used. A sequence-capture, nested PCR and sequence-capture, reverse transcription-nested PCR (Allen et al., 2008) and a real-time Taq-Man PCR (Pusterla et al., 2009b) were used in the studies which demonstrated both EHV-1 genotypes. Furthermore, in these two studies the viral DNA was directly extracted from the tissue. In contrast, in the study with no evidence of dual infection, virus growing in cell culture, isolation of viral DNA and then a real-time Taq-Man PCR was performed (Smith et al., 2009).
In experimentally infected horses, the neurologic strains in comparison to the abortigenic EHV-1 strains express a more pronounced cell-associated viraemia. Without exception, the foals infected with the neuropathogenic genotype exposed an earlier and higher increase of virus-infected PBMC in the blood, which plays a crucial role for viral spread and the cell tropism. In neuropathogenic strains, the nasal shedding of infectious viruses increases and thereby the risk of transmission is increased (Allen and Timoney, 2007).
Horses naturally infected with the neuropathogenic EHV-1 shed higher loads of replicating EHV-1 in nasopharyngeal secretions (NPS) and were a possible risk to other horses. In an natural occurring outbreak of EHV-1 at a racetrack in California higher viral loads and transcriptional activity for the first 60 hours of hospitalization in the index case were recorded. In this case the antiviral drug valacyclovir seemed to reduce viral loads and shedding (Pusterla et al., 2009b). Interestingly, in an *in vitro* experiment it was shown that D 752 ($G_{2254}$) was much more resistant to the antiviral drug aphidicolin and only the N 752 ($A_{2254}$) strain has a drug sensitive phenotype (Ma et al., 2010). Ma et al. (2010) concluded that the amino acid residue 752 in the EHV-1 ORF 30 was not required for virus growth *in vitro*.
Van de Walle et al. (2009) showed that the non-neuropathogenic strain $A_{2254}$ could be transformed into a neuropathogenic strain by a mutation to $G_{2254}$. The $G_{2254}$ strains showed a stronger neuropathogenicity, but the virus shedding was comparable between both variants (Van de Walle et al., 2009).

**Nucleotide change at position 2258** At nucleotide position 2258 a change from A to $C_{2258}$ was reported (Nugent et al., 2006). Smith et al. (2009) confirmed this additional exchange in the viral polymerase that leads to the replacement of the amino acid tyrosine (Y753) with serine

(S753). Furthermore, the change is located in the palm domain of the polymerase protein and is flanking the aspartic acid 752 residue with the neuropathogenic potential. This SNP (single nucleotide polymorphism) at nucleotide position 2258 may have an influence on the activity of the polymerase gene and the neuropathogenic potential (Smith et al., 2009), and thereby affect the outcome or severity of the neurological disease.
RacH, used as an attenuated EHV-1 vaccine strain (Prevaccinol, Resequin NN plus, Intervet), contains both substitutions ($G_{2254}$ and $C_{2258}$). Despite several abortions, no reports exist on neurological signs linked with the use of the RacH vaccine (Nugent et al., 2006).

**EHV-1 in stallions** In an intranasally infected stallion, the infectious virus was detected in the semen and the quality of normal sperm was reduced. EHV-1 was able to replicate in the endothelial cells of the testis and a necrotizing vasculitis and thrombosis resulted. Furthermore, in some stallions the balance of normal sperm decreased (Tearle et al., 1996). In France, a study to assess the risk of EHV-1 excretion in semen was made. EHV-4 was found in none of the samples, however, EHV-1 was found in 13% (51 of 390) of the samples (Hebia-Fellah et al., 2009).

**Pathogenesis of EHV-9** EHV-9 was first isolated from a Thomson‘s gazelle during an outbreak of acute encephalitis (Fukushi et al., 1997). The neuropathogenicity in gazelle was confirmed in a study where seven gazelle‘s died after EHV-9 infection and a nonsuppurative encephalitis was found. Clinical signs and pathological findings were distinct from those of EHV-1 in horses (Yanai et al., 1998).
Schrenzel et al. (2008) confirmed the neuropathogenicity of EHV-9 in nonequids. The pathogenesis of EHV-9 was studied using animal models. EHV-9 was inoculated intranasally. The animals (goats and marmosets) developed neurological disorders. The following histological examination revealed damage in nervous tissues like encephalitis, neuronal necrosis and inclusion bodies (Taniguchi et al., 2000b; Kodama et al., 2007), indicating a strong neurotropism of EHV-9. The pathogenicity of EHV-9 in horses was confirmed by intranasal inoculation of EHV-9 in two horses, causing pneumonia and nonsuppurative encephalitis (Taniguchi et al., 2000a).

**Cattle** Only minor differences in the restriction enzyme pattern between bovine isolates and different EHV-1 strains were found (Crandell et al., 1988; Chowdhury et al., 1988). A barrier breaking from horses to infect ruminants as a result of mutation in the EHV-1 genome was assumed (Pagamjav et al., 2007). But the paper revealed no specific mutations associated with an interspecies transmission, though they assumed that EHV-1 has the potential to infect cattle.

### 2.2.10 Detection methods

Infection with equine herpes viruses 1 and 4 can be determined directly through virus propagation in cell culture and different PCR techniques, or indirectly through the detection of virus neutralizing antibodies, immunfluorescence assay (IFA) and ELISA (enzyme-linked immunosorbent assay). For infections of the respiratory tract and neurological diseases, investigations of nasal swabs and blood samples from the affected horses are recommended. Samples should be taken in the febrile phase after the virus shedding peak has passed (Wilson, 1997). In the case of abortion, tissues from the aborted foetus (lung, liver, spleen), placenta, amniotic fluid and, additionally, blood

samples from the mare are recommended to be taken for investigation (Schröer et al., 2000). The examination of the placenta was mentioned as an important diagnostic tool (Gerst et al., 2003). In aborted foetuses the highest virus quantity is in the lung (Hornyák et al., 2006).

**Direct virus detection** The virus propagation in cell culture is characterized by a good sensitivity and specificity, but the long duration until the rating of the cytopathic effects is a disadvantage. RK 13 (rabbit kidney) cells are selective for EHV-1. EHV-4 does not grow on RK 13.
In the early 1980s, the restriction enzyme analysis allowed differentiation of foetal and respiratory viruses, and distinguishing and defining them as two different virus species EHV-1 and -4 (Studdert et al., 1981; Studdert, 1983). The restriction enzyme analysis with different restriction enzymes can display subtle differences in the restriction pattern of the viruses.
Meanwhile the polymerase chain reaction (PCR) is the first choice in the diagnostic, because of its high sensitivity and specificity. The EHV-1/-4 PCR techniques are mostly more sensitive than the virus isolation (Borchers and Slater, 1993). The conventional PCR protocols can be divided into single step or nested PCRs. Both are non-quantitative PCRs. The real-time-quantitative PCR/qPCR was found to be much more sensitive than the conventional PCR, and the quantitation of virus has been shown (Elia et al., 2006; Hussey et al., 2006). A qPCR of nasal swab samples was recommended as diagnostic tool with a high specificity and sensitivity (Perkins et al., 2008).
A further progression is qPCR that used the minor groove-binding technology, whereby a fluorogenic probe is hydrolysed by Taq polymerase while amplifying the target DNA and the fluorescence is then measured - it was developed to detect EHV-1 glycoprotein B. Other pathogens which are likely to cause abortions were non-reactive in this technique. The authors mentioned that this method provides results in two hours and is highly sensitive (Diallo et al., 2006).

**Indirect virus detection** The neutralization test (NT) is used as a diagnostic tool at the Institute of Virology, Berlin, Germany. The titer is read at a plaque reduction of 50%. Noticeable titer limits are over 1:20. A titer increase in paired sera at intervals of three to four weeks by a factor of four or more was considered as significant (Schröer et al., 2000).
The immunofluorescence assay (IFA) is a common diagnostic agent. IFA titer over 1:1280 are increased. A titer increase in paired sera at intervals of three to four weeks by a factor of four or more was considered as significant (Schröer et al., 2000).
Serological tests ensure no clear differentiation between EHV-1 und EHV-4 caused by the serological cross-reactivity (Allen and Bryans, 1986). Typically older horses have EHV-1/-4 cross-reactive antibodies (Crabb and Studdert, 1995). A type-specific ELISA (enzyme-linked immunosorbent assay) based on glycoproteins (gC and gD), which are both type-specific viral surface antigens, can be used for differentiation of EHV-1 and EHV-4 specific antibodies in the serum (Crabb and Studdert, 1993).

# 3 Objective of the thesis

Some points are still to be clarified, as is evident from the literature review:

1. A single nucleotide polymorphism in the viral polymerase gene (ORF 30) of EHV-1 was described being associated with the outcome of neurological signs (Nugent et al., 2006). My interest was to investigate whether only non-neuropathogenic EHV-1 strains are involved in abortion cases in Germany. To identify the nucleotide at nt position 2254, an ORF 30 nested PCR (Allen, 2006) was performed, followed by a restriction enzyme analysis with *Sal* I. These data were validated by sequencing the nested PCR amplicons.

2. The additional mutation at nt 2258 resulting in an exchange A to C was first described by Nugent et al. in 2006. The impact of the occurrence of this mutation (C $_{2258}$) on the neuropathogenic EHV-1 strains (G $_{2254}$) is still unclear. Thus, I wished to clarify if this additional mutation also exists in Germany.

3. Moreover, the question should be answered whether other mutations in the ORF 30 have been noted.

4. Wild equid and cattle strains were analysed for the mutation in the ORF 30 gene to see if the mutation in the ORF 30 also occurred in cattle and archived wild equid strains.

5. In two independent abortion outbreaks blood samples from the horses were taken at intervals of several weeks for a serological survey.
   In addition to the serological survey, viral DNA was isolated from aborted tissue and PBMC. The viral DNA was investigated by ORF 30 nested PCR and restriction enzyme analysis with *Sal* I. The subject of the examination was to look for a correlation between the occurrence of the nucleotide polymorphism in the ORF 30 as well as antibody titer, vaccination status or age and the emergence of abortions.

# 4 Material and methods

## 4.1 Proof of material

### 4.1.1 Chemicals and solutions

**AppliChem**, Weiterstadt, Germany
Ethanol absolut

**Biochrom AG**, Berlin, Germany
Biocoll seperatin solution (1.077 g/ml)
foetal calf serum
MEM Earle‘s

**Bioline**, Luckenwalde, Germany
dNTP Mix, 2.5 mM each
Hyper Ladder I
Hyper Ladder IV

**Dianova**, Hamburg, Germany
Anti-Horse-IgG (H+L)-fluoresceinisothiocyanat (Anti-Horse-FITC)

**Invitek**, Berlin, Germany
RTP DNA/RNA Virus Mini Kit for simultaneous extraction of nucleic acids from DNA and RNA viruses

**Life Technologies**, Gaithersburg, USA
Eagle‘s minimum essential medium, Dulbecco‘s modification (EDM)

**Merck**, Darmstadt, Germany
Ethidiumbromid (1%)

**Metabion**. Martinsried, Germany
Primer ORF30-F-#8
Primer ORF30-R-#2
Primer ORF30-F-#7
Primer ORF30-R-#3

**New England Biolabs**, Frankfurt am Main, Germany
Restriction enzyme *Sal* I

**Roche Diagnostics**, Mannheim, Germany
Proteinase K, recombinant PCR Grade, 5 mg

**Roth**, Karlsruhe, Germany
Agarose for DNA/RNA gel electrophoresis
Water, molecular biology tested

**Sigma Aldrich**, Hamburg, Germany
Mineral oil, molecular biology tested
Penicillin G
Streptomycin sulfate

**Quiagen**, Hilden, Germany
PCR buffer 10x, containing 15 mM $MgCl_2$
Q-buffer
Taq DNA polymerase, 5 units/$\mu$l

### 4.1.2 Materials

**Eppendorf**, Hamburg, Germany
MultiGuard Barrier Tips, Pipette tips 0.5-10 $\mu$l
MultiGuard Barrier Tips, Pipette tips 10-200 $\mu$l
MultiGuard Barrier Tips, Pipette tips 100-1000 $\mu$l

**Roth**, Karlsruhe, Germany
Multi-tubes, 1.7 ml, DNA-RNA-free
Ultra PCR tubes, 0.65 ml

**Sorenson BioScience**, Salt lake city, USA
Eppendorf, Pipette tips 0.5-10 $\mu$l
Eppendorf, Pipette tips 10-100 $\mu$l
Eppendorf, Pipette tips 500 $\mu$l

**TPP/Techno plastic products**, Trasadingen, Switzerland
24-well-plates
Cell culture flasks (75 $cm^2$)
Centrifuge tubes (50 ml)
Petri dishes of various sizes

### 4.1.3 Equipment

**4peaks**, http://www.macupdate.com/app/mac/14120/4peaks
Visualize and edit DNA sequence files, version 1.7.2

**Bender & Hobein AG**, Zurich, Switzerland
Vortex Genie 2

**Biometra**, Göttingen, Germany
UNO-Thermoblock, thermal cycler

**Bio-Rad**, Munich, Germany
Migration chamber, model 1000/500, power supply

**Biotechnologie**, Erlangen, Germany
Vision Capt software
Quantum Fluoreszenz Imaging System

**Bleymehl clean room technologie**, Inden-Pier, Germany
Laminar flow bench, model ASW-UP-1270, year of construction 1994

**Eppendorf**, Hamburg, Germany
Centrifuge 5403, Rotor 16F24-11
Eppendorf, Pipette Research 0.5-10 $\mu$l
Eppendorf, Pipette Research 10-100 $\mu$l
Eppendorf, Pipette Research 500 $\mu$l

**GFL**, Grossburgwedel, Germany
Incubation water bath

**Hellma**, Müllheim, Germany
Absorption cells, Quarzglas, type 104.002-QS, 10.00 mm

**Heraeus**, Osterode, Germany
Centifuge Biofuge A, type 1217, rotor 1378
Centrifuge Minifuge II, type 4123, rotor 1664
Humified incubator

**HLC**, Bovenden, Germany
Thermo mixer

**H. Saur Laborbedarf**, Reutingen, Germany
Draw-off pump Vakumat 130

**Integra bioscience**, Fernwald, Germany
Pipetboy plus

**Mitsubishi electrics**, Ratingen, Germany
Printer Mitsubishi P93D

**Olympus**, Hamburg, Germany

Microscope, Olympus CK 2

**Pegasus**, Rockville, USA
Hybaid Omni Gene thermal cycler

**Shimadzu**, Duisburg, Germany
UV-VIS spectrophotometer, UV-1202

**Zeiss**, Jena, Germany
Fluorescence microscope, model Axiovert 100

## 4.2 EHV-1 and -4 reference strains

Six EHV-1 reference strains and two EHV-4 reference strains were used in this study, which were provided out of the viral stock of the Institute of Virology, Berlin, Germany.

### 4.2.1 EHV-1 reference strains

The EHV-1 reference strains were kindly provided by various scientists to the Institute of Virology, FU Berlin (see Tab. 4).
EHV-1 strain **Ab4** was isolated from a horse with paresis (Edington et al., 1985). This strain was already investigated by Nugent et al. in 2006, when they described the exchanges from A to $G_{2254}$ (described as neurological) and from A to $C_{2285}$ in the ORF 30.
**Austria IV (AIV)** originated from an EHV-1 outbreak at a Lipizzaner stud farm in Austria. This abortigenic EHV-1 strain was isolated from the lung of an aborted foal in 1983 (Chowdhury et al., 1986). At the Lipizzaner stud farm 30 cases of abortions and perinatal death occurred, followed by the death of 10 mares showing neurological disorders.
**Mar 87** was isolated in 1987 from a horse with rhinopneumonitis and determined as EHV-1 (Thein and Huselstein, 2000).
EHV-1 strain **Army 183** was isolated 1941 in Virginia (USA) from the respiratory tract of a young horse (Jones et al., 1948), causing abortion. In an infection experiment, two horses were infected with the strain Army 183 with both animals developing severe neurological signs (Thein, 1996).
Doll et al. (1954) isolated EHV-1 strain **Kentucky D** which was defined as an abortigenic EHV-1 strain (Doll et al., 1954).
The abortion strain **RacH** has been determined as EHV-1 (Mayr et al., 1965). Nugent et al. (2006) have already investigated the ORF 30 region of this strain and found $G_{2254}$ described as the neuropathogenic genotype. A further substitution at nucleotide position 2258 of adenine for cytosine was found in the open reading frame 30.

### 4.2.2 EHV-4 reference strains

The EHV-4 strain **KT-4** was discovered in the case of a horse with respiratory symptoms in 2005 (Borchers et al., 2005a). Respiratory EHV-4 strain **T252** was discovered in 1975 (Thein and Härtel, 1976) in Germany (see Tab. 5). EHV-4 strains were not detectable in the ORF 30 nested

Table 4: EHV-1 reference strains

| Identity | Source | Genotype | Pathotype |
|---|---|---|---|
| Ab4 | Edington et al., 1985 | EHV-1 | neurological disorders |
| Austria IV / AIV | Chowdhury et al., 1986 | EHV-1 | abortion |
| Mar 87 | Thein P., 1987 | EHV-1 | rhinopneumonitis |
| Army 183 | Jones et al., 1948 | EHV-1 | respiratory |
| Kentucky D | Doll et al., 1954 | EHV-1 | abortion |
| RacH | Mayr et al., 1965 | EHV-1 | abortion |

PCR (see section 4.11.1). Consequently, KT-4 was used as negative control in the ORF 30 nested PCR.

Table 5: EHV-4 reference strains

| Identity | Source | Genotype | Pathotype |
|---|---|---|---|
| KT-4 | Borchers et al., 2005a | EHV-4 | respiratory |
| T252 | Thein and Härtel, 1976 | EHV-4 | respiratory |

## 4.3 Wild animal and cattle EHV strains

Six wild animal EHV strains and two EHV-1 strains isolated from cattle were investigated. They were provided out of the viral stock of the Institute of Virology, Berlin, Germany.

### 4.3.1 Wild animal EHV strains

All samples were derived from the viral stock of the Institute of Virology, Berlin, and were kindly supplied to the Institute of Virology (University of Berlin) as noted in Tab. 6.
Three strains were isolated from animals with neurological signs. The **Ro-1** strain was isolated from a captive blackbuck that showed malignant catarrhalic fever and paresis and classified as related to EHV-1 (Borchers et al., 2006a).
A gazelle showed neurological signs and the isolate (**94-137**) was classified as related to EHV-1 (Kennedy et al., 1996; Borchers et al., 2008; Ghanem et al., 2008).
In a Thomson‘s gazelle with acute encephalitis (isolate **49 800**), the causative virus was classified as EHV-9 (Fukushi et al., 1997).
Three isolates from wild equids were from aborted foetuses. In the Lincoln Park Zoo, a Grevy zebra aborted and the isolate **T 965** was determined as being related to EHV-1 (Borchers et al., 2005b).
Strain **T 529** originated from an aborted onager foetus and was classified in 1985 as related to EHV-1 (Montali et al., 1985).
Strain **T 616** was an isolate from an aborted Grevy zebra foetus in 1986 and documented as being related to EHV-1 (Wolff et al., 1986).

Three wild animal isolates (94-137, T 616 and 49 800) were investigated and classified to have the neuropathogenic genotype $G_{2254}$ in the ORF 30 (Yamada et al., 2008).

Table 6: EHV isolates from different wild animals.

| Identity | Animal | Source | Genotype | Pathotype |
|---|---|---|---|---|
| Ro-1 | blackbuck | Dr. Rockborn, 1988 Kolmarden, Sweden | related to EHV-1 | malignant catarrhal fever |
| 94-137 | gazelle | Kennedy et al., 1996, USA | related to EHV-1 | neurological disorders |
| 49 800 | gazelle | Fukushi et al., 1997, Japan | EHV-9 | neurological disorders |
| T 529 | onager | Montali et al., 1985, USA | related to EHV-1 | abortion |
| T 616 | Grevy zebra | Wolff et al., 1986, USA | related to EHV-1 | abortion |
| T 965 | Grevy zebra | USA, Chicago, 1996 | related to EHV-1 | abortion |

### 4.3.2 Cattle EHV-1 strains

Both cattle strains caused abortion (see Tab. 7). They were classified as EHV-1 in two works by Chowdhury: **136 A** (Chowdhury et al., 1986) and **136 B** (Chowdhury et al., 1988).

Table 7: EHV isolates from cattle.

| Identity | Animal | Source | Genotype | Pathotype |
|---|---|---|---|---|
| 136 A | cattle | Dr. McFerran, Northern Ireland, 1986 | EHV-1 | abortion |
| 136 B | cattle | Dr. McFerran, Northern Ireland, 1988 | EHV-1 | abortion |

## 4.4 Sample origin

The samples listed below were made available for this work by PD Dr. Kerstin Borchers (Institute of Virology, University of Berlin). The abortion cases were divided in single abortion cases and abortion outbreaks. In this context, an abortion outbreak is defined as more than one abort at a stud farm in the same breeding season.

The provided samples were either virus isolates or original tissues. They were already classified as EHV-1 in the diagnostic department of the Institute of Virology (University of Berlin) by cell culture and EHV-1/EHV-4 gB nested PCR (Borchers and Slater, 1993).

Virus isolates were obtained by culture of diagnostic material (foetal lung, foetal liver, foetal spleen, placenta) on RK 13 cells. Only EHV-1 is able to grow in RK 13 cells. The virus isolates were stored at -70°C.

### 4.4.1 Single abortion cases

From the period 1987 to 2009, 38 single abortion cases were provided for this study. They were already classified as EHV-1 (see Tab. 8). All of them were obtained by culture of diagnostic material in cell culture. The detailed origin of the single abortion cases listed according to the year is printed in the annex (see section 10.2.1).

Table 8: EHV-1 isolates from single abortion cases listed by source.

| Source | Years | Case number | Genotype | Sample character |
|---|---|---|---|---|
| Neubauer, LMU | 1987 - 2006 | 17 | EHV-1 | virus isolate |
| Kilian, LUA-RLP | 2009 | 2 | EHV-1 | virus isolate |
| Reckling, LAV | 2009 | 3 | EHV-1 | virus isolate |
| FU Berlin | 2004 -2009 | 16 | EHV-1 | virus isolate |
| Total number | | 38 | | |
| Key: | | | | |
| Neubauer, LMU: | Antonie Neubauer, Institute of Medical Microbiology Ludwig-Maximilians-University, Germany | | | |
| Kilian, LUA-RLP: | Dr. Alexandra Kilian (Landesuntersuchungsamt Koblenz, Rheinland-Pfalz) | | | |
| Reckling, LAV: | Dr. Karl-Friedrich Reckling (Landesamt für Verbraucherschutz, Sachsen-Anhalt) | | | |
| FU Berlin: | Diagnostic department of the Institute of Virology (Free University Berlin) | | | |

**P5276** The EHV-1 abortion isolate P5276 was provided by Peter Hübert, Veterinäruntersuchungsamt, Neumünster, Germany to PD Dr. Kerstin Borchers. As background information is missing, the isolate was examined but not considered in the evaluation.

### 4.4.2 Abortion outbreaks in Germany

In this study four abortion outbreaks with 29 abortion cases were investigated in the period 2007 to 2009 (see Tab. 9). They were classified as EHV-1 in the diagnostic department of the Institute of Virology (Free University Berlin) by cell culture and EHV-1/EHV-4 gB nested PCR (Borchers and Slater, 1993) and provided for this work. The abortion outbreaks were observed in different provinces of Germany, Mecklenburg-Vorpommern/stud farm 1, Brandenburg/stud farm 2, Baden-Württemberg/stud farm 3 and Saxony-Anhalt/stud farm 4. The detailed origin of the abortion outbreaks is described in the following sections 4.4.3, 4.4.4, 4.4.5 and 4.4.6.

### 4.4.3 Abortion outbreak stud farm 1

37 mares aborted in 2007 at the stud farm in Mecklenburg-Vorpommern/Germany. The pathologists at the Institute of Animal Pathology, Free University Berlin reported typical changes in foetal lungs and livers of 18 of the aborted foetuses. The foetal lungs showed multifocal, acute parenchym necrosis, scattered intranuclear, eosinophile inclusion bodies and also atelectasis. The foetal livers showed multifocal and acute necrosis of the parenchym. Thus, the pathologists came

Table 9: Abortion outbreaks on four different stud farms listed by source.

| Source | Years | Case number | Genotype | Sample character |
|---|---|---|---|---|
| Stud farm 1 | 2007 | 18 | EHV-1 | virus isolate |
| Stud farm 2 | 2008 | 6 | EHV-1 | virus isolate |
| Stud farm 3 | 2009 | 2 | EHV-1 | original samples:<br>amniotic fluid<br>nasal swabs<br>lung fluids |
| Stud farm 4 | 2009 | 2 | EHV-1 | virus isolate |
| | | 1 | EHV-1 | placenta |
| Total number | | 29 | | |

to the conclusion a Herpesvirus 1 infection could be the reason for the abortions. The pathological researchers sent tissue samples (foetal lung) from 18 abortion cases for further investigation to the diagnostic laboratory of the Institute of Virology (Free University Berlin). The vaccination status of the mares was unknown.
The virus growing in cell culture (rabbit kidney/RK 13), as well as the EHV-1 gB nested PCR, was EHV-1 positive. The virus isolates of 18 abortion cases were provided for this study (details in annex 10.2.1).

### 4.4.4 Abortion outbreak stud farm 2

In 2008, six mares aborted at the stud farm located in Brandenburg/Germany. The pathologists found effusions in the body cavity and foetal atelektasis and multifocal, petechial haemorrhage in the lungs. Samples from the lungs were sent to the diagnostic laboratory of the Institute of Virology (Free University Berlin). Before the outbreak all mares had been vaccinated with Resequin and two of them were again vaccinated with Duvaxyn EHV 1,4 four month later.
The virus growing in cell culture, as well as the EHV-1 gB nested PCR, was for all six lung samples EHV-1 positive. The six virus isolates from the cell cultures were provided for this study (details in annex 10.2.1).

### 4.4.5 Abortion outbreak stud farm 3

In 2009, two mares (Mare I and Mare II) aborted at a stud farm in Baden-Württemberg/Germany with 150 horses and 10 breeding mares. Seventy-five horses developed fever, six horses showed initial neurological signs, with two of these being pregnant mares. The two mares which aborted afterwards developed neurological signs.
Mare I had been vaccinated with Duvaxyn EHV 1,4 in the 3./4. gestation month, but the second vaccination was not done. It aborted, afterwards the mare was vaccinated clinically healthy with Duvaxyn EHV 1,4. After 12 hours the mare developed neurological symptoms without fever.
Mare II was vaccinated with Duvaxyn EHV 1,4 in gestation month 3./4. and 7./8.. It aborted and developed neurological signs one day after Mare I, but also without fever.
From these two abortions overall five samples (one amniotic fluid, two nasal swabs, two lung fluid

samples) were sent to the Institute of Virology. For all of them the isolation of the virus in cell culture failed. They were directly investigated by EHV-1/EHV-4 gB nested PCR and typed EHV-1. The original samples (two nasal swabs, two lung fluid samples, one amniotic fluid) from both abortions were provided for this study (details in annex 10.2.1). The results of all five samples will be discussed, in evaluation however, only the number of abortion cases was counted.
Additionally, the semen of a clinically healthy stallion from the same stud farm was typed EHV-1. The stallion was not vaccinated against herpes. The original semen sample was provided for this study.

**Blood samples from 11 horses at stud farm 3:** In addition to the samples described above, the stud veterinarian took blood samples from 11 horses in three and a half week intervals and sent them to the diagnostic laboratory of the Institute of Virology (Free University Berlin). Blood samples were serologically investigated with the neutralization test and the immunofluorescence assay for EHV-1 and EHV-4. The clinical signs of the sampled horses are reported in the annex (see section 10.4).

### 4.4.6 Abortion outbreak stud farm 4

In 2009, a stud farm in Saxony-Anhalt/Germany cared for 16 pregnant mares. None of them was vaccinated against EHV-1/-4. One of them resorbed the foetus, seven suffered an abortion and nine delivered healthy foals.
Abortion samples were only obtained in three cases. The pathology of the Landesamt für Verbraucherschutz, Saxony-Anhalt sent virus isolates from two aborted foetuses. The placenta of one aborted foetus was recovered at the stud farm while I took the first blood samples. They were investigated by EHV-1/EHV-4 gB nested PCR in the diagnostic department of the Institute of Virology and typed EHV-1.
The two virus isolates and the placenta were provided for this study (details in annex 10.2.1).

**Blood samples from mares at stud farm 4:** Blood samples were taken from 13 mares, six of them aborted and seven had a regular partus, shortly after the abortions occurred and five and a half weeks after the first sampling. The first blood sampling was performed by myself, the second was made by the stud veterinarian.
EHV-1 and EHV-4 neutralization tests, PBMC isolation out of the citrated blood, viral DNA preparation and ORF 30 nested PCR followed by restriction analysis with *Sal* I were accomplished.
The stud farm precincts was divided into two parts, barn 1 and barn 2. Barn 1 accommodated, without separation, the riding stable, breeding and foaling areas. The mares were living in boxes with free run. Commonly, artificial insemination was used - exceptional however, in 2009 seven mares were fertilized by the stallion. Every week horses left the stud farm to participate in the local tournaments. Two days before abortion Mare E suffered a mild convulsion colic which was treated with metamizol. It was symptom-free one hour after medication. The data from the sampled horses in barn 1 are listed in Tab. 10.

Barn 2 was located approximately 10 minutes walk from barn 1 and contained breeding and foaling areas. The mares were kept in boxes with free run area.
All mares, except Mare C, were vaccinated against influenza and tetanus. Mare J was vaccinated

Table 10: Report of the clinical signs of the sampled horses in barn 1 from stud farm 4

| Mare | Age | Number of pregnancies | Kind of fertilization | Abort | Clinical signs |
|---|---|---|---|---|---|
| Mare A | 4 years | 1 | inseminated artificial | 05.04.09 | swollen limbs in febr./mars |
| Mare B | 4 years | 1 | inseminated artificial | 03.04.09 | swollen limbs in febr./mars |
| Mare C | unknown | unknown | natural mating | 20.02.09 | foreign keeping mare |
| Mare D | 7 years | 2 | natural mating | 23.02.09 | no clinical signs |
| Mare E | 5 years | 2 | inseminated artificial | 01.03.09 | colic |
| Mare F | 12 years | 4 | natural mating | 13.03.09 | no clinical signs |

two years previously, once against Herpesvirus infection (unknown vaccine). In Tab. 11 the background from the horses sampled in barn 2 is listed.

Table 11: Report of the sampled horses in barn 2 from stud farm 4

| Mare | Age | Number of pregnancies | Kind of fertilization | Birth date | Foal |
|---|---|---|---|---|---|
| Mare G | 8 years | 4 | artificial insemination | 02.05.09 | healthy colt |
| Mare H | 7 years | 3 | natural mating | 11.06.09 | healthy colt |
| Mare I | 5 years | 3 | inseminated artificial | 11.05.09 | healthy filly |
| Mare J | 13 years | 1 | natural mating | 14.05.09 | healthy filly |
| Mare K | 22 years | 8 | natural mating | 28.06.09 | healthy colt |
| Mare L | 14 years | 10 | artificial insemination | 01.06.09 | healthy colt |
| Mare M | 20 years | 3 | natural mating | 17.06.09 | healthy colt |

#### 4.4.7 Neurological cases

The diagnostic laboratory of the Institute of Virology (Free University Berlin) obtained three different tissue samples (spinal cord, brain and an organ mixture) from a horse with neurological signs in 2008, kindly provided from Dr. Karl-Friedrich Reckling (Landesamt für Verbraucherschutz Sachsen-Anhalt).

In 2002, Stierstorfer et al. isolated from a horse with neurological signs an EHV-1 strain. This isolate was provided by Antonie Neubauer, Institute of Medical Microbiology, Infectious and Epidemic Diseases, Ludwig-Maximilians-Universität München to the Institute of Virology (University of Berlin).

Virus cultivation in ED cells of the CNS samples and virus isolation were performed parallel to the EHV-1/-4 gB nested PCR (Borchers and Slater, 1993) by the employees of the diagnostic laboratory of the Institute of Virology, University of Berlin (see Tab. 12).

The isolates 3318/2, 3318/3 and 834 were typed EHV-1 and had to be further investigated by ORF 30 nested PCR and restriction fragment analysis with *Sal* I.

Table 12: Results of virus cultivation and EHV-1/-4 gB nested PCR of the neurological cases.

| Designation | Year | Source | Sample character | Virus cultivation | EHV-1/EHV-4 gB nested PCR |
|---|---|---|---|---|---|
| 3318/1 | 2008 | Landesamt für | spinal cord | not possible | negative |
| 3318/2 | 2008 | Verbraucherschutz | brain | not possible | EHV-1 |
| 3318/3 | 2008 | Sachsen-Anhalt | organ mix | not possible | EHV-1 |
| 834 | 2002 | Stiersdorfer et al. (2002) | virus isolate | positive | EHV-1 |

## 4.5 Cell cultures

**Supplemented Eagle's minimum essential medium, Dulbecco's modification (EDM)**

| EDM dry chemical for 10 l | |
|---|---|
| $NaHCO_3$ | 37 g |
| Penicillin G | 0.2 g |
| Streptomycin sulfate | 0.2 g |
| Aqua bidest | ad 10 l |
| adjust pH 7.2 | |
| foetal calf serum 5% | |

**Trypsin solution**

| | |
|---|---|
| Trypsin | 2.5 g |
| EDTA-Dihydrat | 0.744 g (2.0 mM) |
| PBS | ad 1000 ml |

**PBS (phosphate buffered saline)**

| | |
|---|---|
| NaCl | 8.0 g (137.0 mM) |
| KCl | 0.2 g (2.7 mM) |
| $Na_2HPO_4$x2 $H_2O$ | 1.42 g (8.0 mM) |
| $KH_2PO_4$ | 0.24 g (1.8 mM) |
| Aqua bidest | ad 1000 ml |
| adjust pH 7.4 | |

For serological tests (neutralization tests/NT and immunofluorescence assays/IFA) equine dermal cells (ED) were used. ED cell lines were kept permanently in the Institute of Virology, Berlin, Germany. The cells were grown in supplemented EDM and incubated in humid incubators at 37°C and 5% $CO_2$.

For this study virus infected RK 13 cell culture supernatants were used. They had been already classified as EHV-1 positive by EHV-1 gB nested PCR (Borchers and Slater, 1993) by the employees of the Institute of Virology.

### 4.5.1 Cell passaging

A confluent ED cell monolayer in the plastic cell culture dish (diameter 10 cm) was required. The medium was sucked off. 1.5 ml trypsin solution was added for two minutes and mixed gently

during the exposure. Afterwards the trypsin was dismissed. The cells were incubated at room temperature for 10 minutes. Under microscopical observation, single cells had to be noticed and the cells had to be resuspended in supplemented EDM and passaged in the ratio 1:2 into new petri dishes. Then 10 ml supplemented EDM was pipetted per cell culture dish. The cell cultures were incubated (Heraeus, incubator) in a 5% $CO_2$ air atmosphere at 37°C for 24 to 48 hours.

## 4.6 Serological tests

The neutralization test (NT) and the immunofluorescence assay (IFA) were used to determine antibody titers. For the serological tests, blood plasma which was obtained from citrated blood samples or sera from coagulated blood samples were used.

The used virus strains were provided out of the viral stock of the Institute of Virology, Berlin, Germany:
**Virus strains**
AIV, EHV-1, ED, 2.8 x $10^5$ PFU/ml
KT-4, EHV-4, ED, 4 x $10^6$ PFU/ml

For neutralization tests (NT) and immunofluorescence assays (IFA) equine dermal cells (ED) were used. These cell lines were kept permanently in the Institute of Virology, Berlin, Germany.

### 4.6.1 Neutralization test (NT)

**Carboxy-Methylcellulose-Overlaymedium/CMC**

| | |
|---|---|
| Carboxymethylcellulose-sodium salt | 8.0 g |
| MEM Earle‘s | 500 ml |
| FCS | 10 ml FCS |

**Supplemented EDM**

**4% formalin in PBS**

**Giemsa staining solution**

The neutralization test (NT) was performed to investigate the virus neutralizing activity of horse plasma or sera. Ideally, the NT should be performed on blood plasma or sera pairs taken at four-week intervals to determine a noticeable increase or decrease by a factor of 4 or more. Stud farm 3 sent blood samples (sera) at intervals of three and a half weeks, stud farm 4 sent citrated blood samples (plasma) at intervals of five and a half weeks for investigation.
First, sera were heated for 30 minutes at 56°C in a water bath to inactivate the complement.
In each first well 160 $\mu$l and in every other well of the 24-well plate 100 $\mu$l supplemented EDM were pipetted. To obtain a logarithmic dilution series to the base 2, 40 $\mu$l of the sample was added to the first well, mixed and then 100 $\mu$l were transferred to the next well and so on to the dilution step 1:80. Each plate contained a cell control without virus, a virus control and a negative (pool of negative tested sera) and positive (pool of positive tested sera with high titer)

control serum used in the diagnostic laboratory of the Institute of Virology, FU Berlin, Germany. Subsequently, in each well, except the cell control well, 100 $\mu$l of virus suspension containing $10^3$ PFU/ml were pipetted and the plates were incubated for one hour at 37°C to allow existing neutralizing antibodies to bind to the virus and neutralize it. ED (equine dermal) cells (see 4.5) were needed for the next step. The ED cell suspension was produced by removal of the medium from the cell culture dish and treatment of the cell monolayer with trypsin. The detached cells were resuspended in supplemented EDM. Afterwards, 200 $\mu$l ED cell suspension (5 x $10^4$ cells/ml in supplemented EDM) was added to each well.
After two hours of incubation under the same conditions, 400 $\mu$l of overlay medium was added per well to avoid an extracellular virus spread and the plates were further incubated for two days.
After the incubation period, the 24-well plates were fixed and virus inactivated with 4% formalin, followed by staining with Giemsa solution. The evaluation was performed by counting the plaques under the light microscope. If no virus-neutralizing antibodies were present, the virus should multiply in the cells and cause typical plaques in the cell monolayer. The neutralization titer was deduced at the dilution causing a 50% plaque reduction in comparison with the negative serum control.

### 4.6.2 Immunofluorescence assay (IFA)

**Trypsin solution**

| | |
|---|---|
| Trypsin | 2.5 g |
| EDTA-Dihydrat | 0.744 g (2,0 mM) |
| PBS | ad 1000 ml |

**3% formalin**

**1% triton in PBS**

**Anti-Horse-FITC**

**Aqua dest.**

**1x PBS with 1% FCS**

The immunofluorescence assay (IFA) detected the presence of antibodies. We needed $10^6$ ED (equine dermal) cells (see 4.5). The medium was removed from the cell culture dish and then the cell monolayer treated with trypsin. The trypsin was immediately removed. A volume of 1 ml of trypsin remained on the cell culture dish for about five to 10 minutes to detach the cell layer. The detached cells were removed and then infected with a $10^3$ PFU/ml EDM-virus suspension (EHV-1 reference strain AIV or EHV-4 reference strain KT-4). 100 $\mu$l of this virus-cell suspension was added in each well of a 96-well microtiter plate. After two days of incubation at 37°C (Heraeus, incubator, 5% $CO_2$ air atmosphere) the cells were fixed with formalin. The plates could be stored at 4°C until the next use. The formalin in the wells was poured out and 200 $\mu$l 1% triton/PBS were pipetted per well for 30 minutes at room temperature to enhance the permeability of the cell

membranes.
In that time, the dilution series was prepared. The dilution series was performed in duplicate. On a 96-well plate in the first well a 1:10 predilution (90 $\mu$l PBS and 10 $\mu$l serum) was prepared. In the following wells a 1:2 dilution series was prepared (50 $\mu$l PBS and 50 $\mu$l from the previous well). The dilution series was performed to the dilution stage 1:20480. A positive and negative serum control provided by the Diagnostic Laboratory of the Institute of Virology was analysed in parallel.
The IFA plate was cleaned of triton by tapping out and the plate was washed several times with 1% FCS/PBS and tapped out. Then 50 $\mu$l of each prepared serum dilution were pipetted into the corresponding cavity of the IFA plate.
The IFA plate was then incubated for one hour at room temperature and afterwards washed twice with FCS/PBS to remove serum remains. In each well 35 $\mu$l Anti-Horse-FITC-dilution (1:100) was pipetted.
The plate was incubated at room temperature for one hour and then washed three times with FCS/PBS and the residual liquid was tapped out.
In each well 50 $\mu$l of distilled water was pipetted for microscopy. The analysis was conducted under a fluorescence microscope. The infected plaques appeared green. Only coloured plaques were positive. The last dilution step which still showed an antigen-specific fluorescence was defined as IFA titer.

## 4.7 PBMC isolation from citrated blood

**citrated blood**

| | |
|---|---|
| venous blood | 9 ml |
| 3.8% sodium-citrate solution in Aqua dest | 1 ml |

**ficoll**

| | |
|---|---|
| specific gravity | 1.077 g/ml |

**PBS (phosphate buffered saline)**

Based on differences in specific density, the peripheral blood mononuclear cells (PBMC) were separated from the other blood components by overlaying equal volumes (10 ml) ficoll with citrated blood. The tubes were centrifuged (Eppendorf centrifuge type 5043, rotor 16F24-11) for 15 minutes at 4°C and 1.900 rpm. By centrifugation, the mononuclear cells (buffy coat) formed the interphase above the ficoll coat, with the other cells with higher specific gravity sedimenting at the bottom of the tube. In the centrifuged tube there was a four-layered fractionation recognizable, from the bottom to the top: erythrocytes, ficoll, buffy coat and plasma.
The plasma was pipetted into two eppendorf tubes. Then the buffy coat was aspirated with a pipette and transferred into a fresh 10 ml tube and with PBS filled up to 10 ml. Subsequently, a centrifugation (Eppendorf centrifuge type 5043, rotor 16F24-11) for four minutes, 4°C and 1.500 rpm was performed. The supernatant was discarded and the cell pellet was located at the bottom of the tube. The washing step was performed twice before the cell pellet was resuspended in 1 ml PBS and transferred into a 2 ml tube. The tube was centrifugated (Eppendorf centrifuge type

5043, rotor 16F24-11) by the following parameters: (1) 1.000 rpm (2) 4 minutes and (3) 4°C. The supernatant was again discarded. The pellet was frozen at -20°C until the DNA isolation.

## 4.8 DNA preparation

### 4.8.1 DNA preparation from tissue samples and PBMCs

**Digestion buffer**

| | |
|---|---|
| Tween | 2.5 ml |
| EDTA | 0.179 g |
| Tris | 3.025 g |
| Aqua dest. | ad 500 ml |

**Proteinase K**

| | |
|---|---|
| concentration | 15.1 $\mu$g/$\mu$l |
| pH | 7.5; in 10 mM Tris-HCl |

For DNA preparation from tissue samples, two small pieces were cut with a scalpel from the centre of the tissue sample and placed in a tube. 500 $\mu$l digestion buffer and 10 $\mu$l proteinase K were added. For DNA isolation from the PBMC, the thawed pellets (see 4.7) were resuspended in 150 $\mu$l digestion buffer. After pellet resuspension 3 $\mu$l proteinase K (15,1 $\mu$g/$\mu$l) were added. As a negative control, 150 $\mu$l digest buffer and 3 $\mu$l proteinase K were pipetted into another tube. The samples were incubated in a water bath (Thermo Mixer, HLC, Bovenden, Germany) at 56°C for 12 hours, so that the proteinase K could digest the cell components. After the incubation time the lid was punctured with a cannula and proteinase K was inactivated at 95°C for 10 minutes. The DNA was frozen in -20°C. In the ORF 30 nested PCR 1 $\mu$l was used.

### 4.8.2 Viral DNA preparation from virus stocks, infected cell culture supernatants, nasal swabs, lung fluids, amniotic fluid and semen

**Eagle‘s minimum essential medium/EDM**

**RTP DNA/RNA Virus Mini Kit**
Invitek, Berlin, Germany

The viral DNA preparation was performed with archived viral stocks and infected cell culture supernatants. The supernatants of virus infected cell lines were centrifuged (Heraeus Christ Minifuge, type 4123, rotor 1664) first 10 minutes at 1.000 rpm and then 10 minutes for 3.000 rpm to separate the debris from the viral particles.
Semen, lung fluids and amniotic fluids were cleaned from debris by centrifugation (one minute at 1.000 rpm, then for 10 minutes at 3.000 rpm; Heraeus Christ, Minifuge, type 4123, rotor 1664) and used for viral DNA extraction.
Nasal swabs were put in a tube, 2 ml EDM was added and shaken after two hours of incubation at 4°C. After a centrifugation for five minutes at 5.000 rpm (Heraeus Christ, Minifuge, type 4123 rotor 1664) was done.
For viral DNA preparation the RTP DNA/RNA Virus Mini Kit from Invitek (Invitek, Berlin,

Germany) was used. The following extraction parameters were the same as described by the manufacturer. The following centrifugation steps were made using the centrifuge Heraeus Biofuge A, type 1217, rotor 1378, Osterode, Germany.
200 $\mu$l of each obtained viral supernatant, as well as 200 $\mu$l DNA-RNA-free water, were transferred into extraction tubes. The pre-filled extraction tubes contained lyophilized lysis reagents (non chaotropic lysis buffer and Proteinase K). Proteinase K digests the cell components. The viral nucleic acids were stabilized, desoxyribonucleases and ribonucleases inactivated and the selective DNA adsorption to the membrane of the spin filter later on was enhanced. The elution buffer was heated at 80°C in the incubation water bath (GFL, Großburgwedel, Germany). The filled extraction tube was heated and softly vibrated at (1) 65°C 15 minutes and (2) 95°C 10 minutes in a thermo mixer (HLC, Bovenden, Germany). To each extraction tube, 400 $\mu$l of binding buffer were added and the fluids mixed by vortexing (Bender & Hobein AG, Zurich, Switzerland). The 800 $\mu$l fluid-virus mixture were transferred to the spin filter in a receiver tube and centrifuged for one minute at 10.000 rpm. Before the viral nucleic acids were eluted, the membrane of the spin filter was efficiently washed in order to remove all PCR inhibitors. The spin filter was transported in a new receiver tube and 500 $\mu$l of buffer R1 added. The centrifugation was performed at 10.000 rpm for 30 seconds, the spin filter was again transferred in a new receiver tube and 800 $\mu$l of buffer R2 added. Thirty seconds centrifugation at 10.000 rpm were conducted, the filtrate was discarded and the tube was centrifuged for four minutes at 15.000 rpm. The wash buffers R1 and R2 washed all contaminants away and the viral DNA remained bound to the membrane of the spin filter. The spin filter was transferred to the elution tube and 60 $\mu$l of the heated elution buffer were added for an incubation time of 10 minutes at room temperature. This was then followed by centrifugation for one minute at 10.000 rpm and the removal of the filter. The elution buffer released the DNA from the filter membrane and centrifugation obtained the DNA solution. Residual ethanol evaporated in an open lid for 10 minutes in a water bath at 37°C. The extracted DNA (final volume 50 to 55 $\mu$l) was stored at -20°C until use in the PCR. In the ORF 30 nested PCR 3 $\mu$l DNA were used.

## 4.9 Analytical gel electrophoresis

**Loading buffer**

| | |
|---|---|
| Ficoll 400 | 1.5 g |
| Bromphenolblue | 25 mg |
| Aqua dest. | ad 10 ml |

**TAE buffer (10x)/Tris-Acetat-EDTA**

| | |
|---|---|
| Tris | 96.90 g (40 mM) |
| Sodium-acetate | 8.2 g (5 mM) |
| EDTA | 7.4 g (1 mM) |
| Aqua dest. | ad 2 l |
| adjust pH 7.9 | |

**electrophoresis buffer**

| | |
|---|---|
| TAE buffer 10 x | 200 ml |
| Aqua dest. | ad 2 l |
| Ethidiumbromid | 80 $\mu$l |

**2% agarose in TAE**

| | |
|---|---|
| agarose | 4.0 g |
| TAE | 200 ml |

The horizontal gel electrophoresis was used for the separation of DNA accordingly to determine the molecular weight and size of DNA fragments. In the case of nucleic acids, the direction of migration, from negative to positive electrodes, was due to the naturally-occurring negative charge. The speed with which the DNA moved through the gel was directly correlated to its size.
The buffer used was Tris-Acetat-EDTA (TAE). Agarose was dissolved in an electrophoresis buffer by calefaction through microwave. Before pouring the gel, the solution was cooled down. Gels were run in horizontal chambers. The gel concentration was 2%. Standard molecular weight markers were used for every gel. The gels were photographed by Vision Capt.

## 4.10 Determining the molecular masses and concentration of DNA

### 4.10.1 Gel electrophoresis

A determination of the amount of a substance in a band or the relative proportion of a band was possible after staining of gels and subsequent densitometric evaluation. To estimate the molecular masses and concentration of DNA the mass standard HyperLadder IV with a separation range from 100 to 1013 bp for ORF 30 nested PCR amplicons and the restriction enzyme *Sal* I products was used.

### 4.10.2 Photometry

The DNA concentration was determined using the UV-VIS spectrophotometer. A 1:100 dilution was created from the DNA sample for the measurement. Determining of the DNA concentration was carried out at a wavelength of 260 nm.

## 4.11 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a molecular biological technique used to amplify single copies of a chosen DNA fragment. The method is based on thermal cycling. Each cycle consisted of three steps. The first step is the denaturation, when heating the DNA double strands separate. The heating breaks the hydrogen bonds which hold together the two strands of DNA. The second step is called annealing, when the temperature has been chosen specific to the primer, the primer anneal at the specific sites of single stranded DNA. The third step, elongation, refers to the filling of missing strands by the DNA polymerase. The beginning is at the 3 'end of the annealed primer and then follows the DNA strand. These steps will be repeated a different number of times depending on the specific PCR protocol.
Primers are synthetic, single-stranded oligonucleotides which are complementary to a selected sequence of the target DNA region and determine the starting and end point for the amplification.

The other reaction components are the nucleotides, PCR buffer and a heat-stable DNA polymerase (Taq-polymerase) to assemble the DNA.

A modification of the polymerase chain reaction is the nested polymerase chain reaction, intended to increase the specificity and sensitivity. Nested PCR involves two sets of primers, used in two successive runs, the second set amplifies a secondary target within the first run product.

### 4.11.1 ORF 30 nested PCR

RNAseDNAse free water
Roth, Karlsruhe, Germany

dNTPs (0.2 mM dNTPs)
Bioline, Luckenwalde, Germany

Primer ORF30-F-#8, concentration: 0.4 $\mu$M
Primer ORF30-R-#2, concentration: 0.4 $\mu$M
Primer ORF30-F-#7, concentration: 0.4 $\mu$M
Primer ORF30-R-#3, concentration: 0.4 $\mu$M
Metabion, Martinsried, Germany

Taq-Polymerase (1.5 U)
10x PCR reaction buffer
Quiagen, Hilden, Germany

A single nucleotide polymorphism within the EHV-1 gene ORF 30, which encodes the viral DNA polymerase, allowed the differentiation of the neuropathogenic ($G_{2254}$) from the non-neuropathogenic genotype ($A_{2254}$). The EHV-1 $G_{2254}$ polymorphism in the EHV-1 polymerase gene was found associated with the neurological disease in horses.
The ORF 30 EHV-1 sequence ranges from 55.184 to 51.501 bp. ORF 30 is located in the minus strand. The neuropathogenic mutation is located at genome position 52.931 bp (see Tab. 13).

To identify the mutation at the polymorphic site of the polymerase gene, an ORF 30 specific nested PCR was performed. The mutation was located at position 2254 of the EHV-1 ORF 30 gene (Fig. 1).

The primers (see Tab. 14) were selected based on the published paper by Allen (2006). The chosen primers were tested using the program Mac Vector with the gene sequences from the data bank of the "National Center for Biotechnology Information" (NCBI). The selected primers were ordered lyophilized and HPLC purified. The PCR parameters were tested according to the manufacturer‘s specifications and the settings with the best results were used for this work.
In the case of EHV-4, the primer pairs would not generate a PCR product.

The PCR reaction mixture (50 $\mu$l) of the first round each consisted of 1 $\mu$l forward primer (ORF 30 F-8, concentration: 0.4 $\mu$M) and 1 $\mu$l reverse primer (ORF 30 R-2, concentration: 0.4

Table 13: Excerpt of the EHV-1 ORF 30 gene sequence (source: GenBank www.ncbi.nlm.nih.gov, GeneID: 1487570, strain: Ab4, EHV-1) and position of the ORF30 nested primer. The ORF 30 is located in the minus strand.
blue=outer primer F-8 and R-2, red=inner primer F-2 and R-3, magenta=Position 2254, green=Position 2258

| EHV-1 sequence, ORF 30 minus strand, ← direction | | |
|---|---|---|
| 52791 | 5‘-GGGGGTGCTG GTGGGGATTC GCGCCCTCAC CGCCTTTCGC-3‘ | 52830 |
| ← | 3‘-CCCCCACGAC CACCCCTAAG CGCGGGAGTG GCGGAAAGCG-5´ | ← |
| 52831 | 5´-ATGGCCAGCC AGTCGCGCAG CAAGATGCCA AGCAGGCTTT-3´ | 52870 |
| ← | 3‘ TACCGGTCGG TCAGCGCGTC GTTCTACGGT TCGTCCGAAA-5‘ | ← |
| 52871 | 5‘-CGCGAATATG GGCGTGGACA AAAAATAACT TTTGGTCACC-3‘ | 52910 |
| ← | 3‘-GCGCTTATAC CCGCACCTGT TTTTTATTGA AAACCAGTGG-5‘ | ← |
| 52911 | 5‘-CACCTCGAAC GTCGAGTAGT CGACGGATGG TTGAAGCCCG-3‘ | 52950 |
| ← | 3‘-GTGGAGCTTG CAGCTCATCA GCTGCCTACC AACTTCGGGC-5‘ | ← |
| 52951 | 5‘-GCCAGATCCA CTTCATCGAG CGCCAGGGTG GTGAAACAGA-3‘ | 52990 |
| ← | 3‘-CGGTCTAGGT GAAGTAGCTC GCGGTCCCAC CACTTTGTCT-5‘ | ← |
| 52991 | 5‘-GGTTATGGGC CTGGATAATG CTTGGGTATA AGCTAGCGAA-3‘ | 53030 |
| ← | 3‘-CCAATACCCG GACCTATTAC GAACCCATAT TCGATCGCTT-5‘ | ← |
| 53031 | 5‘-GTCAAACACA ACCACGGGGT CCACATGAAA GCCGGATACG-3‘ | 53070 |
| ← | 3‘-CAGTTTGTGT TGGTGCCCCA GGTGTACTTT CGGCCTATGC-5‘ | ← |
| 53071 | 5‘-GGGTCTAGAA CCTTTGCTCC CTGGTAGCCC ACGGCCCTCC-3‘ | 53110 |
| ← | 3‘-CCCAGATCTT GGAAACGAGG GACCATCGGG TGCCGGGAGG-5‘ | ← |
| 53111 | 5‘-CGACGCCGGG CTTCCCGCCT CCGTTTTCAG AAGTAGCGCC-3‘ | 53150 |
| ← | 3‘-GCTGCGGCCC GAAGGGCGGA GGCAAAAGTC TTCATCGCGG-5‘ | ← |
| 53151 | 5‘-AGATCCTGCG GCGTCCGGGG TACCGTCCAC ACCGTCGGGT-3‘ | 53190 |
| ← | 3‘-TCTAGGACGC CGCAGGCCCC ATGGCAGGTG TGGCAGCCCA-5‘ | ← |

$\mu$M), 5 $\mu$l dNTPs (0.2 mM dNTPs), 5 $\mu$l 10 x PCR reaction buffer, 0.3 $\mu$l Taq polymerase (1.5 U) and the viral DNA sample. In case of DNA preparation from abortion tissue by proteinase K, 1 $\mu$l DNA was used. 3 $\mu$l viral DNA was used isolated from archived viral stocks and infected cell culture supernatants prepared by RTP DNA/RNA Virus Mini Kit. The total volume of PCR reaction mixture was then filled up with RNase-DNAse-free water to 50 $\mu$l.
The amplification parameters of the first round ORF 30 PCR were 35 cycles of: (1) denaturation: 94°C for 30 seconds, (2) annealing: 66°C for 30 seconds, (3) elongation: 72°C for 60 seconds.
With each PCR approach a substance-preparation control and reference strains were carried. Four reference strains were initially included as controls: one neurological EHV-1 strain (Ab4, positive control), one abortigenic EHV-1 strain (AIV, positive control), one respiratory EHV-1 strain (Mar87, positive control) and a respiratory EHV-4 strain (KT-4, negative control). These purified viral DNA controls were from the Institute of Virology.

The nested PCR reaction mixture (50 $\mu$l) for the second round each consisted of 1 $\mu$l forward primer (ORF 30 F-7, concentration: 0.4 $\mu$M) and 1 $\mu$l reverse primer (ORF 30 R-3, concentration: 0.4 $\mu$M), 5 $\mu$l dNTPs (0,2 mM dNTPs), 5 $\mu$l 10 x PCR reaction buffer, 0.3 $\mu$l Taq polymerase (1.5 U) and 1 $\mu$l amplicon of the first round. The total volume of nested PCR reaction mixture was

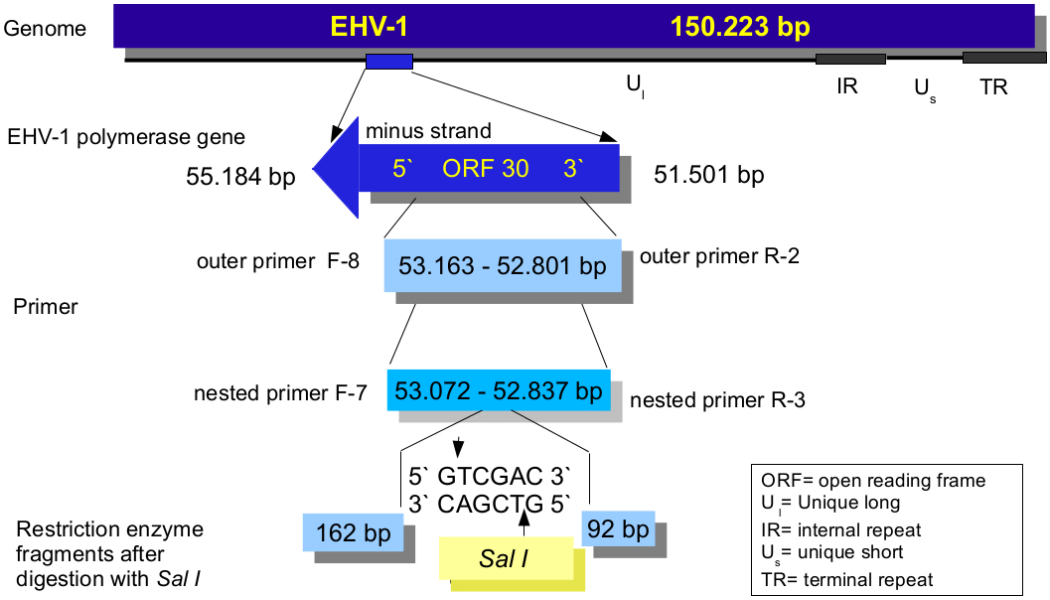


Figure 1: Genome position of the nested primer pairs and restriction site of *Sal* I

Table 14: ORF 30 nested PCR primer sequences

| Outer PCR primer | Primer sequences | Genome localisation |
|---|---|---|
| ORF30-F-8 | 5‘-GTG GAC GGT ACC CCG GAC-3‘ | 53163 |
| ORF30-R-2 | 5‘-GTG GGG ATT CGC GCC CTC ACC-3‘ | 52801 |
| Inner PCR primer | | |
| ORF30-F-7 | 5‘-GGG AGC AAA GGT TCT AGA CC-3‘ | 53072 |
| ORF30-R-3 | 5‘-AGC CAG TCG CGC AGC AAG ATG-3‘ | 52837 |

filled up with RNAse-DNAse free water to 50 $\mu$l.
The nested ORF 30 PCR parameters were 35 cycles of: (1) denaturation: 94°C for 30 seconds, (2) annealing: 68°C for 30 seconds, (3) elongation: 72°C for 60 seconds.
The reactions were conducted in the Hybaid Omni Gene PCR System (Pegasus, Rockville, USA).

### 4.11.2 Evaluation of ORF 30 nested PCR

A 2% agarose gel stained with ethidiumbromide was used.
The marker contained the following components: 2 $\mu$l loading buffer, 5 $\mu$l Hyperladder IV (100 bp marker, Bioline, Luckenwalde, Germany) and 10 $\mu$l RNAse-DNAse-free water. From each amplicon 15 $\mu$l were mixed with 2 $\mu$l loading buffer.
15 $\mu$l were pipetted into each slot and the chamber (Bio-Rad Migration chamber, model 1000/500, power supply, Munich, Germany) was sealed. Gelelectrophoresis was carried out at 100 V (10 V/cm) for one hour.
To establish the ORF 30 nested PCR, the amplified products of both rounds were applied to the gel and analysed. The resulting fragment sizes were 380 bp for ORF 30 PCR product and 254 bp for the ORF 30 nested PCR fragment.

### 4.11.3 Sensitivity of the ORF 30 nested PCR

A dilution series with a selected reference strain was made for the determination of the ORF 30 nested PCR (first and second round) sensitivity. The highly concentrated viral DNA (AIV) used in the diagnostic laboratory was tested by a dilution series in the first round of the ORF 30 nested PCR. The dilution steps were 3 $\mu$l DNA (12 ng DNA), 2 $\mu$l DNA (8 ng DNA), 1 $\mu$l DNA (4 ng DNA) and then in 1:10 dilution steps to $10^{-3}$ (0.004 ng DNA). The amount of DNA was determined photometrically (see 4.10.2). RNAse-DNAse-free water was used for the dilution. The amplicons of the first round were used as templates for the second round ORF 30 nested PCR. The gel from the first and second round was evaluated (see 5.1.2).

## 4.12 Restriction enzyme analysis (REA)

Restriction enzymes cut DNA segments at a specific nucleotide sequence, the restriction site.

### 4.12.1 REA *Sal* I of ORF 30 nested PCR products

*Sal* I HF-high frequency (20.000 U/ml)
New England Biolabs, Frankfurt am Main, Germany

10x NEBuffer 4

| | |
|---|---|
| Potassium acetate | 50 mM |
| Tris-acetate | 20 mM |
| Magnesium acetate | 10 mM |
| Dithiothreitol | 1 mM (pH 7.9, 25°C) |

New England Biolabs, Frankfurt am Main, Germany

The restriction enzyme *Sal* I was used to differentiate the non-neuropathogenic and the neuropathogenic EHV-1 strains by cleaving the ORF 30 nested PCR amplicon into different fragments as Allen (2006) described. The *Sal* I cleavage site

5‘...G↓TCGAC...3‘
3‘...CAGCT↑G...5‘

was located at position 92 of the 254 bp ORF 30 nested PCR amplicon, in the case of the neuropathogenic genotype, two cleavage fragments resulted.
Three reference strains were initially included as controls. The reference strains were amplified with the isolates to be tested in the ORF 30 nested PCR. The neurological EHV-1 strain (Ab4) was expected to be cut into two fragments (positive control) and the abortigenic (AIV) as well as the respiratory EHV-1 strains (Mar87) were expected not to be cleaved by the enzyme (negative controls).
For the restriction enzyme digest, a 100 ng amplicon of the ORF 30 nested PCR product needed to be used. The amount needed to be determined by a comparison of the bands of molecular weight marker and the amplicons. While pipetting, one must take care not to raise the cover of the mineral oil.

The restriction enzyme *Sal* I mixture consisted of 2 $\mu$l 10x NEBuffer 4, 15 to 17 $\mu$l ORF nested PCR ORF 30 amplicon (corresponds to 100 ng), 1 $\mu$l restriction enzyme *Sal* I. The total volume was filled up with RNase-DNAse-free water to 20 $\mu$l.
The components were added in the following order on ice: RNAse-DNAse-free water, buffer, ORF 30 nested PCR product and lastly the restriction enzyme. The tubes were incubated at 37°C for 60 minutes in a thermoblock (Block Thermostate , HLC Biotech, Bovenden, Germany).

### 4.12.2 Evaluation of the REA *Sal* I digestion

The DNA band after *Sal* I restriction analysis was compared to the molecular weight marker HyperLadder IV. The untreated ORF 30 amplicon should be localized at the position of 254 bp in gel electrophoresis. However, the treated neuropathogenic ORF 30 amplicon had one restriction site resulting in two smaller fragments (162 bp and 92 bp) (Allen, 2006; Fritsche and Borchers, 2011). A 2% agarose gel was used stained with ethidiumbromide.
The marker was prepared as described (see 4.11.1). 15 $\mu$l ORF 30 amplicon treated with *Sal* I were added to 2 $\mu$l loading buffer. The untreated ORF 30 amplicon was used (see 4.11.1) as negative control.
Into each slot, 15 $\mu$l of the amplicon-loading buffer-mixture was pipetted and the chamber (Bio-Rad Migration chamber, model 1000/500, power supply, Munich, Germany) was sealed to run for one hour at 100 Volt (10 V/cm).

## 4.13 Sequence analysis

To confirm the restriction enzyme analysis results, the ORF 30 nested PCR products (254 bp) were sequenced by Sequence Laboratories Göttingen GmbH, Germany.
The ORF 30 nested PCR products were prepared with ExoSAP-IT (USB, Ohio, USA). To remove dNTPs and primers ExoSAP-IT utilizes two hydrolytic enzymes in a special formulated buffer: (1) Exonuclease I, removes single-stranded primers and single-stranded DNA and (2) Shrimp Alkaline Phosphatase, removes the remaining dNTPs from the PCR product. The ExoSAP-IT protocol was adopted as follows: 5 $\mu$l ORF 30 nested PCR product with 2 $\mu$l of ExoSAP-IT for a combined 7 $\mu$l volume. Two incubation steps were followed, (1) at 37°C for 15 minutes to degrade remaining primers and nucleotides, (2) at 80°C for 15 minutes to inactivate the ExoSAP-IT enzymes. Afterwards, the PCR product was ready for the DNA sequencing.
The Hot Shot sequencing (300 bp) from Seqlab was chosen. For HotShot sequencing, PCR-products with these conditions are required: 64 ng DNA (the amount of DNA in nanograms was calculated by dividing the PCR product-length 254 bp by four) plus 20 pmol ORF 30 nested primer. The total volume should be 7 $\mu$l with 5-10 mM Tris pH 8.0 in the sample.

The sequencing chromatograms give an overview of how clean the sequence is, this includes mis-calls and errors in the sequence. The examination of the sequencing chromatograms was made with the program 4peaks (Version 1.7.2).

## 4.14 Statistics

The scope of the thesis and the limited sample size can only result in a descriptive analysis. This decision was made in collaboration with the Institute of Biometrics, Free University Berlin.

# 5 Results

A single nucleotide polymorphism in ORF 30 at position 2254 was found to be directly related to the neuropathogenic potential (Nugent et al., 2006). EHV-1 positive abortion samples were investigated by ORF 30 nested PCR and REA *Sal* I, and subsequently sequenced to clarify if principally non-neuropathogenic strains ($A_{2254}$) were the cause of abortion cases. It was expected that neuropathogenic strains ($G_{2254}$) causes neurological signs.

From 67 EHV-1 positive abortion cases the isolated viral DNA was investigated by ORF 30 nested PCR, restriction fragment analysis *Sal* I and the resulting ORF 30 nested amplicons were sequenced. In one abortion outbreak (stud farm 3) the isolated viral DNA of the semen of the stallion was also investigated by ORF 30 nested PCR and restriction fragment analysis *Sal* I.

In addition, serology of blood samples from 24 mares and PBMC isolation of blood samples from 13 mares which aborted were done. They came from two different stud farms with abortion outbreaks. The viral DNA of PBMCs was isolated and investigated by ORF 30 nested PCR, REA *Sal* I and the ORF 30 nested fragment was sequenced.

Viral isolates from two cases with neurological signs were investigated by ORF 30 nested PCR, REA *Sal* I and the ORF 30 nested fragments were sequenced.

For this work, six EHV-1 reference strains and two EHV-4 reference strains were used. Furthermore, five wild equid strains related to EHV-1, one EHV-9 wild equid strain and two cattle EHV-1 strains were examined by ORF 30 nested PCR, REA *Sal* I and the resulting ORF 30 nested fragments were sequenced.

## 5.1 Establishment and evaluation of the ORF 30 nested PCR

The main part of the research was to determine whether the non-neuropathogenic ($A_{2254}$) or neuropathogenic genotype ($G_{2254}$) occurred in virus isolates and tissues from abortion and CNS cases. Allen (2006) described an ORF 30 nested PCR followed by a restriction enzyme analyses with *Sal* I.
The aim was to establish an ORF 30 nested PCR, followed by restriction enzyme analysis, to distinguish between non-neuropathogenic and neuropathogenic EHV-1 strains. The primers were selected based on the published paper by Allen (2006). Subsequently, the chosen primers were tested using the program Mac Vector with the gene sequences from the data bank of the "National Center for Biotechnology Information" (NCBI). PCR parameters were tested according to the manufacturer‘s specifications and the settings with the best results were used for this work (see 4.11.1).
The primer pairs of the ORF 30 nested PCR first and second round were tested separately. It was expected that the primer of the first round (Forward-8/Reverse-2) would have an amplicon of 380 bp. The amplicon from the primer pair of the second round (F-7/R-3) was expected to be 254 bp. As shown in Figure 2, both primer pairs worked as expected.

The reference strains originated from the virus stock of the Institute of Virology and were

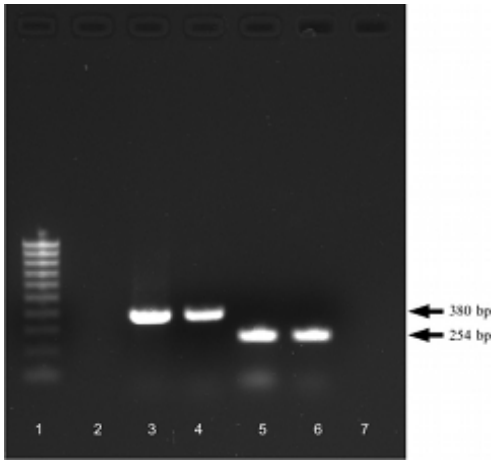


Figure 2: Establishment of the ORF 30 nested PCR: Test of the primers with different reference strains from the first and the second round after agarose gel electrophoresis.

Key:
1. 100 bp DNA ladder HyperLadder IV
2. substance control → negative
3. Ab4, primer 8/2 of the 1. round → positive
4. Mar87, primer 8/2 of the 1. round → positive
5. Ab4, primer 7/3 of the 2. round → positive
6. Mar87, primer 7/3 of the 2. round → positive
7. substance control → negative

grown on ED cells. For viral DNA preparation from the virus-containing supernatants, the RTP DNA/RNA Virus Mini Kit from Invitek (Invitek, Berlin, Germany) was used. The received viral DNA was transferred to tubes and frozen (-20°C) until use.

### 5.1.1 Establishment of the ORF 30 nested PCR with selected EHV-1/-4 reference strains

The three EHV-1 reference strains all have a different pathotype (see Tab. 15). The ORF 30 nested PCR was performed, and the amplicons were tested via restriction enzyme analysis and sequenced.
It was expected for all three EHV-1 reference strains that one amplicon would be the size of 254 bp. For the EHV-4 strains KT-4 and T252 no PCR product resulted, as expected due to the screening with the program MacVector. Since EHV-4 strains had a negative result in the ORF 30 nested PCR, no restriction enzyme analysis or sequencing was performed. It was decided to take KT-4 as a negative ORF 30 reference strain.
Of the substance control, a negative result was expected in the ORF 30 nested PCR.

The results of the agarose gel electrophoresis of the selected EHV-1 and -4 reference strains of the ORF 30 nested PCR can be seen in Fig. 3.

Only EHV-1 strains were amplified in the ORF 30 nested PCR (see Fig. 3). The amplicons had a size of 254 bp. To detect the variation in the polymerase gene that determined neuropathogenic or non-neuropathogenic potential, the resulting nested PCR fragment had to be treated with the restriction enzyme *Sal* I, and for approval, the ORF 30 amplicon had to be sequenced.

Table 15: Background of the selected EHV-1 and -4 reference strains and results for the ORF 30 nested PCR

| Identification | Genotype | Pathotype | ORF 30 nPCR band at height |
|---|---|---|---|
| Ab4 | EHV-1 | neurologic | 254 bp |
| AIV | EHV-1 | abortion | 254 bp |
| Mar87 | EHV-1 | rhinopneumonitis | 254 bp |
| KT-4 | EHV-4 | respiratory | no PCR product |

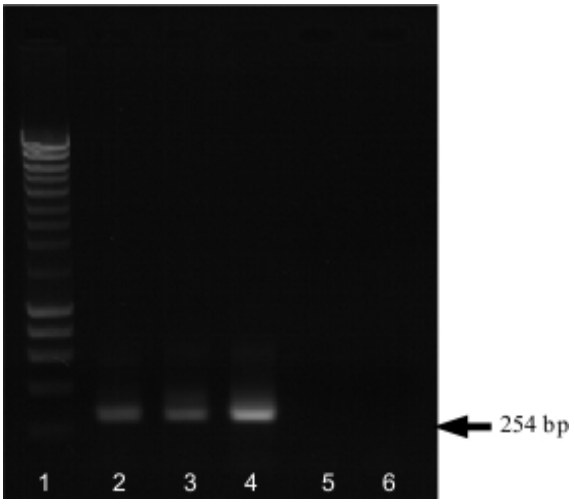


Figure 3: ORF 30 nested PCR: Results of the selected ORF 30 reference strains after agarose gel electrophoresis.

Key:
1. 1000 bp DNA ladder HyperLadder I
2. Ab4, EHV-1, positive ORF 30 reference strain
3. AIV, EHV-1, positive ORF 30 reference strain
4. Mar87, EHV-1, positive ORF 30 reference strain
5. KT-4, EHV-4, negative ORF 30 reference strain
6. substance control, negative

### 5.1.2 Sensitivity of the ORF 30 nested PCR

The highly concentrated EHV-1 reference strain AIV used in the diagnostic laboratory was chosen for the determination of the ORF 30 nested PCR sensitivity by a dilution series. A dilution series beginning with 3 $\mu$l DNA was performed, with RNAse-DNAse-free water used for the dilution. The photometric determination (see 4.10.2 ) of DNA quantity of AIV was 4 ng/$\mu$l.The first round from the ORF 30 nested PCR was evaluated and in the gel electrophoresis a band at the height of 380 bp was seen. The last detectable amplicon was seen at dilution step $10^{-2}$ (0.04 ng DNA) (see Fig. 4).

In the gel electrophoresis of the second round ORF 30 nested PCR a band at the height of 254 bp was seen. From each dilution step of the first round 1 $\mu$l was set in the second round of the ORF 30 nested PCR. In the ORF 30 nested PCR (second round) the last positive amplicon was seen at dilution of $10^{-3}$ (0.004 ng DNA).
To summarize, the sensitivity of the first round was 0.04 ng DNA and for the second round 0.004

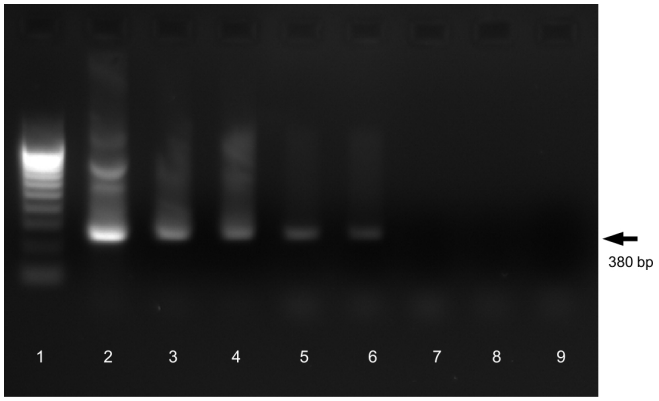


Figure 4: Results of agarose gel electrophoresis of the 1. round ORF 30 nested PCR products in a dilution series.

Key:
1. 100 bp DNA ladder (HyperLadder IV)
2. AIV, 3 $\mu$l (12 ng DNA) $\rightarrow$ positive
3. AIV, 2 $\mu$l (8 ng DNA) $\rightarrow$ positive
4. AIV, 1 $\mu$l (4 ng DNA) $\rightarrow$ positive
5. AIV, $10^{-1}$ (0.4 ng DNA) $\rightarrow$ positive
6. AIV, $10^{-2}$ (0.04 ng DNA) $\rightarrow$ slightly positive
7. AIV, $10^{-3}$ (0.004 ng DNA) $\rightarrow$ negative
8. KT-4 $\rightarrow$ negative control
9. substance control $\rightarrow$ negative

ng DNA.

## 5.2 Test of the restriction enzyme analysis *Sal* I with the ORF 30 fragments of the selected EHV-1 reference strains

All nested PCR amplicons from the ORF 30 nested PCR (second round) were studied with the restriction enzyme *Sal* I (Allen, 2006). The *Sal* I cleavage site is located at position 92 of the 254 bp amplicon. The aim was to differentiate between neuropathogenic and non-neuropathogenic strains. The restriction enzyme *Sal* I digests amplicons from neuropathogenic strains into two fragments (162 and 92 bp), whereas the non-neuropathogenic amplicons remain undigested (see Fig. 6).
The Ab4 strain originated from a horse with paresis (Edington et al., 1985). The ORF 30 amplicon of EHV-1 strain Ab4 was digested into two fragments as expected and was a neuropathogenic strain. Ab4 was chosen as the *Sal* I positive reference strain.
AIV originated from a Lippizaner stud with abortions and neurological signs (Chowdhury et al., 1986). AIV showed unexpectedly a digestion of the ORF 30 amplicon and had to be classified as a neuropathogenic strain with abortigenic potential. It was therefore decided to not take AIV as the *Sal* I reference strain.
The ORF 30 amplicon of the respiratory EHV-1 strain Mar 87 (Thein, 1987) was not digested by the restriction enzyme and defined as a non-neurological/negative *Sal* I reference strain.
The observed incomplete digestion in the restriction enzyme analysis *Sal* I (see Fig. 6) can have

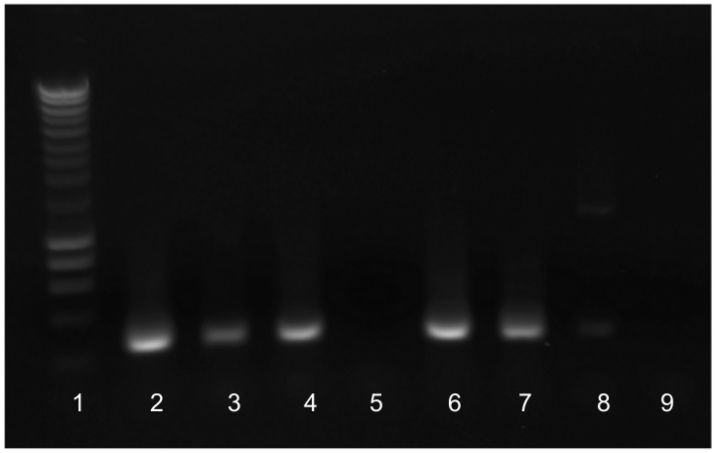


Figure 5: Results of agarose gel electrophoresis of the 2. round ORF 30 nested PCR products in a dilution series.

Key:
1. 1000 bp DNA ladder HyperLadder I
2. AIV, 3 $\mu$l $\rightarrow$ positive
3. AIV, 2 $\mu$l $\rightarrow$ positive
4. AIV, 1 $\mu$l $\rightarrow$ positive
5. KT-4 $\rightarrow$ negative control
6. AIV, $10^{-1}$ $\rightarrow$ positive
7. AIV, $10^{-2}$ $\rightarrow$ positive
8. AIV, $10^{-3}$ $\rightarrow$ slightly positive
9. substance control $\rightarrow$ negative

different reasons like both genotypes exist in the particular ORF 30 amplicons, inhibition by PCR components, too few units of enzyme used or that the incubation time was too short.
For comparison and confirmation a sequencing was performed. The sequencing chromatograms showed clearly that only one genotype was present. Consequently, the results are assessable.

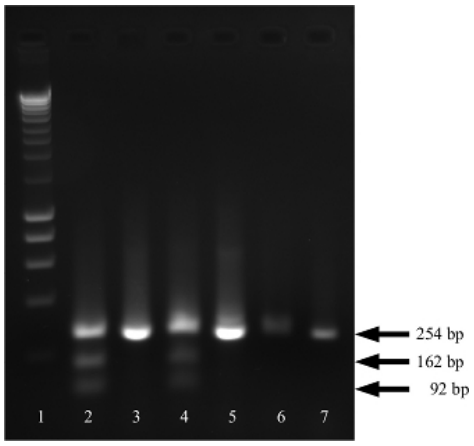


Figure 6: Results of agarose gel electrophoresis of ORF 30 nested PCR fragments after *Sal* I digestion in comparison to non-treated fragments from three selected reference strains.

Key:
1. 100 bp DNA ladder (HyperLadder IV)
2. Ab4, ORF 30 amplicon treated with *Sal* I, positive *Sal* I reference strain
3. Ab4, untreated ORF 30 amplicon
4. AIV, ORF 30 amplicon treated with *Sal* I, positive
5. AIV, untreated ORF 30 amplicon
6. Mar87, ORF 30 amplicon treated with *Sal* I, negative *Sal* I reference strain
7. Mar87, untreated ORF 30 amplicon

## 5.3 Review of the ORF 30 fragments of the selected EHV-1 reference strains by sequencing

The sequencing confirmed the results of the *Sal* I restriction enzyme analysis. EHV-1 strain Ab4 encoded the neuropathogenic genotype $G_{2254}$ as expected. Austria IV (AIV) contained $G_{2254}$ (described as neurological genotype) in the polymerase gene. The respiratory strain Mar87 carry $A_{2254}$, which was described as non-neurological (see Tab. 16).
Ab4 and AIV were separately tested in a second approach and a second sequencing was performed with these amplicons: they showed in both sequencing the same results.
The sequences were translated with the program MacVector into the corresponding amino acid sequence. The nucleotide exchange at position 2254 from adenine to guanine resulted in an amino acid change (Asparagine N 752 / 5‘-AAC-3‘ to aspartic acid D 752 / 5‘-GAC-3‘).
In the EHV-1 strain Ab4, an additional exchange from adenine to cytosine at nucleotide position 2258 ($C_{2258}$) was found (see Tab. 16).

Table 16: EHV-1 reference strains: Excerpt of the sequence alignment of equid herpes virus ORF 30 nested PCR amplicons (second round). blue=$A_{2254}$, red=$G_{2254}$, green=$A_{2258}$, magenta=$C_{2258}$

| Isolate | 3‘- 2271 | Sequence | ← direction | 2236 - 5´ |
|---|---|---|---|---|
| Ab4 | 3‘-GAG CTT GCA GCT CCT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| AIV | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| Mar87 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ | | | |

### 5.3.1 Chromatograms of the ORF 30 fragments of the selected EHV-1 reference strains

The sequencing chromatograms of the ORF 30 amplicons of selected EHV-1 reference strains (see Fig. 7, 8, 9) show evenly-spaced peaks. The peak heights vary, but this is normal up to 3-fold. Baseline peaks, so-called noise, is isolated present, but minimal. The sequences are evaluated.
Only for reference strain Ab4 a double peak with different colour at position 2258 was seen (see Fig. 7). This was confirmed in the second sequenced chromatogram. The dominant peak was C 2258 (blue) and the smaller peak was A 2258 (green). In the sequence only C 2258 was named. A polymorphism at the nucleotide position 2258 was assumed, the nucleotide exchange at position 2258 means an exchange of the amino acid serine (5‘-TCC-3‘/ S 753) to tyrosine (5‘-TAC-3‘ / Y 753).
All chromatograms, examined with the program 4peaks (version 1.7.2), are clean and evaluated.

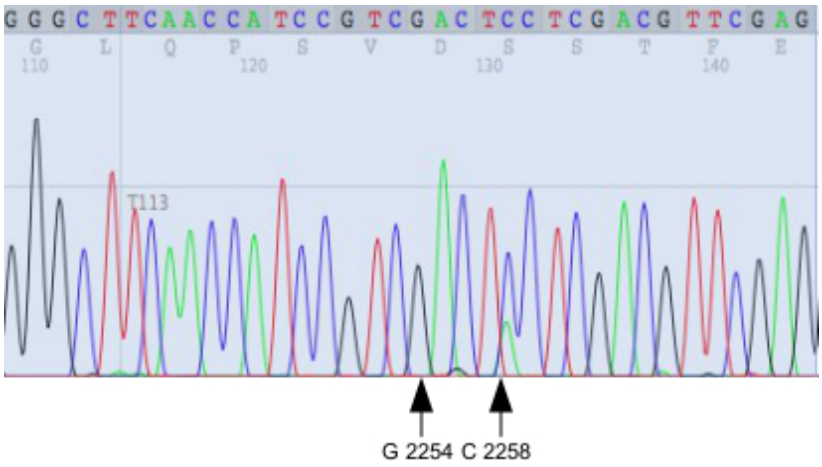


Figure 7: Chromatogram of Ab4, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

## 5.4 Occurrence of the non-neuropathogenic versus the neuropathogenic genotype in EHV-1 reference strains and wild animal and cattle strains

The aim was to screen known EHV-1, wild animal and cattle strains by ORF 30 nested PCR, restriction enzyme analysis *Sal* I and sequencing. The aim of the sequencing of the ORF 30 nested PCR fragments was to verify the results of the *Sal* I restriction enzyme analysis. The received sequences were subsequently translated with the program MacVector into the corresponding amino

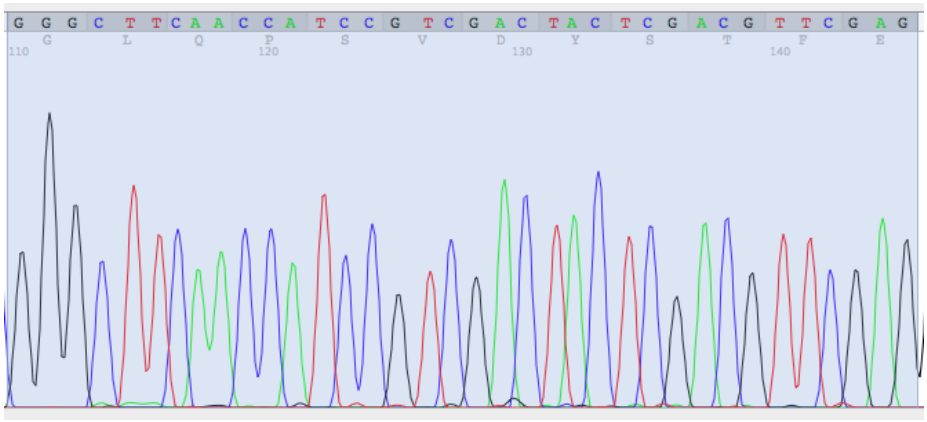


Figure 8: Chromatogram of AIV, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

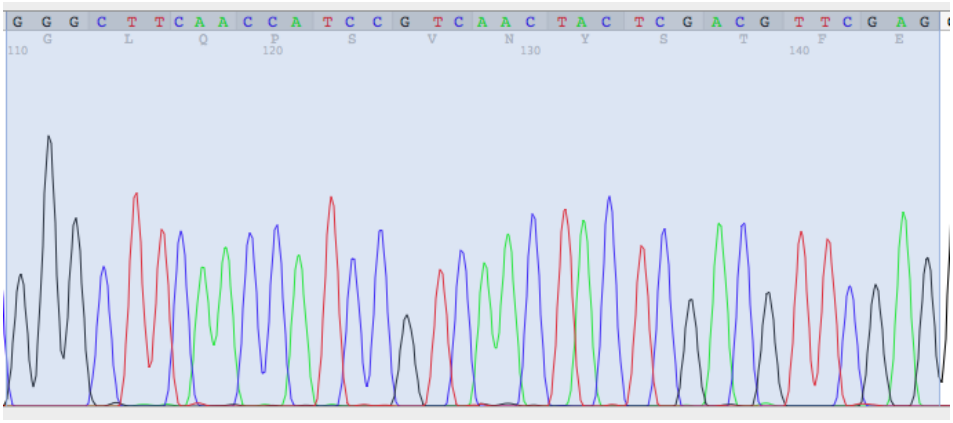


Figure 9: Chromatogram of Mar87, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

acid sequence in order to have a double check.

### 5.4.1 EHV-1 reference strains

**ORF 30 nested PCR** EHV-1 strains Ab4, AIV and Mar87 were already described, see 5.1.1. Army 183 was isolated from an abortion case (Jones at al., 1948), and in an infection experiment the horses developed neurological signs. A 254 bp amplicon resulted in the ORF 30 nested PCR. Kentucky D was from an abortion case (Doll et al., 1954) and positive in the ORF 30 nested PCR. Abortion strain RacH (Mayr et al., 1965) was positive in the ORF 30 nested PCR.

**Restriction enzyme analysis *Sal* I** EHV-1 reference strains Ab4, AIV and Mar87 were described, see 5.2. Army 183 was positive in the *Sal* I restriction enzyme analysis and should be defined as neuropathogenic EHV-1 strain with abortigenic potential.
Kentucky D was positive in the restriction enzyme analysis of the ORF 30 nested PCR fragment. This indicated that Kentucky D might be a neuropathogenic strain with abortigenic potential.
The 254 bp ORF 30 amplicon from the abortion strain RacH was found to be cut into two fragments and therefore defined to have the neuropathogenic genotype.

**Sequencing** The EHV-1 reference strains Ab4, AIV and Mar87 confirmed the results of the *Sal* I restriction enzyme analysis and were described in section 5.3.
The EHV-1 strains Army 183 and Kentucky D both contained the neuropathogenic genotype $G_{2254}$. In abortion strain RacH, Nugents et al.'s (2006) finding on the neuropathogenic genotype $G_{2254}$ was confirmed (see Tab. 17).
The sequences were translated with the program MacVector in the corresponding amino acid sequence. Thus, nucleotide exchange at position 2254 from adenine to guanine resulted in an amino acid change. Asparagine (N 752 / 5'-AAC-3') was exchanged by aspartic acid (D 752 / 5'-GAC-3').

Table 17: EHV-1 reference strains: Excerpt of the sequence alignment of equid herpes virus ORF 30 nested PCR amplicons (second round). blue=$A_{2254}$, red=$G_{2254}$, green=$A_{2258}$, magenta=$C_{2258}$

| Isolate | 3'- 2271 | Sequence | ← direction | 2236 - 5´ |
|---|---|---|---|---|
| Ab4 | 3'-GAG CTT GCA GCT CCT CAG CTG CCT ACC AAC TTC GGG-5' | | | |
| AIV | 3'-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5' | | | |
| Mar87 | 3'-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5' | | | |
| Army 183 | 3'-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5' | | | |
| Kentucky D | 3'-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5' | | | |
| RacH | 3'-GAG CTT GCA GCT CCT CAG CTG CCT ACC AAC TTC GGG-5' | | | |

**Additional mutations in the ORF 30 fragments of EHV-1 reference strains** The sequencing of the ORF 30 amplicons discovered an additional mutation at nt position 2258. The nucleotide exchange $A_{2258}$ (adenine) to $C_{2258}$ (cytosine) resulted in the amino acid exchange tyrosine (Y 753, 5'-TAC-3') to serine (S 753, 5'-TCC-3') - this translation of the received sequences in the corresponding amino acid sequences was done using the program MacVector.

In the EHV-1 strains Ab4 (see Fig. 7) and RacH an additional exchange from adenine to cytosine at nucleotide position 2258 ($C_{2258}$) was confirmed (see Tab. 17).

### 5.4.2 Wild animals and cattle strains

**ORF 30 nested PCR** Ro-1 came from a captive blackbuck with neurological signs. The ORF 30 nested PCR succeeded.
In the ORF 30 nested PCR of the isolate (94-137) from a gazelle with neurological signs a 254 bp amplicon resulted.
Isolate 49800 came from a gazelle with encephalitis and was classified as EHV-9. In the ORF 30 nested PCR, a 254 bp amplicon was demonstrated.
The next three wild equid isolates (T 965, T 529, T 616) all originated from abortion. All three isolates were positive in the ORF 30 nested PCR.

Two strains (136 A and 136 B) from cattle with abortions were positive in the ORF 30 nested PCR, since, for both a 254 bp amplicon resulted.

**Restriction enzyme analysis *Sal* I** The restriction enzyme analysis *Sal* I of the ORF 30 nested PCR fragment from Ro-1 confirmed the neuropathogenic genotype.
For 94-137 the neuropathogenic genotype of the strain was confirmed.
49800 was positive in the restriction enzyme analysis with *Sal* I since two bands (162, 92 bp) were revealed and the isolate was classified as neuropathogenic.
The next three wild animal isolates (T 965, T 529, T 616) all originated from different abortion cases. The 254 bp ORF 30 amplicon of all three isolates showed after *Sal* I digestion two fragments at the heights 162 and 92 bp. All three abortigenic strains also had neuropathogenic potential.

The two strains (136 A and 136 B) from cattle with abortions were classified to carry the neuropathogenic genotype by the restriction enzyme analysis *Sal* I.

**Sequencing** The results of the *Sal* I restriction enzyme analysis was confirmed by the results of sequencing. The sequencing of the ORF 30 nested PCR amplicon of three isolates (Ro-1, 94-137, 49800) from animals with neurological signs as well the three isolates (T 965, T 529, T 616) from the wild equids with abortions, validated the existence of the neuropathogenic genotype $G_{2254}$ in wild equids (see Tab. 18).
Both ORF 30 amplicons from cattle (136 A and 136 B) contained the neuropathogenic genotype $G_{2254}$ (see Tab. 18).
A translation of the received sequences in the corresponding amino acid sequences was done with the program MacVector. The nucleotide exchange at position 2254 from adenine to guanine resulted in the exchange of amino acid asparagine to aspartic acid.
All wild equid isolates were separately tested in a second approach. A second sequencing was performed with these amplicons and they showed the same results.

Table 18: Wild animals and cattle: Excerpt of the sequence alignment of equid herpes virus ORF 30 nested PCR amplicons (second round) in comparison with three EHV-1 reference strains. blue=$A_{2254}$, red=$G_{2254}$, green=$A_{2258}$, magenta=$C_{2258}$, underlines represent unique substitutions.

| Isolate | 3‘- 2271 Sequence ← direction 2236 - 5´ |
|---|---|
| Ab4 | 3‘-GAG CTT GCA GCT CCT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| AIV | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| Mar87 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| Ro-1 | 3‘-GAG CTT GCA ACT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| T 965 | 3‘-GAG CTT GCA ACT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| T 529 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| T 616 | 3‘-GAG CTT GCA ACT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| 94-137 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| 49-800 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| 136 A | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| 136 B | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |

**Additional mutations in the ORF 30 fragments of wild animal and cattle strains** The sequencing of the ORF 30 amplicons of the wild animals uncovered an additional mutation at nt position 2262. The translation of the sequences in the corresponding amino acid sequences was done with the program MacVector and the nucleotide exchange at position 2262 from guanine ($G_{2262}$ / 5‘-TCG-3‘) to adenine ($A_{2262}$ / 5‘-TCA-3‘) did not result in the exchange of the amino acid serine (S 754).

The nucleotide change at position 2262 was found in the following strains: Ro-1, T 965 and T 616 (see Tab. 18). All of them also have the neuropathogenic genotype $G_{2254}$.

No other exchanges were found by the sequencing of the ORF 30 nested PCR amplicons including the EHV-9 isolate 94-137.

**Chromatograms of the ORF 30 fragments of the selected wild animal strains** The sequencing chromatograms of the ORF 30 amplicons of selected wild animal strains show evenly-spaced peaks (see Fig. 11 and Fig. 10). The peak heights vary, but this is normal up to 3-fold. Baseline noise is isolated present. The sequences are evaluated.

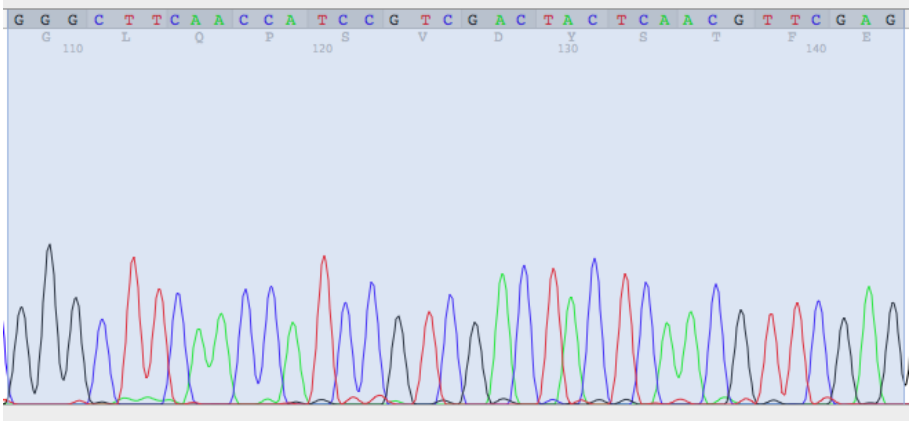


Figure 10: Chromatogram of Ro-1, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

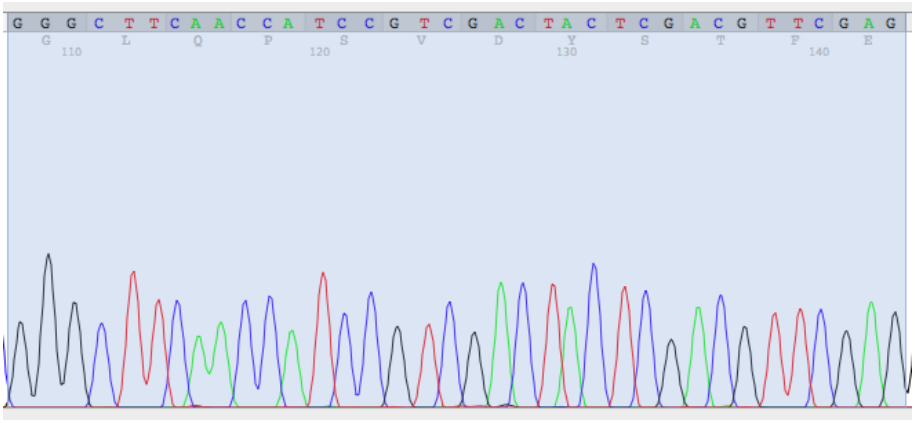

Figure 11: Chromatogram of T529, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

## 5.5 Occurrence of the neuropathogenic EHV-1 genotype in CNS cases

The ORF 30 nested PCR and subsequent *Sal* I REA were performed on viral DNA extracted from three tissue samples and one viral isolate from two horses (see Tab. 19). The isolate 834 originated from a horse with paresis in 2006. The three tissue samples from case 3318 with central nervous system symptoms were collected in 2009.

Table 19: Overview of the investigated CNS cases

| Identification | Genotype | Pathotype | Tissue | Processed sample |
|---|---|---|---|---|
| 834 | EHV-1 | neurologic | isolate | DNA |
| 3318/1 | no result | neurologic | spinal cord | no result |
| 3318 /2 | EHV-1 | neurologic | brain | DNA |
| 3318 /3 | EHV-1 | neurologic | organ mix | DNA |

No amplicon was obtained from the ORF 30 nested PCR for the isolate 3318/1 using the spinal cord DNA as template. The ORF 30 nested PCR and subsequent *Sal* I digestion revealed the neuropathogenic genotype $G_{2254}$ of the isolates 834, 3318/2 and 3318/3. For the three amplicons a confirmation by sequencing was done (see Tab. 20).

Table 20: Excerpt of sequence alignment of the ORF 30 amplicons from CNS isolates in comparison to selected reference strains. blue=$A_{2254}$, red=$G_{2254}$, green=$A_{2258}$, magenta=$C_{2258}$. Substitutions unique indicated underlined.

| Isolate | 3‘- 2271 | Sequence | ← direction | 2236 - 5´ |
|---|---|---|---|---|
| Ab4 | 3‘-GAG CTT GCA GCT CCT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| AIV | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| Mar87 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ | | | |
| 834 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| 3318/2 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| 3318/3 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |

The sequencing discovered no additional mutations in the 254 bp ORF 30 amplicon of the

three sequenced amplicons from the neurological cases (see Tab. 20).
The sequencing chromatograms of the ORF 30 amplicons from the neurological cases show evenly-spaced peaks with normal baseline noise (see Fig. 12).

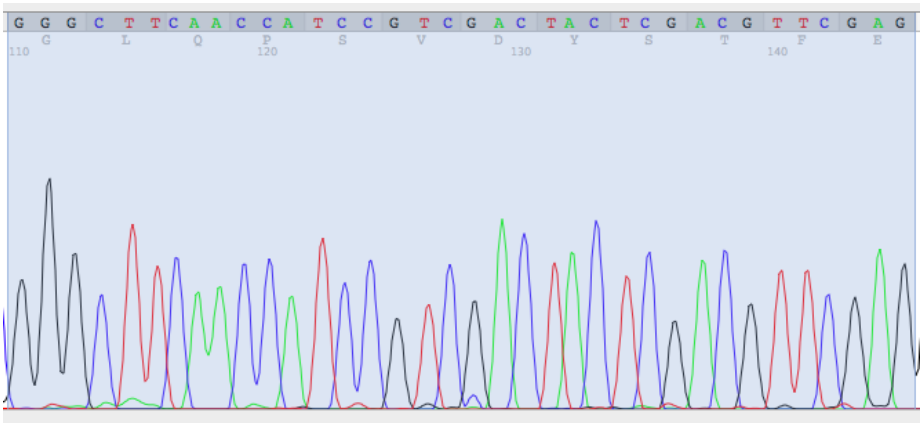

Figure 12: Chromatogram of 834, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

## 5.6 Sample character of the EHV-1 abortion cases

The abortion samples of 67 abortion cases (38 single abortion cases and 29 abortions from four abortion outbreaks) were composed of 64 viral isolates, one tissue sample (Mare A, placenta), two lung fluid samples, two nasal swabs and one amniotic fluid (see Table in Annex 10.2.1). In 2009, from two abortion cases (abortion outbreak 3, Mare I and Mare II), several samples (two lung fluid samples, two nasal swabs and one amniotic fluid, one semen) were received as already mentioned (see Table in Annex 10.2.1). The results of all five samples will be discussed, in evaluation, only the number of abortion cases was counted.
For the DNA preparation, except the placenta of Mare A, the RTP DNA/RNA Virus Mini Kit was used. The tissue sample of the placenta from Mare A (stud farm 4) went after proteinase K digestion in the ORF 30 nested PCR.

## 5.7 Occurrence of the non-neuropathogenic versus the neuropathogenic genotype in EHV-1 abortion cases

It was investigated whether the non-neuropathogenic or neuropathogenic EHV-1 genotype occurred in abortions. ORF 30 nested PCR, restriction enzyme analysis *Sal* I and sequencing of the nested ORF 30 amplicon were used.

### 5.7.1 Investigation of abortion strains by ORF 30 nested PCR

The ORF 30 nested PCR positive controls were the EHV-1 strains Ab4, Mar87 and negative control EHV-4 strain KT-4. A substance control was carried in each approach.

The agarose gel electrophoresis picture from four abortion isolates of 2004, and one from 2006, showed the expected heights of the ORF 30 nested PCR amplicons (see Fig. 13).

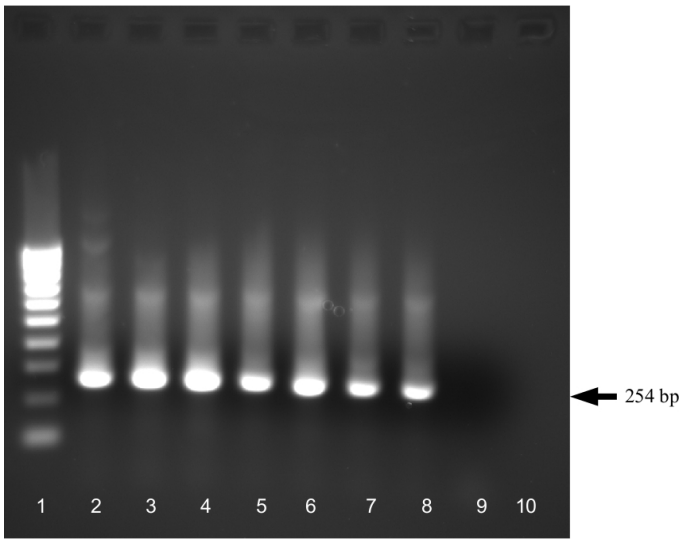


Figure 13: Results of agarose gel electrophoresis of ORF 30 nested PCR amplicons: ORF 30 reference strains and abortion isolates of the years 2004 and 2006

Key:
1. 100 bp DNA ladder (HyperladderIV)
2. Ab4, positive ORF 30 control strain
3. S138/04 → positive
4. S155/04 → positive
5. S415/04 → positive
6. S587/04 → positive
7. S65/06 → positive
8. Mar87, positive ORF 30 control strain
9. KT-4, negative ORF 30 control strain
10. substance control

| Number of ORF 30 nested PCR positive EHV-1 abortion cases: | | | | | |
|---|---|---|---|---|---|
| Year | Single abortion cases | Abortion outbreak stud farm 1 | Abortion outbreak stud farm 2 | Abortion outbreak stud farm 3 | Abortion outbreak stud farm 4 |
| 1987 | 1 | 0 | 0 | 0 | 0 |
| 1989 | 2 | 0 | 0 | 0 | 0 |
| 1990 | 2 | 0 | 0 | 0 | 0 |
| 1991 | 2 | 0 | 0 | 0 | 0 |
| 2003 | 4 | 0 | 0 | 0 | 0 |
| 2004 | 6 | 0 | 0 | 0 | 0 |
| 2006 | 7 | 0 | 0 | 0 | 0 |
| 2007 | 4 | 18 | 0 | 0 | 0 |
| 2008 | 1 | 0 | 6 | 0 | 0 |
| 2009 | 9 | 0 | 0 | 2 | 3 |
| total | 38 | 18 | 6 | 2 | 3 |

Table 21: Results by the ORF 30 nested PCR investigated EHV-1 abortion cases from 38 single abortion cases and 29 abortion cases from four abortion outbreaks from 1987 to 2009. They were sent in for investigation to the Institute of Virology, Berlin, Germany.

All the isolated DNA samples from 67 abortion cases were positive in the ORF 30 nested PCR (see Tab. 21). The details are printed in the annex (see section 10.2.1).

With regard to the abortion outbreak at stud farm 3 in 2009, from two abortion cases (Mare I, Mare II) with neurological signs and one stallion, different samples (two lung fluid samples, two nasal swabs, one amniotic fluid and one semen) were received. In all six samples the DNA isolation was successful and the ORF 30 nested PCR was positive for all six (see section 10.2.1).

Additionally, the isolated DNA of the virus isolate P5276 was positive in the ORF 30 nested PCR.

The positive ORF 30 nested PCR amplicons went to the restriction enzyme analysis *Sal* I.

### 5.7.2 Investigation of the ORF 30 amplicons by restriction enzyme analysis *Sal* I

Through the restriction enzyme analysis we could distinguish between neuropathogenic ($G_{2254}$) or non-neuropathogenic ($A_{2254}$) EHV-1 strains. The ORF 30 nested PCR fragment of the non-neuropathogenic strains remained undigested and in the gel electrophoresis a band at 254 bp was seen. The neuropathogenic strains were cleaved into two smaller fragments and in the gel electrophoresis bands at 92 and 162 bp were provable.

As *Sal* I restriction enzyme analysis controls were used as positive control the EHV-1 strain Ab4 and as negative control the EHV-1 strain Mar87.

As an example, the agarose gel electrophoresis picture from the restriction enzyme analysis *Sal* I of the ORF 30 amplicons from abortions in the year 2004 and one from 2006 showed digested (positive/neuropathogenic) and undigested (negative/non-neuropathogenic) ORF 30 fragments

(see Fig. 14).

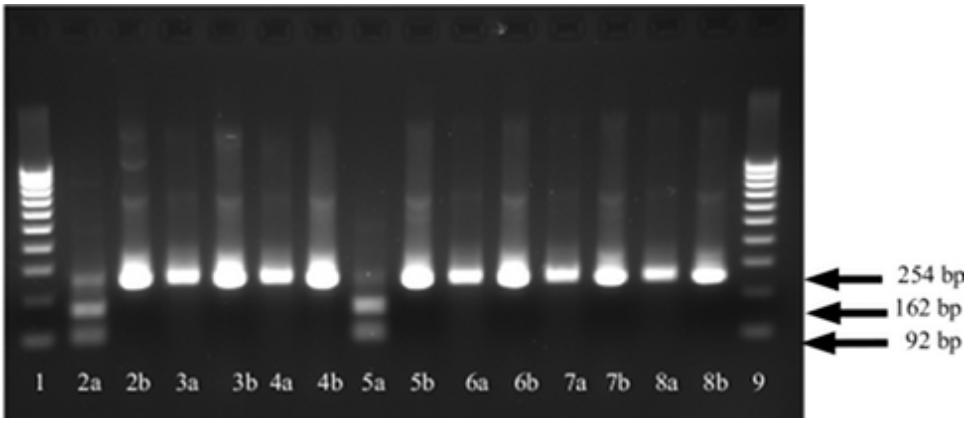


Figure 14: Results of agarose gel electrophoresis of ORF 30 nested PCR amplicons treated with *Sal* I: *Sal* I reference strains and abortion isolates of the years 2004 and 2006

Key:
1. 100 bp DNA ladder (HyperladderIV)
a. ORF 30 nPCR amplicons treated with *Sal* I
b. ORF 30 nPCR amplicons untreated
2. Ab4, positive *Sal* I control strain
3. S138/04 → negative
4. S155/04 → negative
5. S415/04 → positive
6. S587/04 → negative
7. S65/06 → negative
8. Mar87, negative *Sal* I control strain
9. 100 bp DNA ladder (HyperladderIV)

Summarizing, 88.1% (59/67) (see annex 10.2.1) of the abortion cases (single abortion cases and abortions from outbreaks) were classified by restriction enzyme analysis *Sal* I to carry the non-neuropathogenic genotype (N 752/$A_{2254}$). Secondly, 11.9% (8/67) (see annex 10.2.1) of the abortion cases harboured the neuropathogenic genotype (D 752/$G_{2254}$). Six of the eight abortions which expose the neuropathogenic genotype derived from single abortion cases, the remaining two were from the abortion outbreak at stud farm 3 (see Tab. 23).
The number of abortions per year, and the division into neuropathogenic and non-neuropathogenic genotype, is shown in Table 22. Below I refer to the 67 investigated abortion cases and discuss them in more detail. Therefore the following subdivision was taken:

**Single abortion cases** (38 abortion cases)
**Abortion outbreak at stud farm 1** (18 abortion cases)
**Abortion outbreak at stud farm 2** (6 abortion cases)
**Abortion outbreak at stud farm 3** (2 abortion cases)
**Abortion outbreak at stud farm 4** (3 abortion cases)

**Single abortion cases:** Six abortions from single abortion cases were positive in the restriction enzyme analysis *Sal* I of the ORF 30 amplicons and classified as the neuropathogenic genotype. They came from unrelated single abortion cases without neurological signs and occurred in 1987

Table 22: Occurrence of the non-neuropathogenic ($A_{2254}$) and neuropathogenic genotype ($G_{2254}$) associated with abortion. The investigated EHV-1 abortions from 1987 to 2009 were sent for investigation to the Institute of Virology, Berlin, Germany.

| Year | 1987 | 1989 | 1990 | 1991 | 2003 | 2004 | 2006 | 2007 | 2008 | 2009 | total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $A_{2254}$ | 0 | 1 | 1 | 2 | 4 | 3 | 7 | 22 | 7 | 12 | 59 |
| $G_{2254}$ | 1 | 1 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 8 |
| total | 1 | 2 | 2 | 2 | 4 | 6 | 7 | 22 | 7 | 14 | 67 |

Key:
$A_{2254}$ = non-neuropathogenic genotype
$G_{2254}$ = neuropathogenic genotype

Table 23: Occurrence of the non-neuropathogenic ($A_{2254}$) and neuropathogenic genotype ($G_{2254}$) associated with abortion in single abortion cases and abortion outbreaks. The investigated EHV-1 abortions from 1987 to 2009 were sent for investigation to the Institute of Virology, Berlin, Germany.

| Year | 1987 | 1989 | 1990 | 1991 | 2003 | 2004 | 2006 | 2007 | 2008 | 2009 | total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $A_{2254}$ | 0 | 1 | 1 | 2 | 4 | 3 | 7 | 22 | 7 | 12 | 59 |
| SA | 0 | 1 | 1 | 2 | 4 | 3 | 7 | 4 | 1 | 9 | 32 |
| AO 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 18 | 0 | 0 | 18 |
| AO 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 6 |
| AO 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| $G_{2254}$ | 1 | 1 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 8 |
| SA | 1 | 1 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 6 |
| AO 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |

Key:
$A_{2254}$ = non-neuropathogenic genotype
$G_{2254}$ = neuropathogenic genotype
SA = single abortion
AO = abortion outbreak
1 - 4 = studfarm 1 - 4

(T952), 1989 (E525/89), 1990 (F553/89) and three cases in 2004 ((A)E271-3, S415/04, A258). Thirty-two single abortion cases tested negative in the restriction enzyme analysis *Sal* I of the ORF 30 amplicons (see Tab. 24).

Table 24: Overview of the restriction enzyme analysis *Sal* I of the ORF 30 amplicons of the investigated EHV-1 abortion strains from 38 single abortion cases.

| Designation | Year | Origin | ORF 30 nested PCR | REA *Sal* I |
|---|---|---|---|---|
| T952 | 1987 | single case | positive | **positive** |
| E510/89 | 1989 | single case | positive | negative |
| E525/89 | 1989 | single case | positive | **positive** |
| S147/90 | 1990 | single case | positive | negative |

Table 24: Overview of the restriction enzyme analysis *Sal* I of the ORF 30 amplicons of the investigated EHV-1 abortion strains from 38 single abortion cases.

| Designation | Year | Origin | ORF 30 nested PCR | REA *Sal* I |
|---|---|---|---|---|
| F553/89 | 1990 | single case | positive | **positive** |
| KermannNr.1 | 1991 | single case | positive | negative |
| KermannNr.2 | 1991 | single case | positive | negative |
| U169-71 | 2003 | single case | positive | negative |
| 260.3 | 2003 | single case | positive | negative |
| Y30 | 2003 | single case | positive | negative |
| T759/61 | 2003 | single case | positive | negative |
| (A)E271-3 | 2004 | single case | positive | **positive** |
| S138/04 | 2004 | single case | positive | negative |
| S155/04 | 2004 | single case | positive | negative |
| S415/04 | 2004 | single case | positive | **positive** |
| S587/04 | 2004 | single case | positive | negative |
| A258 | 2004 | single case | positive | **positive** |
| E854/6 | 2006 | single case | positive | negative |
| E216-18 | 2006 | single case | positive | negative |
| E573-5 | 2006 | single case | positive | negative |
| E113-115 | 2006 | single case | positive | negative |
| S65/06 | 2006 | single case | positive | negative |
| S173/06 | 2006 | single case | positive | negative |
| S208/06 | 2006 | single case | positive | negative |
| S101/07 | 2007 | single case | positive | negative |
| S144/07 | 2007 | single case | positive | negative |
| S152/07 | 2007 | single case | positive | negative |
| S260/07 | 2007 | single case | positive | negative |
| S261/08 | 2008 | single case | positive | negative |
| S72/09 | 2009 | single case | positive | negative |
| S159/09 | 2009 | single case | positive | negative |
| S238/09 | 2009 | single case | positive | negative |
| S604/09 | 2009 | single case | positive | negative |
| PA284 | 2009 | single case | positive | negative |
| PA332 | 2009 | single case | positive | negative |
| 624 | 2009 | single case | positive | negative |
| 647 | 2009 | single case | positive | negative |
| 681 | 2009 | single case | positive | negative |

**Stud farm 1:** Eighteen abortion cases from the abortion outbreak at stud farm 1 were negative in the restriction enzyme analysis *Sal* I of the ORF 30 amplicons (see Tab. 25).

Table 25: Overview of the restriction enzyme analysis *Sal* I of the ORF 30 amplicons of the investigated EHV-1 abortion strains from the abortion outbreak at stud farm 1.

| Designation | Year | Origin | ORF 30 nested PCR | REA *Sal* I |
|---|---|---|---|---|
| S245/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S275/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S276/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S299/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| V07-5-61 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S309/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S319/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| E1470/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| E1471/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| E1472/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S335/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| E1606/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S384/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| E1730/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S471/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S476/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S477/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |
| S483/07 | 2007 | abortion outbreak stud farm 1 | positive | negative |

**Stud farm 2:** Six abortion cases from the abortion outbreak at stud farm 2 were negative in the restriction enzyme analysis *Sal* I of the ORF 30 amplicons (see Tab. 26).

Table 26: Overview of the restriction enzyme analysis *Sal* I of the ORF 30 amplicons of the investigated EHV-1 abortion strains from the abortion outbreak at stud farm 2.

| Designation | Year | Origin | ORF 30 nested PCR | REA *Sal* I |
|---|---|---|---|---|
| S38/08 | 2008 | abortion outbreak stud farm 2 | positive | negative |
| S45/08 | 2008 | abortion outbreak stud farm 2 | positive | negative |
| S49/08 | 2008 | abortion outbreak stud farm 2 | positive | negative |
| S55/08 | 2008 | abortion outbreak stud farm 2 | positive | negative |
| S59/08 | 2008 | abortion outbreak stud farm 2 | positive | negative |
| S150/08 | 2008 | abortion outbreak stud farm 2 | positive | negative |

**Stud farm 3:** Two abortion cases (Mare I, Mare II) from the abortion outbreak at stud farm 3 with neurological signs prior to abortion were positive in the restriction enzyme analysis *Sal* I of the ORF 30 amplicons. From these two abortion cases (Mare I, Mare II) five samples (V09-3-2, V09-3-12, V09-3-15, V09-3-3, V09-3-23) were investigated and all confirmed the neuropathogenic genotype (see Tab. 27). In evaluation, only the number of abortion cases (2) was counted.
For all of them the isolation of virus in cell culture failed, they were directly investigated by ORF 30 nested PCR.
Additionally, the ORF 30 amplicon of the semen sample (V09-4-1) from stud farm 3 was also tested positive by the restriction enzyme *Sal* I and was classified as neuropathogenic strain (see Tab. 27). However, the semen sample does not count for the evaluation of the abortion cases, it is mentioned here because of the belonging to stud farm 3.

Table 27: Overview of the restriction enzyme analysis *Sal* I of the ORF 30 amplicons from the abortion outbreak at stud farm 3

| Designation | Year | Origin | Sample character | ORF 30 nested PCR | REA *Sal* I |
|---|---|---|---|---|---|
| Mare I | | | | | |
| V09-3-2 | 2009 | abortion outbreak stud farm 3 | nasal swab | positive | **positive** |
| V09-3-12 | 2009 | abortion outbreak stud farm 3 | lung fluid | positive | **positive** |
| V09-3-15 | 2009 | abortion outbreak stud farm 3 | amniotic fluid | positive | **positive** |
| Mare II | | | | | |

Table 27: Overview of the restriction enzyme analysis *Sal* I of the ORF 30 amplicons from the abortion outbreak at stud farm 3

| Designation | Year | Origin | Sample character | ORF 30 nested PCR | REA *Sal* I |
|---|---|---|---|---|---|
| V09-3-3 | 2009 | abortion outbreak stud farm 3 | nasal swab | positive | **positive** |
| V09-3-23 | 2009 | abortion outbreak stud farm 3 | lung fluid | positive | **positive** |
| Stallion | | | | | |
| V09-4-1 | 2009 | abortion outbreak stud farm 3 | semen | positive | **positive** |

**Stud farm 4:** Three abortion cases from the abortion outbreak at stud farm 4 were negative in the restriction enzyme analysis *Sal* I of the ORF 30 amplicons (see Tab. 28). For the placenta of Mare A the isolation of virus in cell culture failed, it was directly investigated by ORF 30 nested PCR.

Table 28: Overview of the restriction enzyme analysis *Sal* I of the ORF 30 amplicons of the investigated EHV-1 abortion strains from the abortion outbreak at stud farm 4.

| Designation | Year | Origin | Sample character | ORF 30 nested PCR | REA *Sal* I |
|---|---|---|---|---|---|
| 470 (Mare C) | 2009 | abortion outbreak stud farm 4 | virus isolate | positive | negative |
| 541 (Mare F) | 2009 | abortion outbreak stud farm 4 | virus isolate | positive | negative |
| Mare A | 2009 | abortion outbreak stud farm 4 | placenta | positive | negative |

**P5276:** Additionally, the ORF 30 amplicon of the virus isolate P5276 was negative in the restriction enzyme analysis *Sal* I and had to be classified as non-neuropathogenic strain. The origin of the isolate P5276 could not be verified. Therefore, it was investigated but not included in the evaluation of the abortion cases.

Subsequently, to confirm the results, a sequencing of selected ORF 30 amplicons was done (see 5.7.3)

### 5.7.3 Sequencing of selected ORF 30 amplicons

**Non-neuropathogenic genotypes from abortion cases:** 12 ORF 30 amplicons were chosen for sequencing (see Tab. 29), they were negative in the restriction enzyme analysis. The selection took into account the year and both single cases and abortion outbreaks.

Single abortion cases:

The sequencing of four ORF 30 amplicons from four single abortion cases of the years 2004 (S587/04), 2006 (S173/06, E113/115) and 2008 (S261/08) confirmed the non-neuropathogenic genotype ($A_{2254}$).

Abortion outbreaks:

The ORF 30 amplicons from three abortions (S275/07, S309/07 and S471/07), abortion outbreak at stud farm 1 (2007), were sequenced and confirmed the non-neuropathogenic genotype ($A_{2254}$). The abortions S45/08 and S150/08 occurred at stud farm 2 (abortion outbreak in 2008). The sequencing of the ORF 30 amplicons from both confirmed the non-neuropathogenic genotype.

Three ORF 30 amplicons (Mare A, 541/Mare F, 470/Mare C), from the abortion outbreak at stud farm 4 in 2009, were sequenced and confirmed the non-neuropathogenic genotype ($A_{2254}$).

The sequencing chromatograms of the ORF 30 amplicons from abortion cases (see Fig. 15) show evenly-spaced peaks. The peak heights vary in a normal range up to 3-fold. Baseline peaks, so-called noise, is isolated present. The sequences are evaluated.

Table 29: Excerpt of sequence alignment of the ORF 30 amplicons from non-neuropathogenic isolates of abortion cases. blue=$A_{2254}$, red=$G_{2254}$, green=$A_{2258}$, magenta=$C_{2258}$.

| Isolate | Origin | 3‘- 2271 Sequence ← direction 2236 - 5´ |
|---|---|---|
| S587/04 | SA | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| S173/06 | SA | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| E113/115 | SA | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| S275/07 | AO 1 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| S309/07 | AO 1 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| S471/07 | AO 1 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| S45/08 | AO 2 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| S150/08 | AO 2 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| S261/08 | SA | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC CCA TTC GGG-5‘ |
| Mare A | AO 4 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| 541/Mare F | AO 4 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| 470/Mare C | AO 4 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ |
| Key: | | |
| | SA | single abortion |
| | AO 1 | abortion outbreak at stud farm 1 |
| | AO 2 | abortion outbreak at stud farm 2 |
| | AO 4 | abortion outbreak at stud farm 4 |

**Neuropathogenic isolates from abortion cases:** Eight abortion cases were tested positive for the neuropathogenic genotype by restriction enzyme analysis *Sal* I and showed a digestion of the ORF 30 amplicons. They were chosen for sequencing (see Tab. 30).

Single abortion cases:

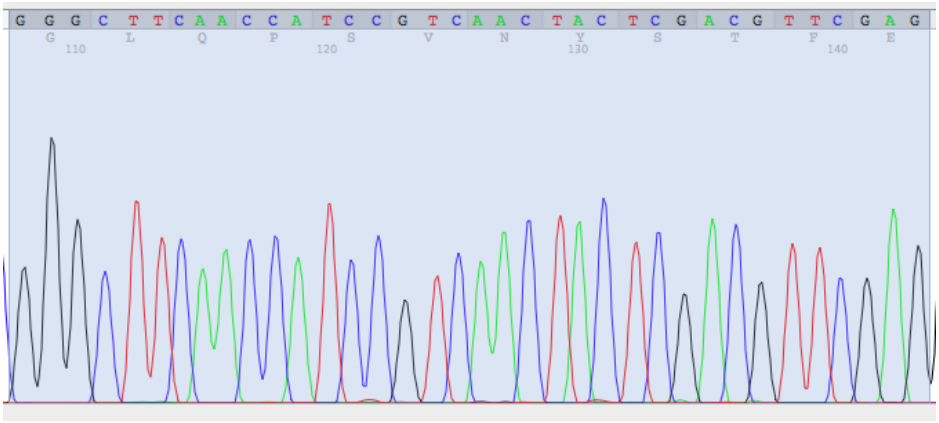

Figure 15: Chromatogram of S45/08, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

The sequencing of six ORF 30 amplicons from six single abortion cases of the years 1987 (T952), 1989 (E525/89), 1990 (F553/89) and 2004 ((A)E271-3, A 258, S415/04) confirmed the neuropathogenic genotype ($G_{2254}$).

Abortion outbreak:

In 2009 at stud farm 3, two mares (Mare I, Mare II) aborted and developed neurological signs prior to the abortion. In both abortion cases the neuropathogenic genotype was confirmed by sequencing (see Tab. 30).

From these two abortion cases (Mare I, Mare II) overall five samples (two nasal swabs, two lung fluids, one amniotic fluid) were investigated by ORF 30 nested PCR and sequenced. All results correlated (see Tab. 31, Fig. 16, Fig. 17) and confirmed the neuropathogenic genotype ($G_{2254}$). Additionally, the semen (V09-4-1) from a symptom-free stallion from the same stud was investigated (see Fig. 18) and was found to contain the neuropathogenic genotype $G_{2254}$ as well.

Table 30: Excerpt of sequence alignment of the ORF 30 amplicons from neuropathogenic isolates of abortion cases. blue=$A_{2254}$, red=$G_{2254}$, green=$A_{2258}$, magenta=$C_{2258}$, underlines marks unique substitutions.

| Isolate | Origin | 3‘- 2271 Sequence ← direction 2236 - 5´ |
|---|---|---|
| T 952 | SA | 3‘-GAG CTT GCA GCT CCT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| E 525/89 | SA | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| F 553/89 | SA | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| S415/04 | SA | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| A(E)271-3 | SA | 3‘-GAA CTT GCA GCT CAT CAG GTC CCT ACC AAC TTC GGG-5‘ |
| A 258 | SA | 3‘-GAA CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| MareI | AO 3 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| MareII | AO 3 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ |
| Key: | | |
| | SA | single abortion |
| | AO 3 | abortion outbreak at stud farm 3 |

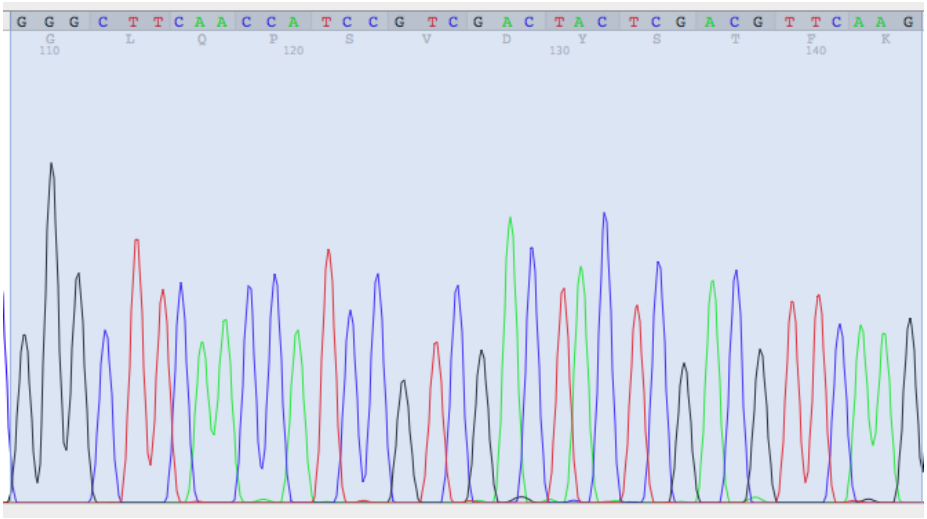

Figure 16: Chromatogram of A(E)271-3, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

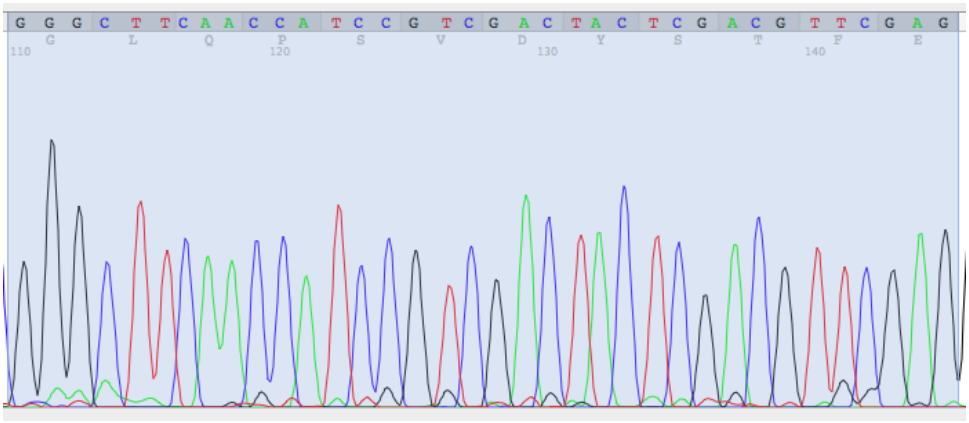

Figure 17: Chromatogram of Mare I (V09-3-2), 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

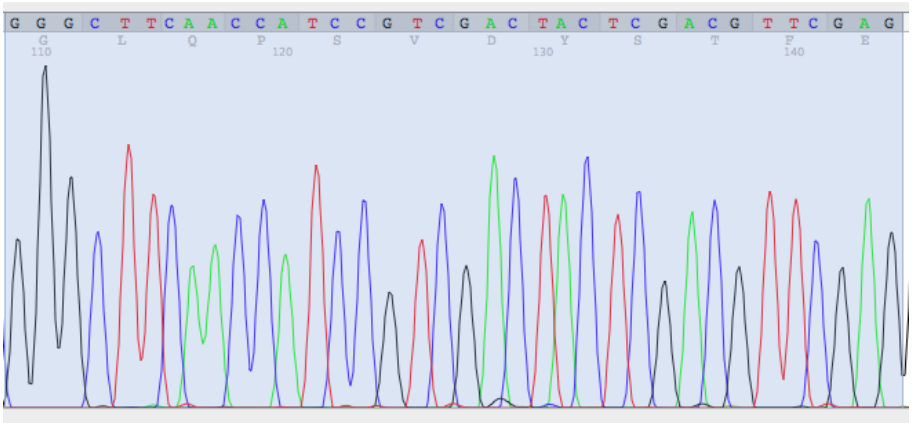

Figure 18: Chromatogram of V09-4-1 (semen sample from a stallion from stud farm 3), 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

Table 31: Excerpt of sequence alignment of the ORF 30 amplicons from stud farm 3 with abortions and neurological signs in 2009. From each abortion case different samples were investigated. blue=$A_{2254}$, red=$G_{2254}$, green=$A_{2258}$, magenta=$C_{2258}$. Substitutions unique indicated underlined.

| | 3‘- 2271 | Sequence | ← direction | 2236 - 5´ |
|---|---|---|---|---|
| Mare I: | | | | |
| V09-3-2 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| V09-3-12 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| V09-3-15 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| Mare II: | | | | |
| V09-3-3 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| V09-3-23 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |
| Stallion: | | | | |
| V09-4-1 | 3‘-GAG CTT GCA GCT CAT CAG CTG CCT ACC AAC TTC GGG-5‘ | | | |

Summarizing, the sequencing confirmed the *Sal* I results. For eight abortions the neuropathogenic genotype ($G_{2254}$) was confirmed and for 12 abortions the non-neuropathogenic genotype ($A_{2254}$) was proven (see Annex 10.2.2). The sequences were translated with the program MacVector in the corresponding amino acid sequence. Therefore, the nucleotide exchange at position 2254 was found to result in the exchange of the amino acid asparagine to aspartic acid.

With regard to the amplicon of strain **P5276** (see 4.4.1), the sequencing confirmed the non-neuropathogenic genotype $A_{2254}$ and the sequencing chromatogram show evenly-spaced peaks and minimal baseline peaks (see Fig. 19).

| Isolate | 3‘- 2271 | Sequence | ← direction | 2236 - 5´ |
|---|---|---|---|---|
| P5276 | 3‘-GAG CTT GCA GCT CAT CAA CTG CCT ACC AAC TTC GGG-5‘ | | | |

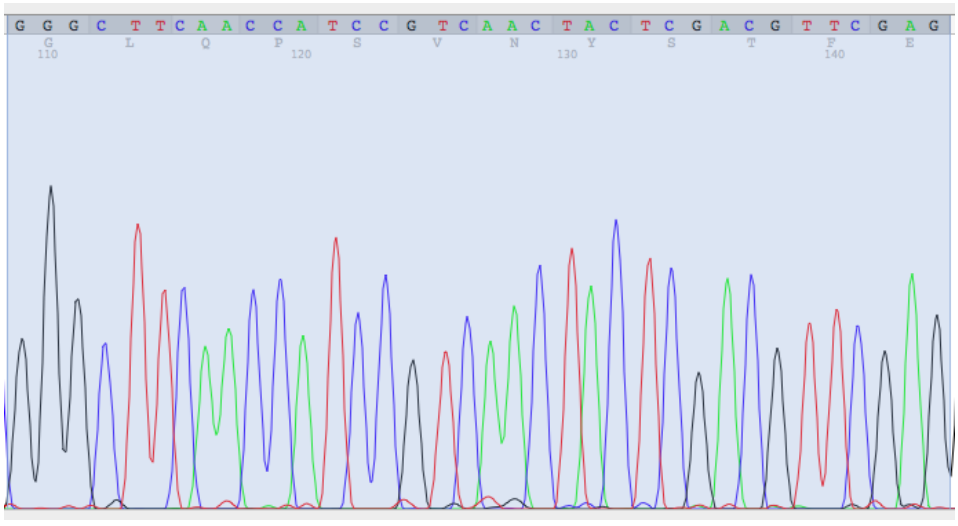


Figure 19: Chromatogram of P5276, 5‘-2236 to 2271-3‘ ORF (open reading frame) 30.

### 5.7.4 Additional mutation in the ORF 30 amplicons from abortion cases

**Nucleotide exchange at nt position 2258** Only the ORF 30 amplicon from the abortion isolate T952 from a single abortion in the year 1987 had at nucleotide position 2258 cytosine ($C_{2258}$) in contrast to $A_{2258}$ (see Tab. 30). The nucleotide exchange from adenine to cytosine at position 2258 led to an exchange of the amino acid tyrosine to serine.
In the ORF 30 amplicon of isolate T952, the neuropathogenic genotype $G_{2254}$ was also found.

**Nucleotide exchange at nt position 2269** Two amplicons (A(E) 2271-3, A 258) from abortion isolates in 2004 revealed at nt position 2269 adenine (A). The other amplicons had at this position guanine ($G_{2269}$) (see Tab. 30). The exchange of guanine with adenine at nucleotide position 2269 led to an amino acid exchange. The amino acid glutamic acid was exchanged by lysine caused by the nucleotide change ($G_{2269}$ to $A_{2269}$). In both amplicons the occurrence of the neuropathogenic genotype was proven ($G_{2254}$). The sequencing chromatogram show evenly-spaced peaks and minimal baseline peaks (see Fig. 16).

### 5.7.5 Stud farm 4: Detection of viral DNA in PBMC and determination of the genotype

PBMCs were isolated (see 4.7) from two blood collection dates (see 4.4.5 and 4.4.6). DNA was isolated out of the PBMCs (see 4.8.1) and was additionally investigated by ORF 30 nested PCR. At the first sampling only Mare A was tested positive in the ORF 30 nested PCR, all others tested negative. The samples four weeks later showed a negative result for Mare A, but Mare E was positive (see Tab. 32).

Table 32: Stud farm 4: ORF 30 nested PCR results of DNA isolated from PBMC

| Mare | Anamnesis | Sample | nPCR ORF 30 15.07.09 | nPCR ORF 30 07.09.09 |
|---|---|---|---|---|
| A | abortion | PBMC | **positive** | negative |
| B | abortion | PBMC | negative | negative |
| C | abortion | PBMC | negative | negative |
| D | abortion | PBMC | negative | negative |
| E | abortion | PBMC | negative | **positive** |
| F | abortion | PBMC | negative | negative |
| G | normal delivery | PBMC | negative | n.d. |
| H | normal delivery | PBMC | negative | n.d. |
| I | normal delivery | PBMC | negative | n.d. |
| J | normal delivery | PBMC | negative | n.d. |
| K | normal delivery | PBMC | negative | n.d. |
| L | normal delivery | PBMC | negative | n.d. |
| M | normal delivery | PBMC | negative | n.d. |
| Key: | | | | |
| n.d. | not done | | | |

The two amplicons from Mare A and E were investigated with restriction enzyme analysis *Sal* I and typed as non-neuropathogenic genotype. The sequencing and chromatogram of the nested

amplicon from Mare A confirmed this result (see Tab. 33 and fig. 20).

Table 33: Restriction enzyme analysis *Sal* I and sequencing results of PBMC of an abortion outbreak of stud farm 4

| Mare | Anamnesis | Sample | nPCR ORF 30 positive | REA *Sal* I | Sequencing |
|---|---|---|---|---|---|
| A | abortion | PBMC | 15.07.09 | negative | A $_{2254}$ A $_{2258}$ |
| E | abortion | PBMC | 07.09.09 | negative | n.d. |

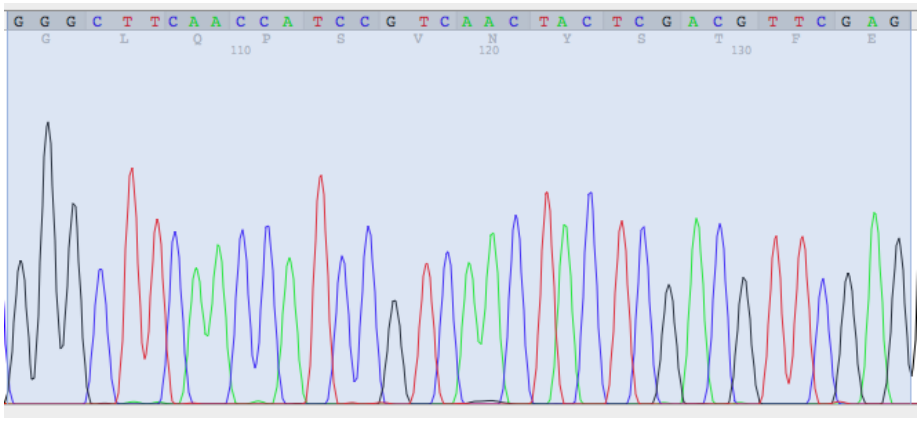


Figure 20: Chromatogram of MareAPBMC (stud farm 4), 5'-2236 to 2271-3' ORF (open reading frame) 30.

## 5.8 Serological studies

On stud farm 3 and stud farm 4, in addition to abortion material, blood samples of horses with and without clinical signs like fever, neurological signs or abortions were taken after an interval of several weeks. The neutralization test (NT) and/or the immunofluorescence assay (IFA) were performed. At the Institute of Virology, Berlin, Germany titer limits NT over 1:20 and IFA over 1:1280 were applied as noticeable. A titer increase at intervals of three to four weeks by a factor of four or more was considered as noticeable.

### 5.8.1 Stud farm 3 with abortions and neurological signs

On stud farm 3 blood samples of horses were taken after an interval of three and a half weeks. The horses were divided according to clinical signs into three different groups, horses with neurological signs, acute febrile horses for less than two days and horses without fever for at least three days.

**Neutralization test**

The results of the neutralization test are presented in Table 34 and Figure 21.

In **group 1**, all four horses showed neurological signs and two mares (Mare I/V09-3-2, Mare II/V09-3-3) also suffered a miscarriage. The initial NT titers of mare I (V09-3-2) were elevated, the titer from the second sampling remained for EHV-1, however, the EHV-4 titer rose. In the

first blood sampling, mare II (V09-3-3) showed no elevation in titer and only a slight increase was noted in the second sampling. However, the titer increases in mare II are more pronounced than in mare I. The sampling of a gelding (V09-3-4) revealed a titer increase. From one mare (V09-4-3) only the second blood sample was received, therefore, a titer increase could not be measured. The neutralization titer for both viruses (EHV-1 and EHV-4) was 1:20.

In **group 2**, the four horses had acute fever for less than two days. Three stallions (V09-3-5, V09-3-6, V09-3-7) had at the first sampling a low titer and also showed no strong titer increase. The pregnant mare V09-3-11 had a low titer, but a titer increase for both viruses. The 8-fold EHV-1 NT titer increase was striking. It was treated with acyclovir to prevent an abortion.

In **group 3**, three horses were summarized, which were at least three days without fever. The gelding V09-3-8 had a low titer and no titer increase. Mare V09-3-9 presented a low titer and only a slight titer increase in comparison to mare V09-3-10 with elevated titers in the beginning and a strong increase.

A stronger antibody response was generally observed in vaccinated horses (see Tab. 34).

Table 34: Stud farm 3: EHV-1 and EHV-4 neutralization test (NT) results from horses of a stud farm with abortions and neurological signs

| Horse | Gender | Signs | Vaccination | EHV-1 NT | | EHV-4 NT | |
|---|---|---|---|---|---|---|---|
| | | | | 08.03.09 | 03.04.09 | 08.03.09 | 03.04.09 |
| Group 1: | Horses with neurological signs. | | | | | | |
| V09-3-2 | mare I | abortion and CNS | Duvaxyn | 1:40 | 1:40 | 1:20 | 1:40 |
| V09-3-3 | mare II | abortion and CNS | Duvaxyn | 1:20 | 1:40 | 1:10 | 1:40 |
| V09-3-4 | gelding | CNS | Resequin | 1:10 | 1:40 | 1:10 | 1:80 |
| V09-4-3 | mare | CNS | Duvaxyn | no serum | 1:20 | no serum | 1:20 |
| Group 2: | Acute febrile horses for less than 2 days. | | | | | | |
| V09-3-5 | stallion | fever | none | ⟨1:10 | 1:20 | ⟨1:10 | 1:20 |
| V09-3-6 | stallion | fever | none | ⟨1:10 | 1:10 | ⟨1:10 | 1:10 |
| V09-3-7 | stallion | fever | none | ⟨1:10 | 1:10 | ⟨1:10 | 1:10 |
| V09-3-11 | pregnant mare | fever | Duvaxyn | ⟨1:10 | 1:80 | ⟨1:10 | 1:40 |
| Group 3: | Horses for at least 3 days without fever. | | | | | | |
| V09-3-8 | gelding | fever | none | 1:10 | 1:10 | 1:10 | 1:10 |
| V09-3-9 | mare | fever | Duvaxyn | 1:10 | 1:40 | ⟨1:10 | 1:20 |
| V09-3-10 | mare | fever | Duvaxyn | 1:20 | 1:40 | 1:40 | 1:80 |

**Immunofluorescence assay**

The results of the immunofluorescence assay are presented in Table 35 and Figure 22.

**Group 1** with neurological signs prior to two abortions: The EHV-1 immunofluorescence titer from mare V09-3-2 was elevated and an increase was detectable. In comparison the first titer from mare V09-3-3 was higher, but no increase was seen. The gelding V09-3-4 showed an elevated titer and an 8-fold titer increase was recognized. From mare V09-4-3 only the second blood sample was

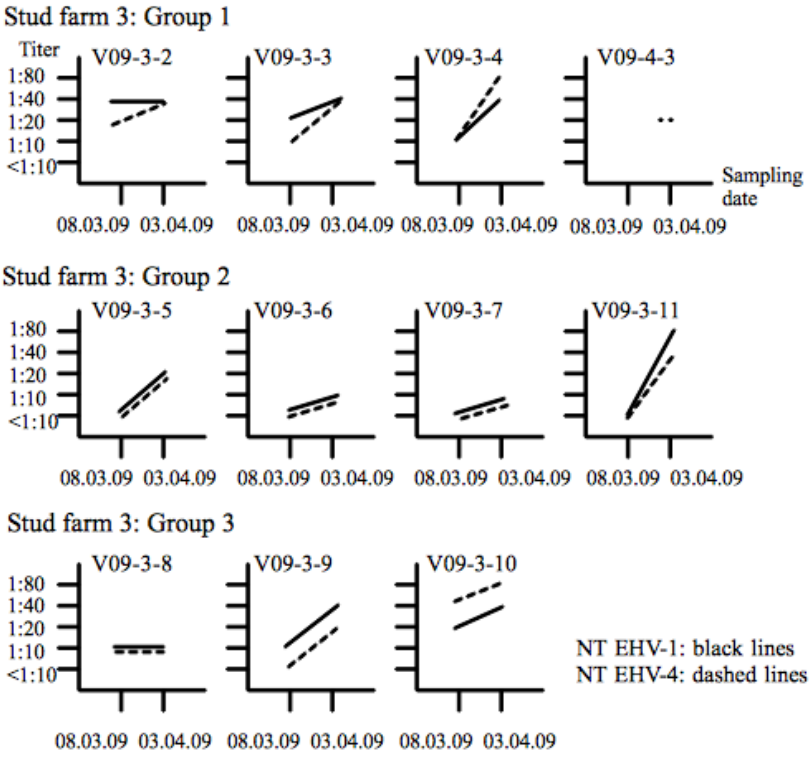


Figure 21: Neutralizing titers of EHV-1 and EHV-4 from the horses of stud farm 3.

received, both IFA titers (EHV-1 and EHV-4) were elevated.

**Group 2** with acute febrile horses: An elevated titer was recognized for the horses V09-3-5, V09-3-6 and V09-3-11. A 4-fold titer increase was seen in all samples (V09-3-5, V09-3-6, V09-3-7 and V09-3-11).

**Group 3** with horses without fever for at least three days: Elevated titers were seen for three horses (V09-3-8, V09-3-9, V09-3-10). The 4-fold titer increase was seen in two cases: V09-3-8, V09-3-9. A 2-fold titer increase was seen for the mare V09-3-10.

In the immunofluorescence assay, no clear difference between vaccinated and unvaccinated horses was found.

Table 35: Stud farm 3: EHV-1 and EHV-4 immunofluorescence assay (IFA) from horses of a stud farm with abortions and neurological signs

| Horse | Gender | Signs | Vaccination | EHV-1 IFA | | EHV-4 IFA | |
|---|---|---|---|---|---|---|---|
| | | | | 08.03.09 | 03.04.09 | 08.03.09 | 03.04.09 |
| Group 1: | Horses with neurological signs. | | | | | | |
| V09-3-2 | mare I | abortion and CNS | Duvaxyn | 1:10240 | 1:20480 | 1:10240 | 1:20480 |
| V09-3-3 | mare II | abortion and CNS | Duvaxyn | 1:20480 | 1:20480 | 1:20480 | 1:20480 |
| V09-3-4 | gelding | CNS | Resequin | 1:2560 | 1:20480 | 1:2560 | 1:20480 |
| V09-4-3 | mare | CNS | Duvaxyn | no serum | 1:20480 | no serum | 1:20480 |
| Group 2: | Acute febrile horses for less than 2 days. | | | | | | |
| V09-3-5 | stallion | fever | none | 1:5120 | 1:20480 | 1:5120 | 1:20480 |
| V09-3-6 | stallion | fever | none | 1:2560 | 1:20480 | 1:2560 | 1:20480 |
| V09-3-7 | stallion | fever | none | 1:1280 | 1:10240 | 1:1280 | 1:10240 |
| V09-3-11 | pregnant mare | fever | Duvaxyn | 1:5120 | 1:20480 | 1:5120 | 1:20480 |
| Group 3: | Horses for at least 3 days without fever. | | | | | | |
| V09-3-8 | gelding | fever | none | 1:5120 | 1:20480 | 1:5120 | 1:20480 |
| V09-3-9 | mare | fever | Duvaxyn | 1:5120 | 1:20480 | 1:5120 | 1:20480 |
| V09-3-10 | mare | fever | Duvaxyn | 1:10240 | 1:20480 | 1:10240 | 1:20480 |

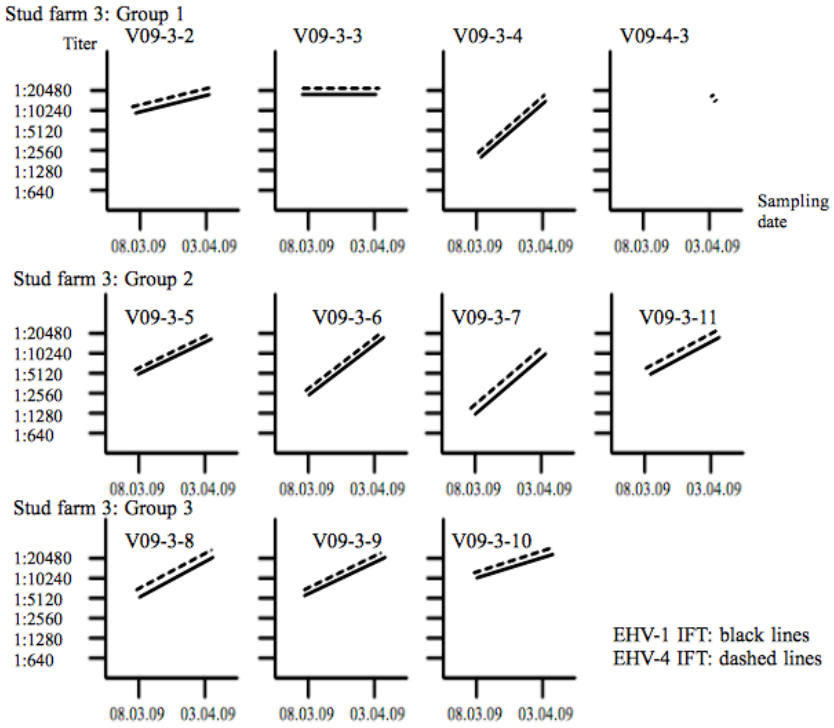


Figure 22: Immunofluorescence titers of EHV-1 and EHV-4 from the horses of stud farm 3.

### 5.8.2 Stud farm 4 with an abortion outbreak

Neutralizing antibodies in paired citrated blood samples at five and a half weeks were investigated from mares with normal delivery and mares that aborted before sampling. The mares were fertilized by artificial insemination or natural mating. In barn 1, all six mares aborted. In contrast, the seven mares in barn 2 had a normal delivery. The type of insemination of mares with abortion or without abortion was distributed approximately equally.
The results were presented in Table 36 and additionally the neutralizing titers of EHV-1 and EHV-4 were plotted for barn 1 (Fig. 23) and barn 2 (Fig. 24).

Table 36: Stud farm 4: EHV-1 and EHV-4 serum neutralization from mares of a stud farm with abortions.

| Mare | Kind of fertilization | EHV-1 neutralization test | | EHV-4 neutralization test | |
|---|---|---|---|---|---|
| | | 13.07.09 | 21.08.09 | 13.07.09 | 21.08.09 |
| | | Barn 1: abort | | | |
| A | artificial | 1:10 | 1:20 | ⟨1:10 | 1:40 |
| B | artificial | 1:10 | 1:10 | ⟨1:10 | 1:20 |
| C | natural | 1:20 | 1:20 | 1:40 | 1:80 |
| D | natural | 1:10 | 1:10 | 1:10 | 1:20 |
| E | artificial | 1:20 | 1:20 | 1:20 | 1:40 |
| F | natural | 1:10 | 1:20 | 1:10 | 1:40 |
| | | Barn 2: normal delivery | | | |
| G | artificial | ⟨1:10 | 1:20 | ⟨1:10 | 1:40 |
| H | natural | 1:10 | 1:20 | 1:10 | 1:40 |
| I | artificial | 1:10 | 1:20 | 1:10 | 1:40 |
| J | natural | 1:20 | 1:40 | 1:10 | 1:40 |
| K | natural | ⟨1:10 | 1:20 | ⟨1:10 | 1:20 |
| L | artificial | 1:10 | 1:20 | 1:10 | 1:40 |
| M | natural | 1:10 | 1:40 | ⟨1:10 | 1:40 |

The EHV-4 NT titers of the six mares in barn 1 showed a more pronounced increase than the EHV-1 neutralizing titers. The EHV-1 titers of four mares (Mare B, C, D, E) remained at the level of the first sampling. The EHV-1 NT titers of the remaining mares A and F showed an increase, but a weaker increase than the EHV-4 titer (Fig. 23). The EHV-4 neutralizing test showed for three mares in barn 1 (Mare A, Mare C and Mare F) an increase of titer by four.
Only mare M in barn 2 showed a 4-fold increase in EHV-1 NT titer. In barn 2 the EHV-4 titer was increased in six cases, namely Mare G, H, I, J, L and M. In barn 2, the titers for EHV-1 and EHV-4 showed an generally increase. The EHV-4 NT titer increase was much stronger except for Mare K, where the EHV-1/-4 titers had the same titer increase (Fig 24).

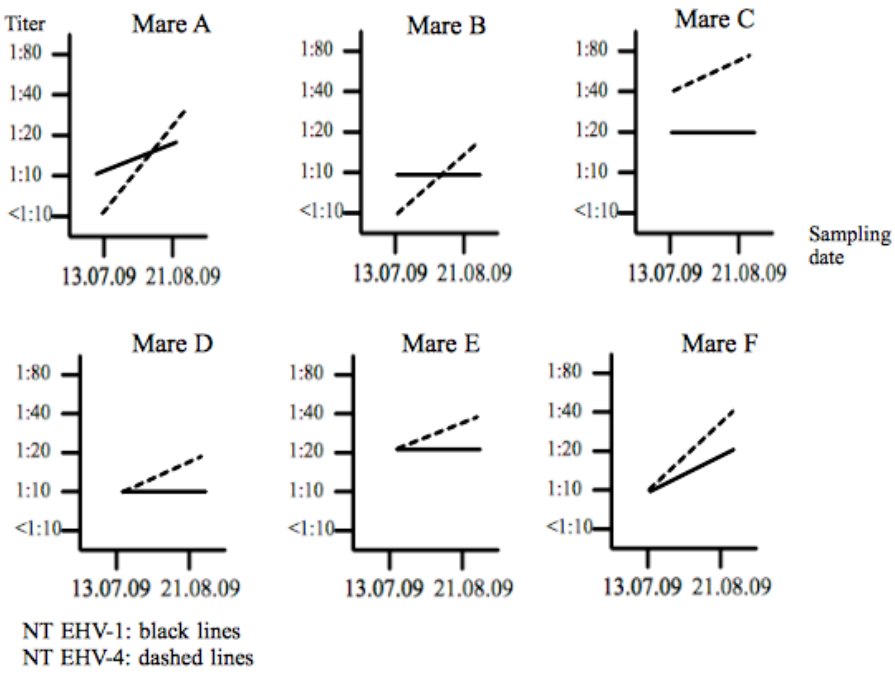


Figure 23: Neutralizing titers of EHV-1 and EHV-4 from the mares of stud farm 4 in barn 1.

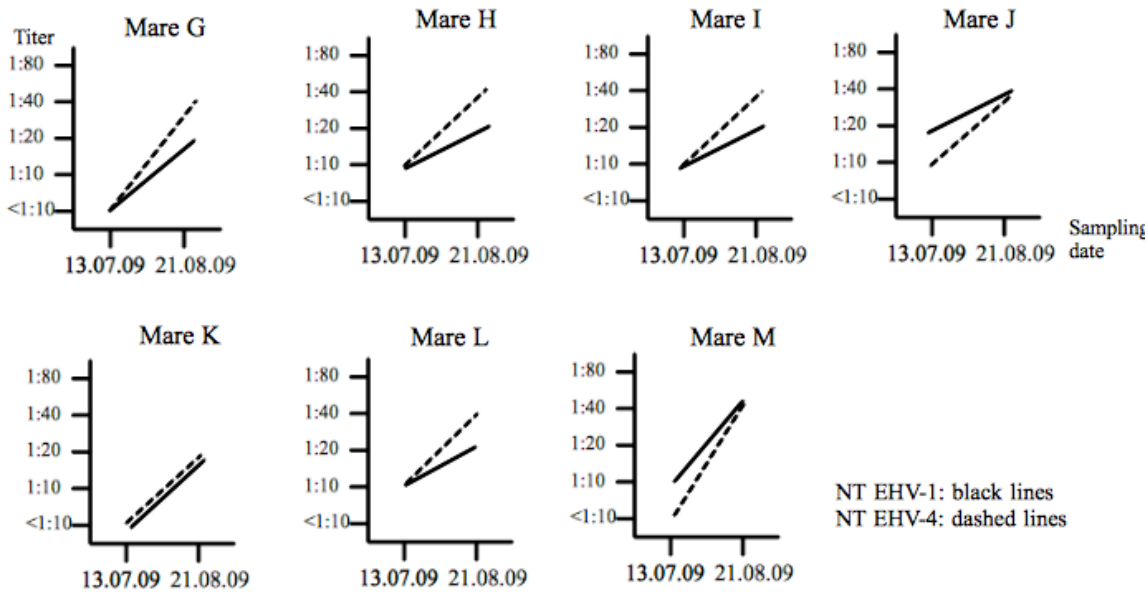


Figure 24: Neutralizing titers of EHV-1 and EHV-4 from the mares of stud farm 4 in barn 2.

# 6 Discussion

## 6.1 Detection of the non-neuropathogenic versus the neuropathogenic EHV-1 genotype in abortion cases in Germany

It was assumed that abortions were predominantly caused by the non-neuropathogenic genotype ($A_{2254}$). Correspondingly, neurological signs were associated with the neuropathogenic genotype ($G_{2254}$).

To identify the non-neuropathogenic or neuropathogenic EHV-1 genotype in abortion cases an ORF 30 nested PCR was performed, followed by a restriction enzyme analysis with *Sal* I. Subsequently, the nested PCR amplicons were sequenced.

The ORF 30 nested PCR was set up according to the paper by Allen (2006). The designed primers were successfully tested (see section 5.1). The ORF 30 nested PCR and subsequent *Sal* I restriction enzyme analysis results of EHV-1 reference strains, abortion and CNS strains correlated with the sequencing results.

88.1% (59/67) of EHV-1 abortion cases in this study exhibited the non-neuropathogenic genotype ($A_{2254}$). 11.9% (8/67) of the EHV-1 abortions in this investigation tested positive for the neuropathogenic genotype ($G_{2254}$).

The eight abortions classified as neuropathogenic were distributed over the years 1987 (one single case), 1989 (one single case), 1990 (one single case), 2004 (three single cases) and 2009 (two cases/abortion outbreak at stud farm 3). No increase or decrease of the occurrence of the neuropathogenic genotype in EHV-1 abortion cases was seen, but on the basis of this small non-representative sample size it cannot be assessed. Only the presence of the neuropathogenic EHV-1 genotype in abortions in Germany was confirmed.

From the eight abortions classified as neuropathogenic genotype, neuropathogenic signs have been reported in two cases. These two derived from the abortion outbreak at stud farm 3. Six of the eight classified as neuropathogenic genotypes came from single abortion cases and showed no neurological signs. They should be classified as abortion isolates containing the neuropathogenic genotype without showing clinical neurological signs. Consequently, an infection with the neuropathogenic EHV-1 strain is not necessarily followed by neurological signs.

In this study, 59 from 67 abortions were classified as the non-neuropathogenic genotype. In the 59 abortions classified as non-neuropathogenic were included 32 single abortion cases and 27 abortions from three abortion outbreaks (18 abortions/stud farm 1, six abortions/stud farm 2, three abortions/stud farm 4). The mares showed no other symptoms. Hence, a coherence between the occurrence of abortions and the non-neuropathogenic EHV-1 ($A_{2254}$) was found.

However, there was no 100% correlation between clinical signs and classification in neuropathogenic versus non-neuropathogenic isolates. In the EHV-1 reference strains Ab4 (pathotype: neurological) and AIV (pathotype: abortion with following neurological disorders), the neuropathogenic genotype was found. The finding of the neuropathogenic genotype and the clinical signs match. EHV-1 strain Mar 87 (pathotype: respiratory) has the non-neuropathogenic genotype and behaves as expected. The EHV-1 strains Army 183 (pathotype: respiratory), Kentucky D (pathotype: abortion) and RacH (pathotype: abortion) unexpectedly all expose the neuropathogenic

genotype. For RacH, the finding was already published by Nugent et al. (2006). It follows from the results of the 67 investigated EHV-1 abortion cases, and the tested EHV-1 reference strains, that it is not only non-neuropathogenic EHV-1 strains that are involved in abortion cases. These results were expected with regard to other studies.

In Argentina, 7% (4/54) of the studied abortion outbreaks were caused by the neuropathogenic genotype, but only 50% (2/4) of them were associated with neurological signs (Vissani et al., 2009). In France, 24% (30 of 125) of the analysed horses showed the neuropathogenic genotype. From the 30 horses which were infected with the neuropathogenic EHV-1 genotype, only seven suffered from neurological disorders, one respiratory case and 22 abortion cases (Pronost et al., 2010b). Both studies confirmed the assumption that the presence of the neuropathogenic genotype does not necessarily causes neurological symptoms.
In one study in Turkey, in the abortion isolates (including foetal lung, liver, spleen, brain and maternal nasal and vaginal swabs) from 12 abortion cases, EHV-1 DNA (5/12 abortion cases) was detected, but no neuropathogenic genotype was found (Turan et al., 2012).
In Uruguay the occurrence of EHV-1 in the horse population was confirmed and additionally, the neurological genotype was found (Easton et al., 2009).
In Japan´s major horse breeding area (Hidaka district) from the years 2001 to 2010 the prevalence in abortion cases was 2.7% (3/113 cases) and only non-neurological outbreaks were observed (Tsujimura et al., 2012).
In addition to Japan, a small number of neurological cases was observed in Brazil. One neurological case was reported in Brazil as carrying the neurological genotype, but in two neurological cases, the non-neurological genotype was also found (Mori et al., 2011).
In a retrospective study (1984-2007) of field isolates from North America, 24% (5/21) of horses with neurological signs were typed to have the non-neurological genotype (Perkins et al., 2009), they stated that other causes had to play a role or influence the development of neurological signs. In contrast, the occurrence of the neuropathogenic genotype from the years 1960 to 2006 in the USA increased from 3.3% in 1960 to 14.4% in 1990, achieving an occurrence of 19.4% (2000-2006). It was hypothesized that the latent reservoir of neuropathogenic viruses will lead on to a continuing increase in prevalence (Smith et al., 2009). In North America, EHV-1 infections with neurological signs have occurred recently more often (Lunn et al., 2009) and the infection has often resulted in devastating losses (Henninger et al., 2007). The neuropathogenic EHV-1 disease has been termed an emerging disease for the horse population in the USA (Allen and Timoney, 2007). Allen et al. (2008) stated that there seemed to be no sustainable trend for one or the other genotype at that time. Another study in the USA showed different results and they concluded that non-neuropathogenic strains were more prevalent (Pusterla et al., 2012).

The formation of neurological signs possibly is not solely dependent only on the presence of the neuropathogenic genotype, like others before mentioned (Pronost et al., 2010b). Further investigations are needed to better understand the relevance of the neuropathogenic genotype. Other factors like age, sex, host immunity, hormonal factors, stud management, hygienic standards, vaccination, stress, reactivation of latent strains and infection dose could play a role and influence the outcome of neurological signs (Allen, 2008; Nugent et al., 2006; Pusterla et al., 2009a; Vissani

et al., 2009). Furthermore, other nucleotide exchanges in the ORF 30 could have an effect on the severity of abortigenic or neuropathogenic potential (see section 6.3).
In a mouse model, another determinant for the neuropathogenic potential was found in the ORF 37, so in addition to the mutation in the ORF 30, this should be taken into account (Kasem et al., 2010). The DNA polymerase is only one compound of the replication cascade, hence, it could not be excluded that other open reading frames with a function in the viral replication have an effect on the neuropathogenic potential (Pronost et al., 2010a). In the mouse encephalitis model, the open reading frame 37 of EHV-1 was found to have a neuropathogenic effect in cultivated neural cells (Kasem et al., 2010). Telford et al. (1992) specified the gene function of ORF 37 as the counterpart of the HSV-1 (Herpes Simplexvirus 1) UL 24, and UL 24 was identified as a tegument protein (Varnum et al., 2004). It is questionable whether this applies only to mice, therefore, in further studies it should be tested for a potential neuropathogenicity in horses.

At stud farm 3, in addition to abortions, fever and neurological symptoms were also observed, and the neuropathogenic genotype was found. It is theoretically possible that both genotypes exist in the same horse. A polymorphism in the presence of the background of the non-neuropathogenic type in the EHV-1 DNA Pol gene was assumed to exist at low frequency in the abortion isolates, and this was adopted to complicate the determination. Unfortunately, it was not possible to detect these mutations at such a low frequency with the techniques (ORF 30 nested PCR, REA *Sal* I and sequencing) used in this thesis.
The incomplete digestion in the restriction enzyme analysis *Sal* I (see Fig. 6) can mean that both genotypes exist in the particular ORF 30 amplicons. Other reason are the inhibition by PCR components, too few units of enzyme used or that the incubation time was too short. The sequencing chromatograms showed clearly that only one genotype at position 2254 was present. Consequently, one of the above mentioned parameters of the restriction enzyme analysis *Sal* I did not fit. A too short incubation period (optimal 1 hour for 1 $\mu$l) or too few units of restriction enzyme can be excluded (4.12.1). The inhibition by PCR components could be possible, since the ORF 30 amplicons were not purified. Nevertheless, the results are assessable.
The sequencing chromatograms confirmed that at the particular position (nt 2254) only one peak was visible. A polymorphic position will show both nucleotides simultaneously by showing heterozygous (double) peaks of different colours in the chromatogram. Only in the chromatogram of Ab4 a double peak at position 2258 was found. The sequencing named C 2258, but the chromatogram revealed a second peak with A 2258 (see Fig. 7). In none of the other chromatograms from the ORF 30 nested PCR fragments double peaks were observed.
Therefore, in cooperation with Prof. Hugh Field and Dr. Soumi Sukla, Department of Veterinary Medicine, University of Cambridge, Cambridge, U.K., intentional mismatch primers were designed (data not presented). Based on the theory that one mismatch in the primer sequence makes no difference to the resulting fragment, but two mismatches will not generate a PCR product (Sukla et al., 2010), the primer for the ORF 30 mismatch PCR were designed using MacVector. A primer with the neuropathogenic genotype (first mismatch for the non-neuropathogenic genotype) and a second mismatch with two nucleotides off were designed with the following PCR conditions: 35 cycles, 94°C 30 s, 52°C 30 s and 72°C 60 s for denaturation, annealing and elongation, respectively. The specific primers were tested successfully using the program MacVector. The expected

fragment size was 320 bp. Despite numerous changes in PCR conditions and primers, a suitable amplicon was not obtained (data not presented), therefore the method could not be established. Although the approach with the ORF 30 mismatch PCR did not work out, in the meantime, great new methods were developed by other working groups. Ultra deep sequencing, based on the sequencing by synthesis method (pyrosequencing), was developed. This new technique permits a detection of mutations at very low levels. Currently ultra deep sequencing is used to determine low frequency mutations for example in HIV (human immunodeficiency virus) samples (Wang et al., 2007). Another option could be real-time PCR using allelic discrimination, named $E_1$ rPCR. This new method allows the discrimination between neuropathogenic and non-neuropathogenic strains, and furthermore, could identify dual infections with a low detection limit (Smith et al., 2012).

The coexistence of both genotypes ($A_{2254}$ and $G_{2254}$) seems to be really rare. In two studies (Allen et al., 2008; Pusterla et al., 2009b), the simultaneous infection with $A_{2254}$ and $G_{2254}$ strains was detected in submandibular lymph nodes, blood and nasopharyngeal secretions. It could not be excluded that both EHV-1 strains coexisted. In contrast, another report found no evidence of a coinfection (Smith et al., 2009). However, the studies used different PCR techniques and samples. Allen et al. (2008) utilized sequence-capture, nested PCR and sequence-capture and reverse transcription-nested PCR, and Pusterla et al. (2009b) used a real-time Taq-Man PCR assay. Both took the isolates directly for PCR. In comparison, Smith et al. (2009) used EHV-1 abortion isolates grown on foetal equine dermis (ED) and a real-time Taq-Man PCR. To clarify if the detection of both variants correlated with the detection method and the sample (cultivated virus or original tissue), additional investigations are needed.

In this study, the EHV-1 abortion tissues or isolates were grown in cell culture, except six samples (two nasal swabs, two lung fluid, one amniotic fluid and one placenta) from three abortions (Mare I, Mare II, Mare A) which were used for ORF 30 nested PCR directly after viral DNA preparation. In the same cell culture it is unlikely that two different types (neuropathogenic and non-neuropathogenic) of EHV-1 can be sustained (Diallo et al., 2006). This fact, along with the conventional ORF 30 nested PCR in this study, meant it was not possible to detect both genotypes in the same sample.

However, double infections can occur by new infection with a neuropathogenic strain and simultaneous reactivation of a latent abortion strain or *vice versa* (Allen et al., 2008).

Summarizing, the ORF 30 nested PCR and *Sal* I restriction analysis was found to be a fast and secure diagnostic tool in the case of an abortion outbreak. For horse breeders it is important to know which EHV-1 strain has caused a current abortion outbreak in order to optimize the management of stud farms and to minimize losses by the more aggressive neuropathogenic genotype ($G_{2254}$) through adequate therapy (Pusterla et al., 2009a). EHM have a higher contagious risk and a strict isolation of affected horses for a minimization of transmission is useful (Goehring et al., 2011). A safer way, however, is that the management of outbreaks of EHV-1-associated diseases should not be influenced by the typing of the EHV-1 strain. Furthermore, all strains have the potential to cause severe disease, whether abortion and/or neurological disease (Lunn et al., 2009). Stress through change in the horse population, unrestricted transport between different barns and reduced ventilation in barns increased the rapid spread of infection (Henninger et al.,

2007). Therefore, these factors can and should be respected.

## 6.2 EHV-1 in stallions

In this study, the semen of a stallion at stud farm 3 was investigated at the same time when the neurological signs and abortion cases occurred. The stallion was symptom-free, but the semen was tested positive for the neuropathogenic EHV-1 genotype ($G_{2254}$). Unfortunately, only the semen of one stallion was investigated and therefore it is the only one that can be reported on in the present study. Virus isolation was not successful.

The role of stallions in the transmission of neuropathogenic EHV-1 strains should be taken into account and further research is necessary. In particular, it should be clarified whether EHV-1 can survive in semen and if its infectivity remains. The possible transmission of EHV-1 through semen by natural mating or by artificial insemination was already suggested (Hebia-Fellah et al., 2009). Virus shedding in semen of naturally infected stallions (with the neuropathogenic EHV-1 genotype) was detected for up to three weeks. But the spermatozoa seemed to be unaffected by EHV-1 (Walter et al., 2012). It is, however, already associated on the clinical side with scrotal oedema and the loss of libido (Greenwood and Simson, 1980). In addition, EHV-1 damaged the sperm development and reduced the normal sperm concentration (Tearle et al., 1996). It should be ascertained out if stallions can infect mares through mating with infected semen.

Other herpes viruses, such as Canine Herpesvirus 1 (CHV-1), can infect the mucosa of the male penis and foreskin, and as in horses it is a latent infection. But viruses are excreted at irregular intervals and without the appearance of lesions on the penis. The affected males can be contagious (Carmichael and Greene, 1998) and dogs infected with herpes virus should be used with caution for breeding, because a herpes virus infection can lead to the death of the puppies.

Similarly, Bovine Herpesvirus-1 (BHV-1) is excreted in the semen (Van Engelenburg et al., 1995). A reduction of the conception rate, followed by endometritis, abortion and infertility can be caused by BHV-1 contaminated semen (Parsonson and Snowdon, 1975).

A case report described a natural EHV-1-infected zebra stallion who suffered rhinitis and pulmonary oedema. In the testis and epididymides, EHV-1 was also localized. The localization in leydig cells and germinal epithelium led to the assumption that the semen could be a carrier for EHV-1 (Blunden et al., 1998). The lesions were comparable to those found in experimentally infected pony colts (Tearle et al, 1996). That supported the conjecture that venereal shedding could be possible as a transmission route (Blunden et al., 1998).

EHV-1 was detected in the semen of stallions and other herpes viruses have the ability to be contagious through the semen. It should be further investigated if EHV-1 can be transmitted via semen.

## 6.3 Additional nucleotide exchanges in the ORF 30 in Germany

In addition to the neuropathogenic genotype at nucleotide position 2254, the sequencing of ORF 30 amplicons revealed more nucleotide changes in the ORF 30.

### 6.3.1 Nucleotide exchange at position 2258

The sequencing discovered a substitution at position 2258 from adenine ($A_{2258}$) to cytosine ($C_{2258}$) in one abortion isolate (T952) from the year 1987, as well in the EHV-1 reference strains RacH and Ab4. The nucleotide exchange at position 2258 led to an exchange of the amino acid tyrosine (Y 753) to serine (S 753).

This exchange at position 2258 was first described by Nugent et al. (2006) in the RacH strain. Additionally, Smith et al. (2009) found the $C_{2258}$ and $G_{2254}$ genotype in two isolates from 1974. The close location to the neuropathogenic genotype at position 752 was suggested to have an influence on the expression of neurological signs induced through the neuropathogenic genotype ($G_{2254}$).

The palm domain of the polymerase gene included the amino acid position 753. Interestingly, the change from tyrosine to serine results in a less hydrophobic residue and could therefore influence the folding ability of the domain (Smith et al., 2009), and maybe this influences the function.

In the attenuated virus vaccine abortion strain RacH both mutations were present ($G_{2254}$ and $C_{2258}$). The identification of $C_{2258}$ in one of the field isolates (T952) in the year 1987 suggested either a natural mutation, or that the abortion isolate may be either derived from RacH vaccine strain or a double infection. Since there was no information regarding the vaccination status of the affected mare, this point could not be clarified.

A new method has already managed to distinguish between neuropathogenic and non-neuropathogenic EHV-1 strains and confirmed the findings in the isolate RacH. The primer-probe energy transfer (PriProET) technique is a PCR technique in which one of the primers is labelled with fluorescent dye. The dye is activated through the hybridization and the fluorescence can be detected and quantified. The fluorescence corresponds to an energy following excitation and a melting point analysis obtains the allele-specific melting temperatures. Strains with the neuropathogenic genotype have higher melting temperatures compared with the non-neuropathogenic isolates. This new primer-probe energy transfer method shows an exact correlation with the sequence variation in the ORF 30 target region. RacH could not be assigned to either two groups, the $G_{2254}$ suggested RacH as a member of the neuropathogenic strains, but the substitution at position 2258 from adenine ($A_{2258}$) to cytosine ($C_{2258}$) caused the unique peak at the intermediate melting point (67.8°C). Consequently, RacH has a unique specified sequence in this region. The peaks of melting temperatures correlated with the sequencing of ORF 30 (Malik et al., 2010).

The identification of $C_{2258}$ in the Ab4 strain was quite surprising in this study. It was compared to the original sequence of Ab4 (Telford et al., 1992), which reported $A_{2258}$ at this position. This was confirmed by the study of Nugent et al. (2006). It is possible that the Ab4 stock used for this study mutated compared to the stocks used by the other groups, possibly during passage in cell culture. Genomic changes and recombinations during passage in cells with non-equine origin have been described (Allen et al., 1983; Kirisawa et al., 1993). Another suggestion is the contamination of the Ab4 virus with a different virus strain. After the first sequencing, an archived Ab4 virus isolate from the first passages out of the Institute‘s stock was investigated and this confirmed the results of the first sequencing.

### 6.3.2 Nucleotide exchange at position 2269

In the ORF 30, another nucleotide exchange was revealed by sequencing of the ORF 30 nested amplicons. The nucleotide guanine ($G_{2269}$) was exchanged to adenine ($A_{2269}$) at nucleotide position 2269 in two abortion isolates (A(E) 271-3, A 258) from single abortion cases from the year 2004. An amino acid exchange from glutamic acid (E 757) to lysine (K 757) resulted. Both isolates were also demonstrated to contain the neuropathogenic genotype ($G_{2254}$). There are no reports so far about this exchange. The influence of the outcome of neuropathogenic or abortigenic potential is quite unclear because of the sample size being too small. In the two mares in which abortion isolates of the $A_{2269}$ genotype were confirmed, both suffered an abortion and no other clinical signs.
Both samples were provided as viral isolates for this study by Dr. Neubauer, Munich. Therefore, the number of cell passages was unknown and a mutation during cell passage could not be excluded. But the other five (four abortion isolates: E510/89, T952, F553/89, E113-115; one CNS isolate: 834) sequenced viral isolates, also provided by Dr. Neubauer, did not carry an exchange at nucleotide position 2269.

## 6.4 Detection of the neuropathogenic genotype in cattle and archived wild equid strains

The neuropathogenic genotype $G_{2254}$ was determined in six archived wild equid isolates and in two cattle isolates. The presence of $G_{2254}$ in the isolates 94-137, T616 and 49 800 was first detected by Yamada et al. (2008) and was confirmed by the presented study (Fritsche, not published).
Eight isolates tested positive for the neuropathogenic genotype. Three from the eight neuropathogenic isolates were from cases with neurological signs and five from the eight isolates derived from abortions. Consequently, there seemed to be no correlation between the neuropathogenic genotype and the clinical signs. For this small sample size it is a daring statement and this assumption necessarily needs further investigations.
Another explanation could be the finding of Nugent et al. (2006), they compared the ORF 30 region with other herpes viruses and the position 2254 was occupied by G (Nugent et al., 2006). It has been suggested that the ancestral EHV-1 encoded $G_{2254}$ and the non-neuropathogenic genotype $A_{2254}$ arose due to a selective advantage (Ocampos et al., 2009). This presumption was justified by the fact that in other herpesviruses, the position 752 was an acidic residue (D) and highly conserved (Nugent et al., 2006). It was suggested that the EHV-1 progenitor may carry D $752/G_{2254}$ (Lunn et al., 2009). In comparison, Van de Walle et al. (2009) stated that the mutation could have arisen from a spontaneous point mutation at nt position 2254, A was replaced by G. However, the possibility that, due to the conserved acidic residue (stable D 752), a G to A change took place was considered as more likely by the authors (Nugent et al., 2006). In this study, the EHV-1 strains Army 183 (1948), Kentucky D (1954), RacH (1965) carried $G_{2254}$, were all isolated almost 50 to 65 years ago and supported the above described hypothesis.
The progenitor of EHV-1 was discussed elsewhere as deriving from the ancestor of the modern horse, and this was justified by the great antigenic and genetic similarity of EHV-1 strains from horses, donkeys, zebra and onagers (Blunden et al., 1998; Crabb and Studdert, 1990; Montali et al., 1985; Wolff et al., 1986). The close relationship between EHV-1 in horses and EHV in zebras,

onager and antelope suggests that EHV-1 was formed from the precursor of the wild animal viruses through evolution.
In this study, in six archived wild equid isolates (two zebra, one onager, three antelope isolates) and in the two cattle isolates (136A, 136B), the neuropathogenic genotype $G_{2254}$ was determined and supported the above described hypothesis.
EHV-1-induced abortions in cattle have long been known (Chowdhury et al., 1988).
In the past there were no natural barriers which prevented virus transmission between wild animals and domesticated horses, therefore, appropriate measures should be taken to ensure that EHV-1 variants with unknown pathogenic potential do not become a threat to the horse industry (Ibrahim et al., 2007).

### 6.4.1 Nucleotide exchange at position 2262

The sequencing of the ORF 30 nested PCR amplicon revealed an additional nucleotide change from G to A at position 2262. The $A_{2262}$ genotype was found in three isolates, Ro-1, T965 and T616. This exchange was not found in any of the equid isolates. At the amino acid level, no amino acid exchange was found. It is questionable whether this mutation has an effect on the outcome or severity of neurological symptoms. On the other hand, the theory that EHV-1 originated from the ancestor of the modern horse (Crabb and Studdert, 1990) could lead to the idea that the G to A (position 2262) exchange could happen *vice versa* and consequently, the $G_{2262}$ genotype represented the mutation.

## 6.5 Detection of the neuropathogenic EHV-1 genotype in neurological cases in Germany

In three EHV-1 isolates from two horses with only neurological illnesses, the neuropathogenic genotype ($G_{2254}$) was confirmed. The ORF 30 nested PCR and subsequent *Sal* I restriction analysis results were in accordance with the sequencing results in all of the three sequenced amplicons from neurological cases.
The infection with the neuropathogenic genotype led to neuropathogenic signs in the affected horses. The number of neurological cases (2) was too low in this study. However, other authors like Leutenegger et al. (2008) detected the neuropathogenic genotype in 86% of the neuropathogenic isolates and Perkins et al. (2009) defined the odds of suffering neurological signs when infected with the EHV-1 $G_{2254}$ strain as 162 times higher in comparison with an infection with the EHV-1 $A_{2254}$ genotype.

## 6.6 Serological and molecular biological detection of EHV-1 in abortion outbreaks on two stud farms

### 6.6.1 Stud farm 3

In stud farm 3, neurological signs and fever were the first clinical signs of infection. They began in February 28, 2009. Two mares aborted and showed neurological signs, two had only neurological signs and seven were febrile.
Group 1 with neurological signs prior to two abortions: For two mares (V09-3-2, V09-3-3) with

abortion and neurological signs the titers were elevated, but a strong increase was missing. The high initial titers were attributed to the vaccination, but the low increase later on presumably allowed the abortions. The abortion samples from both mares were examined by ORF 30 nested PCR and tested positive for the neuropathogenic genotype in the *Sal* I digestion and sequencing. A gelding (V09-3-4) with neurological signs showed an elevated titer and a strong titer increase was seen. It was vaccinated with Resequin. According to package insert for the vaccine it only protects against the respiratory EHV-1/-4 diseases. Although the antibody response was strong and a cross reaction could be assumed.

Group 2 with acute febrile horses for less than 2 days: Unfortunately, the start and end of the fever in respect to the date of sampling is not known. Three unvaccinated stallions with fever for one day showed in the NT low titer and no strong titer increase. In contrast, in the IFA elevated titers were recognized and also a 4-fold titer increase. Thus, an antibody response was present. The pregnant mare V09-3-11 had a 8-fold EHV-1 antibody titer increase. This was indicative of an acute infection or reactivation of the virus. The mare suffered only fever for less than two days. It was vaccinated with Duvaxyn1,4 in month 3./4. and 7./8. and was treated with the viral inhibitor acyclovir (since 28 February 2009 treatment three times a day 9600 mg). Acyclovir inhibits the viral polymerase but not the cellular DNA polymerase (Henninger et al., 2007) and by reducing the viral propagation also the viral shedding should be reduced. The appropriate dose of acyclovir for horses is still unknown, and the bioavailability of the drug orally administered is not sufficient to obtain a drug level in the blood (Garré et al., 2007). No abortion was triggered, it is questionable whether the acyclovir treatment or the antibody response had an effect. Comparisons with other treated horses with different acyclovir concentrations were missing.

Group 3 with horses without fever for at least three days: Three horses, one not vaccinated and two vaccinated with Duvaxyn1,4, showed in the NT low titer and no strong titer increase. But in the IFA, elevated titers were recognized and a slight titer increase.

Interestingly, the horses with the high titers at the first sampling had not shown a strong titer increase. Probably, the horses were at different stages of infection. In the vaccinated horses, a stronger antibody response was generally observed and this supported the advice to vaccine against EHV-1/-4.

### 6.6.2 Stud farm 4

At stud farm 4, 13 mares were sampled, six had aborted and seven had a normal delivery. The non-neuropathogenic EHV-1 genotype was found by ORF 30 nested PCR and restriction enzyme analysis *Sal* I.

The antibody titers in this study supported the statement that the increase of antibody titer after an abortion differs individually (Schröer et al., 2000). The hight of the antibody titer does not allow any statement about the prognosis of abortion. The lower antibody reaction as measured in the NT against EHV-1 possibly resulted in the abortions in barn 1. In barn 2, higher NT titers were seen. Presumably, because of the high antibody titer no abortions occurred in barn 2. The serological examination of paired sera is still the best way to detect an infection or reactivation. But the clinical signs must also be considered, such as the swollen limbs of two mares from stud farm 4 that aborted later.

The PBMCs of the blood samples of the mares from stud farm 4 were isolated to clarify if viraemic

EHV-1 in the blood leukocytes were detectable via ORF 30 nested PCR. The distinction between non-neuropathogenic and neuropathogenic strains in PBMC was also investigated. In two cases from stud farm 4, EHV-1 was detectable in the PBMC fraction and the non-neuropathogenic genotype was found. Interestingly, one (Mare A) was detected at the first blood collection, the second (Mare E) four weeks later. But both mares suffered an abortion and both showed an EHV-4 NT titer increase (Mare A: noticeable titer increase, Mare E: no strong increase), but for EHV-1, no strong titer increase was seen. Allen and Timoney postulated in 2007 that a positive EHV-1 DNA detection by PCR in blood leucocytes correlated with an active EHV-1 infection, therefore Mare A was actively infected at the first blood sampling and then EHV-1 probably went into latency. In contrast, Mare E was infected actively at the second sampling. Although the viral load (different at the beginning and end of acute infection) should be taken into account.
Latent EHV-1 in circulating leukocytes is not detectable with the conventional PCR methods, therefore it could be possible that EHV-1 does not stay in the state of latency in circulating leukocytes (Allen, 2006). PBMCs from 11 latently infected ponies were analysed with real-time PCR and EHV-1 was not found (Hussey et al., 2006). In contrast, in the murine model, EHV-1 in the state of latency was detectable in PBMC using nested PCR (Baxi et al., 1996). Another interesting point was the assumption that positive EHV-1 DNA detection by PCR in PBMC may be caused by the presence of dead virus particles from the last vaccinations (Allen and Timoney, 2007). But the mares from stud farm 4 were not vaccinated against herpesvirus, except Mare J. It suffered no abortion and no EHV-1 in PBMC was detectable.
The mares of stud farm 4 were inseminated artificial or by natural mating. This raised the question whether abortions occurred more often after one type of insemination or the other, because at stud farm 3 a semen sample tested positive for the neuropathogenic genotype of EHV-1 (see section 6.2). The distribution of the type of insemination on mares with abortion or without abortion was found to be approximately the same. A correlation between the occurrence of abortion and the type of insemination was not found at stud farm 4. However, neither the portability of EHV-1 via semen has been demonstrated, nor was stallion sperm of stud farm 4 examined.

In summary, it was found useful to separate the breeding area from the normal stud farm area. In stud farm 4, it was seen that only the mares in barn 1 suffered abortions, but caused by the separation and a higher antibody response of the mares in barn 2, a normal delivery was possible. Barn 1 was in the area with the normal riding stable and a lot of horse transports due to the local tournaments.
The mares at stud farm 4 were not vaccinated against EHV-1/-4. It is useful to vaccinate to reduce the risk of foal losses. Only one vaccine (Duvaxyn1,4, Fort Dodge) claims to work against EHV-1-induced abortion. Goehring et al. (2010) described that vaccinations significantly reduces the severity of symptoms of EHV-1 disease and viral shedding. High concentrations of CTLPs (EHV-1 specific cytotoxic T-lymphocyte precursors) were induced via vaccination and the following immunological mechanism. The CTLPs seemed to have a protective effect against EHV-1-induced neurological disease. The infected horses with preexposure low CTLP concentration showed neurological deficits and a high postexposure viraemic load. Horses with high concentrations of CTLPs were better able to control the development of clinical signs (Allen, 2008). Vaccines which are able to stimulate the immune mechanisms to enhance the level of CTLPs are an objective of further

vaccine development.
In contrast, the mares I and II from stud farm 3 were vaccinated both with Duvaxyn1,4 in gestation month 3./4. and mare II also in month 7./8.. Both aborted and showed neurological signs. The causative agent was the neuropathogenic genotype of EHV-1. It is questionable whether the vaccination protected against the infection with the neuropathogenic EHV-1 genotype. But in vaccinated horses from stud farm 3, a stronger antibody response was observed.

# 7 Summary

In this study, the non-neuropathogenic genotype (N752/$A_{2254}$) was detected in 88.1% (59/67) of the EHV-1 abortion isolates. The neuropathogenic genotype (D752/$G_{2254}$) was detected in 11.9% (8/67).

The infection with the neuropathogenic EHV-1 strain did not necessarily result in neurological signs. Only two of the mares (2/8) with the neuropathogenic genotype showed neurological signs prior to abortion.

Another mutation at nt position 2258 was found, an amino acid change from Y to S753 was recognized in EHV-1 reference strain Ab4, RacH and abortion T952 (single abortion case). The amino acid changes at position 752 and 753 possibly correspond with the outcome of the neuropathogenic potential. Further investigations are needed to clarify if there is a correlation.

In two abortion isolates (A(E) 271-3, A 258) from single abortion cases from the year 2004 the nucleotide guanine ($G_{2269}$) was exchanged to adenine ($A_{2269}$), an amino acid change from glutamic acid to lysine resulted (E 757 to K 757).

In six archived wild equid isolates and in two cattle isolates the neuropathogenic genotype $G_{2254}$ was found. In three wild equid ORF 30 amplicons the sequencing revealed an additional nucleotide change from G to A at position 2262, no amino acid exchange resulted.

In this study, a clinical healthy stallion was found to have the neuropathogenic genotype in semen. Further studies should be performed to examine if EHV-1 was excreted via semen and if the infectivity remained.

The detection of virus in PBMC via ORF 30 nested PCR succeeded in this study only twice and it is questionable whether this low detection rate depends on the stage of infection (active or latent) or the detection method.

The ORF 30 nested PCR was found to be a fast and safe method to differentiate neuropathogenic from non-neuropathogenic EHV-1 strains. However, the typing of the strains should not influence the stud farm management in the case of outbreak, as both strains - neuropathogenic and non-neuropathogenic - have the potential to cause disease.

# 8 Zusammenfassung

**Virologische und molekularbiologische Charakterisierung von equinen Herpesvirus 1 (EHV-1) Isolaten in Deutschland**

In dieser Studie wurde der nicht-neuropathogene Genotyp ($N752/A_{2254}$) in 88.1% (59/67) der EHV-1 Abortisolate gefunden. Der neuropathogene Genotyp ($D752/G_{2254}$) wurde in 11.9% (8/67) gefunden.

Nur zwei der acht Stuten mit dem Nachweis des neuropathogenen Genotyps zeigten zusätzlich zum Abort auch neurologische Symptome. Eine Infektion mit dem neuropathogenen EHV-1 mündet also nicht zwangsläufig in einer neurologischen Verlaufsform.

An der Nukleotidposition 2258 wurde eine weitere Mutation im ORF 30 des EHV-1 Referenzstammes Ab4, RacH und Abortprobe T952 (Einzelabort) gefunden. Ein Aminosäurewechsel von Y zu S 753 ist die Folge. Ein Zusammenhang des Aminosäureaustausches der Positionen 752 und 753 und damit eine Beeinflussung des neuropathogenen Potentials ist in Diskussion. Es sollten sich weitere Untersuchungen anschliessen.

Bei zwei Abortisolaten (A(E) 271-3, A 258) von Einzelaborten aus dem Jahr 2004 wurde anstatt Gunanin ($G_{2269}$) Adenin ($A_{2269}$) gefunden. Dies resultierte im Aminosäurewechsel von Glutaminsäure zu Lysin (E 757 zu K 757).

In sechs archivierten Wildequidenisolaten und 2 Isolaten von Rindern wurde der neuropathogene Genotyp ($G_{2254}$) gefunden. In den ORF 30 Amplikons von 3 Wildequiden wurde durch die Sequenzierung an der Nukleotidposition 2262 ein Wechsel von G auf A festgestellt, daraus resultierte kein Aminosäurewechsel.

Bei einem klinisch unauffälligen Hengst von Gestüt 3 wurde im Sperma der neuropathogene EHV-1 Stamm nachgewiesen. Auf demselben Gestüt wurde bei zwei Stuten mit Aborten und neurologischen Symptomen ebenfalls der neuropathogene Genotyp festgestellt. Es wäre anzuraten weitere Spermaproben zu untersuchen, um festzustellen ob EHV-1 über Sperma ausgeschieden wird und ob EHV-1 infektiös bleibt.

Der Virusnachweis aus PBMCs per ORF 30 nested PCR gelang nur zweimal. Es ist die Frage ob diese niedrige Nachweisrate an dem Stadium der Infektion (aktiv oder latent) liegt oder der Untersuchungsmethode.

Die ORF 30 nested PCR stellt eine schnelle und sichere Methode dar um neuropathogene und nicht-neuropathogene EHV-1 Genotypen zu unterscheiden. Die Kategorisierung in neuropathogene und nicht-neuropathogene EHV-1 Stämme sollte jedoch das Management im EHV-1 Ausbruchsfall nicht beeinflussen. Beide Genotypen haben das Potential schwere Erkrankungen auszulösen und es sollten bei Verdacht strenge Hygiene- und Quarantänemassnahmen getroffen werden.

# 9 Bibliography


Allen, G. P., Yeargan, M. R., Bryans, J. T. (1983). „Alterations in the equine herpesvirus 1 genome after *in vitro* and *in vivo* virus passage.“ Infect Immun **40**(1):436–439.

Allen, G. P., Yeargan, M. R., Turtinen, L. W., Bryans, J. T. (1985). „A new field strain of equine abortion virus (equine herpesvirus-1) among kentucky horses.“ Am J Vet Res **46**(1):138–140.

Allen, G. P. and Bryans, J. T. (1986). „Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections.“ Prog Vet Microbiol Immunol **2**:78–144.

Allen, G. P. (1998). „A portrait of the pathogenesis of equine herpesvirus abortion.“ Equine Dis. Quart **7**:(3).

Allen, G. P., Kydd, J., Slater, J., Smith, K. (2004). „Infectious diseases of livestock.“ Oxford Press **76**.

Allen, G.P. (2006). „Antemortem detection of latent infection with strains of equine herpesvirus-1 in horses.“ Am J Vet Res **67**(8):1401–1405.

Allen, G. P. and Breathnach, C. C. (2006). „Quantification by real-time PCR of the magnitude and duration of leucocyte-associated viraemia in horses infected with neuropathogenic vs. non-neuropathogenic strains of EHV-1.“ Equine Vet J **38**(3):252–257.

Allen, G. P. and Timoney, P. (2007). „Recent advances in our understanding of equine herpesvirus-1 (EHV-1) myeoloencephalopathy.“ Proceedings **Volume 1**:111.

Allen, G. P. (2008). „Risk factors for development of neurologic disease after experimental exposure to equine herpesvirus-1 in horses.“ Am J Vet Res **69**(12): 1595–1600.

Allen, G. P., Bolin, D. C., Bryant, U., Carter, C. N., Giles, R. C., Harrison, L. R., Hong, C. B., Jackson, C. B., Poonacha, K., Wharton, R., Williams, N. M. (2008). „Prevalence of latent, neuropathogenic equine herpesvirus-1 in the thoroughbred broodmare population of central kentucky.“ Equine Vet J **40**(2):105–110.

Baxi, M. K., Borchers, K., Bartels, T., Schellenbach, A., Baxi, S., Field, H. (1996). „Molecular studies of the acute infection, latency and reactivation of equine herpesvirus-1 (EHV-1) in the mouse model.“ Virus Res **40**(1):33–45.

Benetka, V., Brandstätter, M., Högler, S., Möstel, K. (2002). „EHV-4 bedingter Abortus in einem österreichischen Pferdebestand mit respiratorischen und neurologischen Krankheitsfällen.“ Sonderdruck Tierarztl. Umschau **57**(9):464-471.

Blunden, A. S., Smith, K. C., Whitwell, K. E., Dunn, K. A. (1998). „Systemic infection by equid herpesvirus-1 in a grevy's zebra stallion (*Equus grevyi*) with particular reference to genital pathology.“ J Comp Pathol **119**(4):485–493.

Borchers, K. and Slater, J. (1993). „A nested PCR for the detection and differentiation of EHV-1 and EHV-4.“ J Virol Methods **45**(3):331–336.

Borchers, K., Wolfinger, U., Lawrenz, B., Schellenbach, A., Ludwig, H. (1997). „Equine herpesvirus 4 DNA in trigeminal ganglia of naturally infected horses detected by direct in situ PCR.“ J Gen Virol **78**(5):1109–1114.

Borchers, K. (1999). „Infections with EHV-1, -2, -4 and -5: Distribution, pathogenesis, immune response and latency.“ Recent Res. Devel. Virol. **1**:773-788.

Borchers, K., Wolfinger, U., Ludwig, H. (1999). „Latency-associated transcripts of equine herpesvirus type 4 in trigeminal ganglia of naturally infected horses.“ J Gen Virol **80**(8):2165–2171.

Borchers, K., Daly, J., Stiens, G., Kreling, K., Kreling, I., Ludwig, H. (2005a). „Characterisation of three equine influenza A H3N8 viruses from Germany (2000 and 2002): evidence for frozen evolution.“ Vet Microbiol **107**(1-2):13–21.

Borchers, K., Wiik, H., Frölich, K., Ludwig, H., East, M. L. (2005b). „Antibodies against equine herpesviruses and equine arteritis virus in burchell's zebras (*Equus burchelli*) from the serengeti ecosystem.“ J Wildl Dis **41**(1):80–86.

Borchers, K., Böttner, D., Lieckfeldt, D., Ludwig, A., Frölich, K., Klingeborn, B., Widen, F., Allen, G., Ludwig, H. (2006a). „Characterization of equid herpesvirus 1 (EHV-1) related viruses from captive grevy's zebra and blackbuck.“ J Vet Med Sci **68**(7):757–760.

Borchers, K., Thein, R., Sterner-Kock, A. (2006b). „Pathogenesis of equine herpesvirus-associated neurological disease: a revised explanation.“ Equine Vet J **38**(3):283–287.

Borchers, K., Lieckfeldt, D., Ludwig, A., Fukushi, H., Allen, G., Fyumagwa, R., Hoare, R. (2008). „Detection of equid herpesvirus 9 DNA in the trigeminal ganglia of a burchell's zebra from the serengeti ecosystem.“ J Vet Med Sci **70**(12):1377–1381.

Carmichael, L. E. and Greene, C. (1998). „Infectious diseases of the dog and cat - Canine Herpesvirus Infection.“ Saunders: 28-32.

Carvalho, R., Oliveira, A. M., Souza, A. M., Passos, L. M., Martins, A. S. (2000). „Prevalence of equine herpesvirus type 1 latency detected by polymerase chain reaction.“ Arch Virol **145**(9):1773–1787.

Chowdhury, S. I., Kubin, G., Ludwig, H. (1986). „Equine herpesvirus type 1 (EHV-1) induced abortions and paralysis in a lipizzaner stud: a contribution to the classification of equine herpesviruses.“ Arch Virol **90**(3-4):273–288.

Chowdhury, S. I., Ludwig, H., Buhk, H. J. (1988). „Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts.“ Virus Res **11**(2):127–139.

Chowdhury, S. I., Buhk, H. J., Ludwig, H., Hammerschmidt, W. (1990). „Genomic termini of equine herpesvirus 1.“ J Virol **64**(2):873–880.

Crabb, B. S. and Studdert, M. J. (1990). „Comparative studies of the proteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3: antibody response of the natural hosts.“ J Gen Virol **71**(9):2033–2041.

Crabb, B. S. and Studdert, M. J. (1993). „Epitopes of Glycoprotein G of Equine Herpesviruses 4 and 1 Located near the C Termini Elicit Type-Specific Antibody Responses in the Natural Host“ J Virol **67**(10):6332-6338.

Crabb, B. S. and Studdert, M. J. (1995). „Equine herpesviruses 4 (equine rhinopneumonitis virus) and 1 (equine abortion virus).“ Adv Virus Res **45**:153–190.

Crandell, R. A., Ichimura, H., Kit, S. (1988). „Isolation and comparative restriction endonuclease DNA fingerprinting of equine herpesvirus-1 from cattle.“ Am J Vet Res **49**(11):1807–1813.

Crowhurst, F. A., Dickinson, G., Burrows, R. (1981). „An outbreak of paresis in mares and geldings associated with equid herpesvirus 1.“ Vet Rec **109**(24):527–528.

Darlington, R. W. and Randall, C. C. (1963). „The nucleic acid content of equine abortion virus.“ Virology **19**:322–327.

Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellett, P. E., Roizman, B., Studdert, M. J., Thiry, E. (2009). „The order herpesvirales.“ Arch Virol **154**(1):171–177.

Diallo, I. S., Hewitson, G., Wright, L., Rodwell, B. J., Corney, B. G. (2006). „Detection of equine herpesvirus type 1 using a real-time polymerase chain reaction.“ J Virol Methods **131**(1):92–98.

Doll, E. R., Wallace, E., Richards, M. G. (1954). „Thermal, hematological, and serological responses of weanling horses following inoculation with equine abortion virus: its similarity to equine influenza.“ Cornell Vet **44**(2):181–190.

Easton, C., Fuentealba, N. A., Paullier, C., Alonzo, P., Carluccio, J., Galosi, C. M. (2009). „Immunohistochemical and molecular detection of equine herpesvirus 1 in Uruguay.“ Rev Sci Tech **28**(3):1085–1090.

Edington, N., Bridges, C. G., Huckle, A. (1985). „Experimental reactivation of equid herpesvirus 1 (EHV-1) following the administration of corticosteroids.“ Equine Vet J **17**(5):369–372.

Edington, N., Bridges, C. G., Patel, J. R. (1986). „Endothelial cell infection and thrombosis in paralysis caused by equid herpesvirus-1: equine stroke.“ Arch Virol **90**(1-2):111–124.

Edington, N., Smyth, B., Griffiths, L. (1991). „The role of endothelial cell infection in the endometrium, placenta and foetus of equid herpesvirus 1 (EHV-1) abortions.“ J Comp Pathol **104**(4):379–387.

Elia, G., Decaro, N., Martella, V., Campolo, M., Desario, C., Lorusso, E., Cirone, F., Buonavoglia, C. (2006). „Detection of equine herpesvirus type 1 by real time PCR.“ J Virol Methods **133**(1):70–75.

Field, H. J., Biswas, S., Mohammad, I. T. (2006). „Herpesvirus latency and therapy–from a veterinary perspective.“ Antiviral Res **71**(2-3):127– 133.

Fraser, N. W., Block, T. M., Spivack, J. G. (1992). „The latency-associated transcripts of herpes simplex virus: RNA in search of function.“ Virology **191**(1):1–8.

Friday, P. A., Scarratt, W. K., Elvinger, F., Timoney, P. J., Bonda, A. (2000). „Ataxia and paresis with equine herpesvirus type 1 infection in a herd of riding school horses.“ J Vet Intern Med **14**(2):197–201.

Fritsche, A.-K. and Borchers, K. (2011). „Detection of neuropathogenic strains of equid herpesvirus 1 (EHV-1) associated with abortions in Germany.“ Vet Microbiol **147**(1-2):176-80.

Fukushi, H., Tomita, T., Taniguchi, A., Ochiai, Y., Kirisawa, R., Matsumura, T., Yanai, T., Masegi,T., Yamaguchi, T., Hirai, K. (1997). „Gazelle herpesvirus 1: a new neurotropic herpesvirus immunologically related to equine herpesvirus 1.“ Virology **227**(1):34–44.

Fukushi, H., Yamaguchi, T., Yamada, S. (2012). „Complete genome sequence of equine herpesvirus type 9.“ J. Virol. **86**(24):13822.

Gallo, M. L., Dorsky, D. I., Crumpacker, C. S., Parris, D. S. (1989). „The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase.“ J Virol **63**(12):5023–5029.

Garcia-Blanco, M. A. and Cullen, B. R. (1991). „Molecular basis of latency in pathogenic human viruses.“ Science **254**(5033):815–820.

Garré, B., van der Meulen, K., Nugent, J., Neyts, J., Croubels, S., Backer, P. D., Nauwynck, H. (2007). „*In vitro* susceptibility of six isolates of equine herpesvirus 1 to acyclovir, ganciclovir, cidofovir, adefovir, PMEDAP and foscarnet.“ Vet Microbiol **122**(1- 2):43–51.

Gerst, S., Borchers, K., Gower, S. M., Smith, K. C. (2003). „Detection of EHV-1 and EHV-4 in placental sections of naturally occurring EHV-1- and EHV-4-related abortions in the UK: use of the placenta in diagnosis.“ Equine Vet J **35**(5):430–433.

Ghanem, Y. M., Fukushi, H., Ibrahim, E. S. M., Ohya, K., Yamaguchi, T., Kennedy, M. (2008). „Molecular phylogeny of equine herpesvirus 1 isolates from onager, zebra and thomson's gazelle.“ Arch Virol **153**(12):2297–2302.

Gibson, J. S., Slater, J. D., Awan, A. R., Field, H. J. (1992). „Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation.“ Arch Virol **123**(3-4):351–366.

Gilkerson, J. R. and Barrett, E. J. (2008). „Equine herpesvirus neurological disease.“ Equine Vet J **40**(2):102–103.

Goehring, L. S. and van Oldendruitenborgh-Oosterbann M. M. S. (2001). „The mystery of equine herpes myeloencephalopathy.“ Equine Veterinary Education **13**:36-42.

Goehring, L. S., van Maanen, C., van Oldruitenborgh-Oosterbaan, M. M. S. (2005). „Neurological syndromes among horses in the Netherlands. A 5 year retrospective survey (1999- 2004).“ Vet Q **27**(1):11–20.

Goehring, L. S., van Maanen, C., Berendsen, M., Cullinane, A., de Groot, R.J., Rottier, P.J., Wesselingh, J.J., van Oldruitenborgh-Oosterbaan, M. M. S. (2010). „Experimental infection with neuropathogenic equid herpesvirus type 1 (EHV-1) in adult horses.“ Vet J. **186**(2):180-7.

Goehring, L. S., Hussey, G. S., Ashton, L. V., Schenkel, A. R., Lunn, D. P. (2011). „Infection of central nervous system endothelial cells by cell-associated EHV-1.“ Vet Microbiol **148**(2-4):389-395.

Goodman, L. B., Wagner, B., Flaminio, M. J. B. F., Sussman, K. H., Metzger, S. M., Holland, R., Osterrieder, N. (2006). „Comparison of the efficacy of inactivated combination and modified-live virus vaccines against challenge infection with neuropathogenic equine herpesvirus type 1 (EHV-1).“ Vaccine **24**(17):3636– 3645.

Goodman, L. B., Loregian, A., Perkins, G. A., Nugent, J., Buckles, E. L., Mercorelli, B., Kydd, J. H., Palu, G., Smith, K. C., Osterrieder, N., Davis-Poynter, N. (2007). „A point mutation in a herpesvirus polymerase determines neuropathogenicity.“ PLoS Pathog **3**(11):e160.

Gosztonyi, G., Borchers, K., Ludwig, H. (2009). „Pathogenesis of equine herpesvirus-1 infection in the mouse model.“ APMIS **117**(1):10–21.

Greenwood, R. E. and Simson, A. R. (1980). „Clinical report of a paralytic syndrome affecting stallions, mares and foals on a thoroughbred studfarm.“ Equine Vet J **12**(3):113–117.

Hasebe, R., Kimura, T., Sato, E., Okazaki, K., Ochiai, K., Wada, R., Umemura, T. (2002). „Equine herpesvirus-1-induced encephalomyelitis in mice: a comparative study of neuroadapted virus and its parental strain.“ J Comp Pathol **127**(2-3):118–125.

Hebia-Fellah, I., Léauté, A., Fiéni, F., Zientara, S., Imbert-Marcille, B.-M., Besse, B., Fortier, G., Pronost, S., Miszczak, F., Ferry, B., Thorin, C. Pellerin, J.-L., Bruyas, J.-F. (2009). „Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR).“ Theriogenology **71**(9):1381–1389.

Heldens, J. G., Hannant, D., Cullinane, A. A., Prendergast, M. J., Mumford, J. A., Nelly, M., Kydd, J. H., Weststrate, M. W, van den Hoven, R. (2001). „Clinical and virological evaluation of the efficacy of an inactivated EHV-1 and EHV-4 whole virus vaccine (Duvaxyn EHV1,4). Vaccination/challenge experiments in foals and pregnant mares.“ Vaccine **19**(30):4307–4317.

Henninger, R. W., Reed, S. M., Saville, W. J., Allen, G. P., Hass, G. F., Kohn, C. W., Sofaly, C. (2007). „Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian center.“ J Vet Intern Med **21**(1):157–165.

Hornyák, A., Bakonyi, T., Kulik, M., Kecskeméti, S., Rusvai, M. (2006). „Application of polymerase chain reaction and virus isolation techniques for the detection of viruses in aborted and newborn foals.“ Acta Vet Hung **54**(2):271–279.

Hussey, S. B., Clark, R., Lunn, K. F., Breathnach, C., Soboll, G., Whalley, J. M., Lunn, D. P. (2006). „Detection and quantification of equine herpesvirus-1 viremia and nasal shedding by real-time polymerase chain reaction.“ J Vet Diagn Invest **18**(4):335–342.

Ibrahim, E. S. M., Kinoh, M., Matsumura, T., Kennedy, M., Allen, G. P., Yamaguchi, T., Fukushi, H. (2007). „Genetic relatedness and pathogenicity of equine herpesvirus 1 isolated from onager, zebra and gazelle.“ Arch Virol **152**(2):245–255.

Jones, T. C., Gleiser, C. A., Maurer, F. D., Hale, M. W., Roby, T. O. (1948). „Transmission and immunisation studies on equine influenza.“ J Am vet med Ass **9**:243-253.

Kasem, S., Yamada, S., Kiupel, M., Woodruff, M., Ohya, K., Fukushi, H. (2008). „Equine herpesvirus type 9 in giraffe with encephalitis.“ Emerg Infect Dis **14**(12):1948–1949.

Kasem, S., Yu, M. H. H., Yamada, S., Kodaira, A., Matsumura, T., Tsujimura, K., Madbouly, H., Yamaguchi, T., Ohya, K., Fukushi, H. (2010). „The ORF37 (UL24) is a neuropathogenicity determinant of equine herpesvirus 1 (EHV-1) in the mouse encephalitis model.“ Virology **400**(2):259–270.

Kennedy, M., Ramsay, E., Diderrich, V., Richmann, L., Allen, G., Potgieter, L. (1996). „Encephalitis associated with a variant of equine herpesvirus 1 in a thomson‘s gazelle (*Gazella thomsoni*).“ Journal of Zoo and Wildlife Medicine **27**(4):533-538.

Kirisawa, R., Ohmori, H., Iwai, H., Kawakami, Y. (1993). „The genomic diversity among equine herpesvirus-1 strains isolated in Japan.“ Arch Virol **129**(1-4):11–22.

Kodama, A., Yanai, T., Yomemaru, K., Sakai, H., Masegi, T., Yamada, S., Fukushi, H., Kuraishi, T., Hattori, S., Kai, C. (2007). „Acute neuropathogenicity with experimental infection of equine herpesvirus 9 in common marmosets (*Callithrix jacchus*).“ J Med Primatol **36**(6):335–342.

Leutenegger, C. M., Madigan, J. E., Mapes, S., Thao, M., Estrada, M., Pusterla, N. (2008). „Detection of EHV-1 neuropathogenic strains using real-time PCR in the neural tissue of horses with myeloencephalopathy.“ Vet Rec **162**(21):688–690.

Loregian, A., Case, A., Cancellotti, E., Valente, C., Marsden, H. S., Palu, G. (2006). „Cloning, expression, and functional characterization of the equine herpesvirus 1 DNA polymerase and its accessory subunit.“ J Virol **80**(13):6247–6258.

Lunn, D. P., Davis-Poynter, N., Flaminio, M. J. B. F., Horohov, D. W., Osterrieder, K., Pusterla, N., Townsend, H. G. G. (2009). „Equine herpesvirus-1 consensus statement.“ J Vet Intern Med **23**(3):450–461.

Ma, G., Lu, C., Osterrieder, N. (2010). „Residue 752 in DNA polymerase of equine herpesvirus type 1 is non-essential for virus growth *in vitro*.“ J Gen Virol **91**(7):1817–1822.

Malik, P., Pálfi, V., Bálint, A. (2010). „Development of a new primer- probe energy transfer method for the differentiation of neuropathogenic and non-neuropathogenic strains of equine herpesvirus-1.“ J Virol Methods **169**(2):425– 427.

Matsumura, T., Sugiura, T., Imagawa, H., Fukunaga, Y., Kamada, M. (1992). „Epizootiological aspects of type 1 and type 4 equine herpesvirus infections among horse populations.“ J Vet Med Sci **54**(2):207–211.

Mayr, A., Böhm, H. O., Brill, J., Woyciechowska, S. (1965). „Characterization of an equine abortion virus from Poland and comparison with known equine rhinopneumonitis virus strains.“ Arch Gesamte Virusforsch **17**(2):216–230.

Montali, R. J., Allen, G. P., Bryans, J. T., Phillips, L. G., Bush, M. (1985). „Equine herpesvirus type 1 abortion in an onager and suspected herpesvirus myelitis in a zebra.“ J Am Vet Med Assoc **187**(11):1248–1249.

Mori, E., Borges, A. S., Delfiol, D. J. Z., Filho, J. P. O., Goncalves, R. C., Cagnini, D. Q., Lara, M. C. C. S. H., Cunha, E. M. S., Villalobos, E. M. C., Nassar, A. F. C., Castro, A. M. M. G., Brandao, P. E., Richtzenhain, L. J. (2011). „First detection of the equine herpesvirus 1 neuropathogenic variant in Brazil.“ Rev Sci Tech **30**(3):949–954.

Nowotny, N., Burtscher, H., Bürki, F. (1987). „Neuropathogenicity for suckling mice of equine herpesvirus 1 from the lipizzan outbreak 1983 and of selected other EHV-1 strains.“ Zentralbl Veterinarmed B **34**(6):441–448.

Nugent, J., Birch-Machin, I., Smith, K. C., Mumford, J. A., Swann, Z., Newton, J. R., Bowden, R. J., Allen, G. P., Davis-Poynter, N. (2006). „Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks.“ J Virol **80**(8):4047–4060.

Ocampos, G. P. M., Fuentealba, N. A., Sguazza, G. H., Jones, L. R., Cigliano, M. M., Barbeito, C. G., Galosi, C. M. (2009). „Genomic and phylogenetic analysis of Argentinian equid herpesvirus 1 strains.“ Virus Genes **38**(1):113–117.

Ostlund, E. N. (1993). „The equine herpesviruses.“ Vet Clin North Am Equine Pract **9**(2):283–294.

Pagamjav, O., Yamada, S., Ibrahim, E.-S. M., Crandell, R. A., Matsumura, T., Yamaguchi, T., Fukushi, H. (2007). „Molecular characterization of equine herpesvirus 1 (EHV-1) isolated from cattle indicating no specific mutations associated with the interspecies transmission.“ Microbiol Immunol **51**(3):313–319.

Parsonson, I. M. and Snowdon, W. A. (1975). „The effect of natural and artificial breeding using bulls infected with, or semen contaminated with, infectious bovine rhinotracheitis virus.“ Aust Vet J **51**(8):365–369.

Patel, J. R., Edington, N., Mumford, J. A. (1982). „Variation in cellular tropism between isolates of equine herpesvirus-1 in foals.“ Arch Virol **74**(1):41–51.

Patel, J. R. and Heldens, J. (2005). „Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)–epidemiology, disease and immunoprophylaxis: a brief review.“ Vet J **170**(1):14–23.

Perkins, G. A., Goodman, L. B., Dubovi, E. J., Kim, S. G., Osterrieder, N. (2008). „Detection of equine herpesvirus-1 in nasal swabs of horses by quantitative real-time PCR.“ J Vet Intern Med **22**(5):1234–1238.

Perkins, G. A., Goodman, L. B., Tsujimura, K., Van de Walle, G. R., Kim, S. G., Dubovi, E. J., Osterrieder, N. (2009). „Investigation of the prevalence of neurologic equine herpes virus type 1 (EHV-1) in a 23-year retrospective analysis (1984-2007).“ Vet Microbiol **139**(3-4):375–378.

Petzoldt, K., Rosenbruch, M., Thein, P., Merkt, H., Schulze-Spüntrup, J. (1982). „Cases of paresis and paralysis in a german thoroughbred stud.“ Berl Münch Tierärztl Wochenschr **95**(5):81–85.

Pronost, S., Cook, R. F., Fortier, G., Timoney, P. J., Balasuriya, U. B. R. (2010a). „Relationship between equine herpesvirus-1 myeloencephalopathy and viral genotype.“ Equine Vet J **42**(8):672–674.

Pronost, S., Leon, A., Legrand, L., Fortier, C., Miszczak, F., Freymuth, F., Fortier, G. (2010b). „Neuropathogenic and non-neuropathogenic variants of equine herpesvirus 1 in France.“ Vet Microbiol **145**(3-4):329–333.

Pronost, S., Legrand, L., Pitel, P.-H., Wegge, B., Lissens, J., Freymuth, F., Richard, E., Fortier, G. (2012) „Outbreak of equine herpesvirus myeloencephalopathy in France: a clinical and molecular investigation.“ Transbound Emerg Dis **59**(3):256–263.

Purifoy, D. J., Lewis, R. B., Powell, K. L. (1977). „Identification of the herpes simplex virus DNA polymerase gene.“ Nature **269**:621–623.

Pusterla, N., Wilson, W. D., Madigan, J. E., Ferraro, G. L. (2009a). „Equine herpesvirus-1 myeloencephalopathy: a review of recent developments.“ Vet J **180**(3):279–289.

Pusterla, N., Wilson, W. D., Mapes, S., Finno, C., Isbell, D., Arthur, R. M., Ferraro, G. L. (2009b). „Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in california.“ Vet J **179**(2):230–239.

Pusterla, N., Hussey, S. B., Mapes, S., Johnson, C., Collier, J. R., Hill, J., Lunn, D. P., Wilson, W. D. (2010a). „Molecular investigation of the viral kinetics of equine herpesvirus-1 in blood and nasal secretions of horses after corticosteroid-induced recrudescence of latent infection.“ J Vet Intern Med **24**(5):1153–1157.

Pusterla, N., Mapes, S., Wilson, W. D. (2010b). „Prevalence of equine herpesvirus type 1 in trigeminal ganglia and submandibular lymph nodes of equids examined postmortem.“ Vet Rec **167**(10):376–378.

Pusterla, N., Mapes, S., Wilson, W. D. (2012). „Prevalence of latent alpha-herpesviruses in thoroughbred racing horses.“ Vet J **193**(2):579-82.

Roizman, B. and Sears, A. E. (1987). „An inquiry into the mechanisms of herpes simplex virus latency.“ Annu Rev Microbiol **41**:543–571.

Schrenzel, M. D., Tucker, T. A., Donovan, T. A., Busch, M. D. M., Wise, A. G., Maes, R. K., Kiupel, M. (2008). „New hosts for equine herpesvirus 9.“ Emerg Infect Dis **14**(10):1616–1619.

Schröer, U., Lange, A., Glatzel, P., Ludwig, H., Borchers, K. (2000). „Relevance of infection with equine herpesvirus 1 (EHV-1) in a german thoroughbred stud: vaccination, abortion and diagnosis.“ Berl Münch Tierärztl Wochenschr **113**(2):53–59.

Slater, J. D., Borchers, K., Thackray, A. M., Field, H. J. (1994). „The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse.“ J Gen Virol **75**(8):2007–2016.

Slater, J. D., Lunn, D. P., Horohov, D. W., Antczak, D. F., Babiuk, L., Breathnach, C., Chang, Y.-W., Davis-Poynter, N., Edington, N., Ellis, S., Foote, C., Goehring, L., Kohn, C. W., Kydd, J., Matsumura, T., Minke, J., Morley, P., Mumford, J., Neubauer, T., O'Callaghan, D., Osterrieder, K., Reed, S., Smith, K., Townsend, H., van der Meulen, K. Whalley, M., Wilson, W. D. (2006). „Report of the equine herpesvirus-1 havermeyer workshop, San Gimignano, Tuscany, June 2004.“ Vet Immunol Immunopathol **111**(1- 2):3–13.

Smith, P. C. (1976). „The bovine herpesviruses: an overview.“ Proc Annu Meet U S Anim Health Assoc **80**:149–158.

Smith, K. C., Mumford, J. A., Lakhani, K. (1996). „A comparison of equid herpesvirus-1 (EHV-1) vascular lesions in the early versus late pregnant equine uterus.“ J Comp Pathol **114**(3):231–247.

Smith, K. C., Whitwell, K. E., Mumford, J. A., Hannant, D., Blunden, A. S., Tearle, J. P. (2000). „Virulence of the V592 isolate of equid herpesvirus-1 in ponies.“ J Comp Pathol **122**(4):288–297.

Smith, K. C. and Borchers, K. (2001). „A study of the pathogenesis of equid herpesvirus-1 (EHV-1) abortion by in-situ Hybridization.“ J. Comp. Pathol **125**:304-310.

Smith, K. L., Allen, G. P., Branscum, A. J., Cook, R. F., Vickers, M. L., Timoney, P. J., Balasuriya, U. B. R. (2009). „The increased prevalence of neuropathogenic strains of EHV-1 in equine abortions.“ Vet Microbiol **141**(1-2):5-11.

Smith, K. L., Li, Y., Breheny, P., Cook, R. F., Henney, P. J., Sells, S., Pronost, S., Lu, Z., Crossley, B. M., Timoney, P. J., Balasuriya, U. B. R. (2012) „New real-time PCR assay using allelic discrimination for detection and differentiation of equine herpesvirus-1 strains with A2254 and G2254 polymorphisms.“ J Clin Microbiol **50**(6):1981–1988.

Soehner, R. L., Gentry, G. A., Randall, C. C. (1965). „Some physicochemical characteristics of equine abortion virus nucleic acid.“ Virology **26**:394–405.

Stierstorfer, B., Eichhorn, W., Schmahl, W., Brandmüller, C., Kaaden, O. R., Neubauer, A. (2002). „Equine herpesvirus type 1 (EHV-1) myeloencephalopathy: a case report.“ J Vet Med B Infect Dis Vet Public Health **49**(1):37–41.

Studdert, M. J., Simpson, T., Roizman, B. (1981). „Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases.“ Science **214**(4520):562–564.

Studdert, M. J. (1983). „Restriction endonuclease DNA fingerprinting of respiratory, foetal and perinatal foal isolates of equine herpesvirus type 1.“ Arch Virol **77**(2-4):249–258.

Studdert, M. J., Fitzpatrick, D. R., Horner, G. W., Westbury, H. A., Gleeson, L. J. (1984). „Molecular epidemiology and pathogenesis of some equine herpesvirus type 1 (equine abortion virus) and type 4 (equine rhinopneumonitis virus) isolates.“ Aust Vet J **61**(11):345–348.

Studdert, M. J., Crabb, B. S., Ficorilli, N. (1992). „The molecular epidemiology of equine herpesvirus 1 (equine abortion virus) in Australasia 1975 to 1989.“ Aust Vet J **69** (5), 104–111.

Studdert, M. J., Hartley, C. A., Dynon, K., Sandy, J. R., Slocombe, R. F., Charles, J. A., Milne, M. E., Clarke, A. F., El-Hage, C. (2003). „Outbreak of equine herpesvirus type 1 myeloencephalitis: new insights from virus identification by PCR and the application of an EHV-1-specific antibody detection ELISA.“ Vet Rec **153**(14):417–423.

Sukla, S., Biswas, S., Birkmann, A., Lischka, P., Zimmermann, H., Field, H. J. (2010). „Mismatch primer-based PCR reveals that helicase-primase inhibitor resistance mutations preexist in herpes simplex virus type 1 clinical isolates and are not induced during incubation with the inhibitor.“ J Antimicrob Chemother **65**(7):1347–1352.

Taniguchi, A., Fukushi, H., Matsumura, T., Yanai, T., Masegi, T., Hirai, K. (2000a). „Pathogenicity of a new neurotropic equine herpesvirus 9 (gazelle herpesvirus 1) in horses.“ J Vet Med Sci **62**(2):215–218.

Taniguchi, A., Fukushi, H., Yanai, T., Masegi, T., Yamaguchi, T., Hirai, K. (2000b). „Equine herpesvirus 9 induced lethal encephalomyelitis in experimentally infected goats.“ Arch Virol **145**(12):2619–2627.

Tearle, J. P., Smith, K. C., Boyle, M. S., Binns, M. M., Livesay, G. J., Mumford, J. A. (1996). „Replication of equid herpesvirus-1 (EHV-1) in the testes and epididymides of ponies and venereal shedding of infectious virus.“ J Comp Pathol **115**(4):385–397.

Telford, E. A., Watson, M. S., McBride, K., Davison, A. J. (1992). „The DNA sequence of equine herpesvirus-1.“ Virology **189**(1):304–316.

Telford, E. A., Watson, M. S., Perry, J., Cullinane, A. A., Davison, A. J. (1998). „The DNA sequence of equine herpesvirus-4.“ J Gen Virol **79**(5):1197–1203.

Thein, P. and Härtel, G. (1976). „Untersuchungen zur Virusätiologie respiratorischer Erkrankungen des Pferdes.“ Prakt Tierarzt, Colleg Vet **57**:24–29.

Thein, P. (1996). „Herpesvirusinfektionen des Pferdes.“ Handbuch der tierärztlichen Praxis, Enke Verlag, Stuttgart: 349.

Thein, P. and Huselstein, P. (2000). „Untersuchungen zur Stammvariabilität von Pferdeherpesviren des Typs EHV-4.“ Pferdeheilkunde **16**:479-486.

Tsujimura, K., Oyama, T., Katayama, Y., Muranaka, M., Bannai, H., Nemoto, M., Yamanaka, T., Kondo, T., Kato, M., Matsumura, T. (2012). „Prevalence of equine herpesvirus type 1 strains of neuropathogenic genotype in a major breeding area of Japan.“ J Vet Med Sci **73**(12):1663–1667.

Turan, N., Yildirim, F., Altan, E., Sennazli, G., Gurel, A., Diallo, I., Yilmaz, H. (2012). „Molecular and pathological investigations of EHV-1 and EHV-4 infections in horses in Turkey.“ Res Vet Sci **93**(3):1504–1507.

Vandekerckhove, A. P., Glorieux, S., Gryspeerdt, A. C., Steukers, L., Duchateau, L., Osterrieder, N., de Walle, G.R.V., Nauwynck, H. J. (2010). „Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants.“ J Gen Virol **91**(8):2019–2028.

Van de Walle, G. R., Goupil, R., Wishon, C., Damiani, A., Perkins, G. A., Osterrieder, N. (2009). „A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease.“ J Infect Dis **200**(1):20–25.

Van de Walle, G. R., May, M. A., Peters, S. T., Metzger, S. M., Rosas, C. T., Osterrieder, N. (2010). „A vectored equine herpesvirus type 1 (EHV-1) vaccine elicits protective immune responses against EHV-1 and H3N8 equine influenza virus.“ Vaccine **28**(4):1048–1055.

Van Engelenburg, F. A., Van Schie, F. W., Rijsewijk, F. A., Van Oirschot, J. T. (1995). „Excretion of bovine herpesvirus 1 in semen is detected much longer by PCR than by virus isolation.“ J Clin Microbiol **33**(2):308–312.

Van Maanen, C., Willink, D.L., Smeenk, L.A., Brinkhof, J., Terpstra, C. (2000). „An equine herpesvirus 1 (EHV-1) abortion storm at a riding school.“ Vet Q **22** (2), 83–87.

Van Maanen, C. (2002). „Equine herpesvirus 1 and 4 infections: an update.“ Vet Q **24**(2):58–78.

Varnum, S .M., Streblow, D. N., Monroe, M. E., Smith, P., Auberry, K. J., Pasa-Tolic, L., Wang, D., Camp, 2nd, D. G., Rodland, K., Wiley, S., Britt, W., Shenk, T., Smith, R. D., Nelson, J. A. (2004). „Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome.“ J Virol **78**(20):10960–10966.

Vissani, M. A., Becerra, M. L., Perglione, C. O., Tordoya, M. S., Mino, S., Barrandeguy, M. (2009). „Neuropathogenic and non-neuropathogenic genotypes of equid herpesvirus type 1 in Argentina.“ Vet Microbiol **139** (3-4), 361–364.

Walter, J., Balzer, H.-J., Seeh, C., Fey, K., Bleul, U., Osterrieder, N. (2012). „Venereal shedding of equid herpesvirus-1 (EHV-1) in naturally infected stallions.“ J Vet Intern Med **26**(6):1500–1504.

Wang, C., Mitsuya, Y., Gharizadeh, B., Ronaghi, M., Shafer, R. W. (2007). „Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance.“ Genome Res **17**(8):1195–1201.

Welch, H. M., Bridges, C. G., Lyon, A. M., Griffiths, L., Edington, N. (1992). „Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues.“ J Gen Virol **73**(2):261–268.

Whalley, J. M., Robertson, G. R., Davison, A. J. (1981). „Analysis of the genome of equine herpesvirus type 1: arrangement of cleavage sites for restriction endonucleases EcoRI, BglII and BamHI.“ J Gen Virol **57**(2):307–323.

Wilson, W. D. (1997). „Equine herpesvirus 1 myeloencephalopathy.“ Vet Clin North Am Equine Pract **13**(1):53–72.

Wilsterman, S., Soboll-Hussey, G., Lunn, D. P., Ashton, L. V., Callan, R. J., Hussey, S. B., Rao, S., Goehring, L. S. (2011). „Equine herpesvirus-1 infected peripheral blood mononuclear cell subpopulations during viremia.“ Vet Microbiol **149**(1-2):40–47.

Wohlsein, P., Lehmbecker, A., Spitzbarth, I., Algermissen, D., Baumgärtner, W., Böer, M., Kummrow, M., Haas, L., Grummer, B. (2011). „Fatal epizootic equine herpesvirus 1 infections in new and unnatural hosts.“ Vet Microbiol **149**(3-4):456-60.

Wolff, P. L., Meehan, T. P., Basgall, E. J., Allen, G. P., Sundberg, J. P. (1986). „Abortion and perinatal foal mortality associated with equine herpesvirus type 1 in a herd of grevy's zebra.“ J Am Vet Med Assoc **189**(9):1185–1186.

Yamada, S., Matsumura, T., Tsujimura, K., Yamaguchi, T., Ohya, K., Fukushi, H. (2008). „Comparison of the growth kinetics of neuropathogenic and nonneuropathogenic equid herpesvirus type 1 (EHV-1) strains in cultured murine neuronal cells and the relevance of the D/N(752) coding change in DNA polymerase gene (ORF30).“ J Vet Med Sci **70**(5):505–511.

Yanai, T., Sakai, T., Fukushi, H., Hirai, K., Narita, M., Sakai, H., Masegi, T. (1998). „Neuropathological study of gazelle herpesvirus 1 (equine herpesvirus 9) infection in thomson's gazelles (*Gazella thomsoni*).“ J Comp Pathol **119**(2):159–168.

# 10 Annex

## 10.1 Reference strains

### 10.1.1 Origin of the EHV-1/-4 reference strains and wild animal strains, sample character, EHV-1/-4 classification and processed sample character

| Designation | Year | Source | Sample character | Processed sample | Genotype |
|---|---|---|---|---|---|
| | EHV-1 reference strains | | | | |
| Ab4 | 1985 | Edington et al. (1985) | virus isolate | DNA | EHV-1 |
| AIV | 1986 | Chowdhury et al. (1986) | virus isolate | DNA | EHV-1 |
| Mar87 | 1987 | Thein P. (1987) | virus isolate | DNA | EHV-1 |
| RacH | 1965 | Mayr et al. (1965) | virus isolate | DNA | EHV-1 |
| KentD | 1954 | Doll et al. (1954) | virus isolate | DNA | EHV-1 |
| Army 183 | 1987 | Jones et al. (1948) | virus isolate | DNA | EHV-1 |
| | EHV-4 reference strains | | | | |
| T252 | 1975 | Thein and Härtel (1976) | virus isolate | DNA | EHV-4 |
| KT-4 | 2006 | Borchers et al. (2005) | virus isolate | DNA | EHV-4 |
| | Wild animal EHV strains | | | | |
| Ro-1 | 1988 | Rockborn (1988) | virus isolate | DNA | related to EHV-1 [2,4] |
| T965 | 1996 | Chicago (1986) | virus isolate | DNA | related to EHV-1 [1,4] |
| T529 | 1985 | Montali et al. (1985) | virus isolate | DNA | related to EHV-1 [1,4] |
| T616 | 1986 | Wolff et al. (1986) | virus isolate | DNA | related to EHV-1 [1,4] |
| 94-137 | 1996 | Kennedy et al. (1996) | virus isolate | DNA | related to EHV-1 [2,4] |
| 49 800 | 1996 | Fukushi et al. (1996) | virus isolate | DNA | EHV-9 [3,4] |
| [1] | EHV-1 gB sequence showed 98% homology to that of onager and zebra (Ibrahim et al., 2007) | | | | |
| [2] | EHV-1 showed 97.8 % homology to gB sequence of gazelle (Ibrahim et al., 2007) | | | | |
| [3] | The gB region of EHV-9 showed a 97% homology to EHV-1 (Fukushi et al., 1997) | | | | |
| [4] | EHV isolates (from the zebra, antelope and onager) being close to EHV-1 or EHV-9 (Borchers et al., 2006a; Ghanem et al., 2008) | | | | |
| | EHV-1 strains in cattle | | | | |
| 136A | 1986 | McFerran (1986) | virus isolate | DNA | EHV-1 |
| 136B | 1988 | McFerran (1986) | virus isolate | DNA | EHV-1 |

### 10.1.2 Results of the ORF 30 nested PCR, *Sal*I restriction enzyme analysis and sequencing of the reference strains and wild animal strains

| Isolate | Year | ORF 30 nPCR | REA *Sal*I | Base at ORF 30 position 2254 | Base at ORF 30 position 2258 |
|---|---|---|---|---|---|
| | EHV-1 reference strains | | | | |
| Ab4 | 1985 | positive | positive | G | C |
| AIV | 1986 | positive | positive | G | A |
| Mar87 | 1987 | positive | negative | A | A |
| RacH | 1965 | positive | positive | G | C |
| KentD | 1954 | positive | positive | G | A |
| Army 183 | 1987 | positive | positive | G | A |
| | EHV-4 reference strains | | | | |
| T252 | 1975 | negative | not done | | |
| KT-4 | 2006 | negative | not done | | |
| | Wild animal EHV strains | | | | |
| Ro-1 | 1988 | positive | positive | G | A |
| T965 | 1996 | positive | positive | G | A |
| T529 | 1985 | positive | positive | G | A |
| T616 | 1986 | positive | positive | G | A |
| 94-137 | 1996 | positive | positive | G | A |
| 49 800 | 1996 | positive | positive | G | A |
| | EHV-1 strains in cattle | | | | |
| 136A | 1986 | positive | positive | G | A |
| 136B | 1988 | positive | positive | G | A |

### 10.1.3 Sequences of the reference strains and wild animal strains

Alignment of the ORF 30 sequences for the investigated reference strains. blue=$N_{752}/A_{2254}$, red=$D_{752}/G_{2254}$, green=$Y_{753}/A_{2258}$, magenta=$S_{753}/C_{2258}$, underlines represent unique substitutions. Combined with the alignment of the amino acid sequences ORF 30 EHV-1 investigated strains, generated using MacVector. blue=$N_{752}$, red=$D_{752}$, green=$Y_{753}$, magenta=$S_{753}$:

| Isolate | 3´-2271 | Sequence | | | | ← direction | | | | | 2242-5´ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | EHV-1 reference strains | | | | | | | | | | | |
| Ab4 | 3‘- | GAG | CTT | GCA | GCT | CCT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | S | D | V | S | P | Q | |
| AIV | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |

| Isolate | 3´-2271 | | | Sequence | | | ← direction | | | | 2242-5´ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mar87 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q |
| Army183 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| Kent D | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| RacH | 3‘- | GAG | CTT | GCA | GCT | CCT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | S | D | V | S | P | Q |
| | Wild equid strains | | | | | | | | | | |
| Ro-1 | 3‘- | GAG | CTT | GCA | ACT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| T 965 | 3‘- | GAG | CTT | GCA | ACT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| T 529 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| T 616 | 3‘- | GAG | CTT | GCA | ACT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| 94-137 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| D 49800 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| | EHV-1 cattle strains | | | | | | | | | | |
| 136 A | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |
| 136 B | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q |

## 10.2 Abortion cases

### 10.2.1 Origin of the 67 EHV-1 abortion cases, sample character, processed sample character, ORF 30 nested PCR and *Sal* I restriction enzyme results. Selection of the ORF 30 amplicons for sequencing

| Designation | Year | Source | Origin | Sample character | Processed sample | ORF 30 nested PCR | REA *Sal* I | Sequencing |
|---|---|---|---|---|---|---|---|---|
| T952 | 1987 | Neubauer | single case | virus isolate | DNA | positive | **positive** | **yes** |
| E510/89 | 1989 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| E525/89 | 1989 | Neubauer | single case | virus isolate | DNA | positive | **positive** | **yes** |
| S147/90 | 1990 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| F553/89 | 1990 | Neubauer | single case | virus isolate | DNA | positive | **positive** | **yes** |
| KermannNr.1 | 1991 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| KermannNr.2 | 1991 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| U169-71 | 2003 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| 260.3 | 2003 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| Y30 | 2003 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| T759/61 | 2003 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| (A)E271-3 | 2004 | Neubauer | single case | virus isolate | DNA | positive | **positive** | **yes** |
| S138/04 | 2004 | Virology FU Berlin | single case | virus isolate | DNA | positive | negative | no |
| S155/04 | 2004 | Virology FU Berlin | single case | virus isolate | DNA | positive | negative | no |
| S415/04 | 2004 | Virology FU Berlin | single case | virus isolate | DNA | positive | **positive** | **yes** |
| S587/04 | 2004 | Virology FU Berlin | single case | virus isolate | DNA | positive | negative | **yes** |
| A258 | 2004 | Neubauer | single case | virus isolate | DNA | positive | **positive** | **yes** |
| E854/6 | 2006 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| E216-18 | 2006 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| E573-5 | 2006 | Neubauer | single case | virus isolate | DNA | positive | negative | no |
| E113-115 | 2006 | Neubauer | single case | virus isolate | DNA | positive | negative | **yes** |
| S65/06 | 2006 | Virology | single case | virus isolate | DNA | positive | negative | no |

| Designation | Year | Source | Origin | Sample character | Processed sample | ORF 30 nested PCR | REA *Sal* I | Sequencing |
|---|---|---|---|---|---|---|---|---|
| S173/06 | 2006 | FU Berlin Virology | single case | virus isolate | DNA | positive | negative | **yes** |
| S208/06 | 2006 | FU Berlin Virology | single case | virus isolate | DNA | positive | negative | no |
| S101/07 | 2007 | FU Berlin Pathology | single case | virus isolate | DNA | positive | negative | no |
| S144/07 | 2007 | Dr. Haider Pathology | single case | virus isolate | DNA | positive | negative | no |
| S152/07 | 2007 | FU Berlin Pathology | single case | virus isolate | DNA | positive | negative | no |
| S245/07 | 2007 | Dr. Haider Pathology | outbreak stud farm 1 | virus isolate | DNA | positive | negative | no |
| S260/07 | 2007 | FU Berlin Pathology | single case | virus isolate | DNA | positive | negative | no |
| S275/07 | 2007 | FU Berlin Pathology | outbreak stud farm 1 | virus isolate | DNA | positive | negative | **yes** |
| S276/07 | 2007 | FU Berlin Pathology | outbreak stud farm 1 | virus isolate | DNA | positive | negative | no |
| S299/07 | 2007 | FU Berlin Pathology | outbreak stud farm 1 | virus isolate | DNA | positive | negative | no |
| V07-5-61 | 2007 | FU Berlin Pathology | outbreak stud farm 1 | virus isolate | DNA | positive | negative | no |
| S309/07 | 2007 | FU Berlin Pathology | outbreak stud farm 1 | virus isolate | DNA | positive | negative | **yes** |
| S319/07 | 2007 | FU Berlin Pathology | outbreak | virus isolate | DNA | positive | negative | no |

| Designation | Year | Source | Origin | Sample character | Processed sample | ORF 30 nested PCR | REA *Sal* I | Sequencing |
|---|---|---|---|---|---|---|---|---|
| | | FU Berlin | stud farm 1 | | | | | |
| E1470/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| E1471/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| E1472/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| S335/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| E1606/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| S384/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| E1730/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| S471/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | **yes** |
| | | FU Berlin | stud farm 1 | | | | | |
| S476/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| S477/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| S483/07 | 2007 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | FU Berlin | stud farm 1 | | | | | |
| S38/08 | 2008 | Pathology | outbreak | virus isolate | DNA | positive | negative | no |
| | | Dr. Haider | stud farm 2 | | | | | |
| S45/08 | 2008 | Pathology | outbreak | virus isolate | DNA | positive | negative | **yes** |

| Designation | Year | Source | Origin | Sample character | Processed sample | ORF 30 nested PCR | REA *Sal* I | Sequencing |
|---|---|---|---|---|---|---|---|---|
| S49/08 | 2008 | Dr. Haider Pathology | stud farm 2 outbreak | virus isolate | DNA | positive | negative | no |
| S55/08 | 2008 | Dr. Haider Pathology | stud farm 2 outbreak | virus isolate | DNA | positive | negative | no |
| S59/08 | 2008 | Dr. Haider Pathology | stud farm 2 outbreak | virus isolate | DNA | positive | negative | no |
| S150/08 | 2008 | Dr. Haider Pathology | stud farm 2 outbreak | virus isolate | DNA | positive | negative | **yes** |
| S261/08 | 2008 | Dr. Haider Pathology | stud farm 2 single case | virus isolate | DNA | positive | negative | **yes** |
| S72/09 | 2009 | Pathology FU Berlin | single case | virus isolate | DNA | positive | negative | no |
| V09-3-2/ Mare I | 2009 | stud veterinarian | outbreak stud farm 3 | nasal swab | DNA | positive | **positive** | **yes** |
| V09-3-3/ Mare II | 2009 | stud veterinarian | outbreak stud farm 3 | nasal swab | DNA | positive | **positive** | **yes** |
| S159/09 | 2009 | Pathology FU Berlin | single case | virus isolate | DNA | positive | negative | no |
| S238/09 | 2009 | Pathology Dr. Haider | single case | virus isolate | DNA | positive | negative | no |
| S604/09 | 2009 | Pathology FU Berlin | single case | virus isolate | DNA | positive | negative | no |
| PA284 | 2009 | Koblenz | single case | virus isolate | DNA | positive | negative | no |
| PA332 | 2009 | Koblenz | single case | virus isolate | DNA | positive | negative | no |
| 624 | 2009 | Saxony-Anhalt | single case | virus isolate | DNA | positive | negative | no |
| 647 | 2009 | Saxony-Anhalt | single case | virus isolate | DNA | positive | negative | no |

| Designation | Year | Source | Origin | Sample character | Processed sample | ORF 30 nested PCR | REA *Sal* I | Sequencing |
|---|---|---|---|---|---|---|---|---|
| 681 | 2009 | Saxony-Anhalt | single case | virus isolate | DNA | positive | negative | no |
| 470/Mare C | 2009 | stud veterinarian | outbreak stud farm 4 | virus isolate | DNA | positive | negative | **yes** |
| 541/Mare F | 2009 | stud veterinarian | outbreak stud farm 4 | virus isolate | DNA | positive | negative | **yes** |
| Mare A | 2009 | stud veterinarian | outbreak stud farm 4 | placenta | DNA | positive | negative | **yes** |

**Stud farm 3:** The samples listed below came from two in the table above already considered abortion cases (V09-3-2/MareI, V09-3-3/MareII). Since different samples from these two abortion cases and one semen sample from a stallion from the same abortion outbreak at stud farm 3 were sent for investigation, it is here more clearly representable.
Additionally the abortion isolate P5276 is listed here:

| Designation | Year | Source | Sample character | Genotype | Processed sample | ORF 30 nested PCR | REA *Sal* I | Sequencing |
|---|---|---|---|---|---|---|---|---|
| Mare I | | | | | | | | |
| V09-3-2 | 2009 | stud farm 3 | nasal swab | EHV-1 | DNA | positive | **positive** | **yes** |
| V09-3-12 | 2009 | stud farm 3 | lung fluid | EHV-1 | DNA | positive | **positive** | **yes** |
| V09-3-15 | 2009 | stud farm 3 | amniotic fluid | EHV-1 | DNA | positive | **positive** | **yes** |
| Mare II | | | | | | | | |
| V09-3-3 | 2009 | stud farm 3 | nasal swab | EHV-1 | DNA | positive | **positive** | **yes** |
| V09-3-23 | 2009 | stud farm 3 | lung fluid | EHV-1 | DNA | positive | **positive** | **yes** |
| Stallion | | | | | | | | |
| V09-4-1 | 2009 | stud farm 3 | sperm | EHV-1 | DNA | positive | **positive** | **yes** |
| | single abortion case | | | | | | | |
| P5276 | not known | Hübert | virus isolate | EHV-1 | DNA | positive | negative | **yes** |

### 10.2.2 Sequences of the abortion strains from 1987 to 2009

Excerpt of the alignment of the ORF 30 sequences for the investigated strains. blue=$N_{752}/A_{2254}$, red=$D_{752}/G_{2254}$, green=$Y_{753}/A_{2258}$, magenta=$S_{753}/C_{2258}$, underlines represent unique substitutions. Combined with the alignment of the amino acid sequences ORF 30 EHV-1 investigated strains, generated using MacVector. blue=$N_{752}$, red=$D_{752}$, green=$Y_{753}$, magenta=$S_{753}$:

| Isolate | 3´-2271 | | | Sequence | | | ← direction | | | | 2242-5´ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T 952 | 3‘- | GAG | CTT | GCA | GCT | CCT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | S | D | V | S | P | Q | |
| E 525/89 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| F 553/89 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| S415/04 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| S587/04 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| A(E)271-3 | 3‘- | GA<u>A</u> | CTT | GCA | GCT | CAT | CAG | GTC | CCT | ACC | AAC | -5‘ |
| | | <u>K</u> | F | T | S | Y | D | V | S | P | Q | |
| A 258 | 3‘- | GA<u>A</u> | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | <u>K</u> | F | T | S | Y | D | V | S | P | Q | |
| S173/06 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| E113/115 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| S275/07 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| S309/07 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| S471/07 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| S45/08 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| S150/08 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| S261/08 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | CCA | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |
| V09-3-2/ | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| Mare I | | E | F | T | S | Y | D | V | S | P | Q | |
| V09-3-3/ | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| Mare II | | E | F | T | S | Y | D | V | S | P | Q | |
| Mare A | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |

| Isolate | 3´-2271 | | | Sequence | | ← direction | | | | 2242-5´ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | E | F | T | S | Y | N | V | S | P | Q | |
| 541/ | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| Mare F | | E | F | T | S | Y | N | V | S | P | Q | |
| 470/ | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | AAC | -5‘ |
| Mare C | | E | F | T | S | Y | N | V | S | P | Q | |

Excerpt of the alignment of the ORF 30 sequences for the different investigated samples from 2 mares and one stallion from the abortion outbreak at stud farm 3 and the abortion isolate P5276: blue=$N_{752}/A_{2254}$, red=$D_{752}/G_{2254}$, green=$Y_{753}/A_{2258}$, magenta=$S_{753}/C_{2258}$, underlines represent unique substitutions. Combined with the alignment of the amino acid sequences ORF 30 EHV-1 investigated strains, generated using MacVector. blue=$N_{752}$, red=$D_{752}$, green=$Y_{753}$, magenta=$S_{753}$:

| Isolate | 3´-2271 | | | Sequence | | ← direction | | | | 2242-5´ |
|---|---|---|---|---|---|---|---|---|---|---|
| Mare I | | | | | stud farm 3 | | | | | |
| V09-3-2 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| V09-3-12 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| V09-3-15 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| Mare II | | | | | stud farm 3 | | | | | |
| V09-3-3 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| V09-3-23 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| Stallion | | | | | stud farm 3 | | | | | |
| V09-4-1 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |
| | | | | | single abortion case | | | | | |
| P5276 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAA | CTG | CCT | ACC | CAA | -5‘ |
| | | E | F | T | S | Y | N | V | S | P | Q | |

## 10.3 Neurological cases

### 10.3.1 Results of the ORF 30 nested PCR, *Sal*I restriction enzyme analysis and selection of the ORF 30 amplicons for sequencing of neurological cases

| Isolate | ORF 30 nPCR | REA *Sal* I | Sequencing |
|---|---|---|---|
| 3318 /1 | negative | n.d. | no |
| 3318 /2 | positive | **positive** | **yes** |
| 3318 /3 | positive | **positive** | **yes** |
| 834 | positive | **positive** | **yes** |

### 10.3.2 Sequences of the neurological EHV strains

Excerpt of the alignment of the ORF 30 sequences for the investigated strains. blue=$N_{752}/A_{2254}$, red=$D_{752}/G_{2254}$, green=$Y_{753}/A_{2258}$, magenta=$S_{753}/C_{2258}$, underlines represent unique substitutions. Combined with the alignment of the amino acid sequences ORF 30 EHV-1 investigated strains, generated using MacVector. blue=$N_{752}$, red=$D_{752}$, green=$Y_{753}$, magenta=$S_{753}$:

| Isolate | 3´-2271 | | | Sequence | | | ← direction | | | | 2242-5´ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3318/2 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| brain | | E | F | T | S | Y | D | V | S | P | Q | |
| 3318/3 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| organ mix | | E | F | T | S | Y | D | V | S | P | Q | |
| 834 | 3‘- | GAG | CTT | GCA | GCT | CAT | CAG | CTG | CCT | ACC | AAC | -5‘ |
| | | E | F | T | S | Y | D | V | S | P | Q | |

## 10.4 Report of the clinical signs of the sampled horses from stud farm 3

| Group 1 | Horses with neurological signs |
|---|---|
| V09-3-2 | Pregnant mare (term 16.06.09), abortion on 06.03.09. Second vaccination during pregnancy is absent. After the abortion occur and the mare was clinically healthy vaccination with $Duvaxyn_{1,4}$. Evolved 12 hours after vaccination neurological signs, but without fever |
| V09-3-3 | Pregnant mare (term 21.04.09), vaccination with $Duvaxyn_{1,4}$ in month 3./4. and 7./8.. Neurological signs since 28.02.09, no fever. Abortion. |
| V09-3-4 | Four year old gelding, regularly vaccinated with Resequin (EHV-1/-4). Neurological signs since 01.03.09. |
| V09-4-3 | Mare, evolved neurological signs. Vaccinated last time at 03.04.09. |
| Group 2 | Acute febrile horses for less than 2 days. |
| V09-3-5 | Nine year old stallion, evolved fever, not currently vaccinated against EHV-1/-4. |

| | |
|---|---|
| V09-3-6 | Three year old stallion, not vaccinated against EHV-1/-4. Fever since one day over 40°C. |
| V09-3-7 | Three year old unvaccinated stallion. Fever over 40°C since one day. |
| V09-3-11 | Four year old pregnant mare (term april 2009), vaccinated with $\text{Duvaxyn}_{1,4}$ in month 3./4. and 7./8.. Since 28.02.09 treatment with Acyclovir (oral 3 times 9600 mg). Fever since one day. |
| Group 3 | Horses for at least 3 days without fever again. |
| V09-3-8 | Elder gelding, not currently vaccinated against herpes. |
| V09-3-9 | Fourteen year old mare, irregularly vaccinated. |
| V09-3-10 | Three year old mare, regularly vaccinated with $\text{Duvaxyn}_{1,4}$, last time in february 2009. |

## 10.5 Serology

### 10.5.1 Stud farm 3: EHV-1/-4 serum neutralization and immunofluorescence assay

EHV-1/-4 neutralization test from paired blood samples of horses of stud farm 3 with abortions and neurological signs:

| Horse | Gender | Signs | EHV-1 08.03.09 | NT 03.04.09 | EHV-4 08.03.09 | NT 03.04.09 |
|---|---|---|---|---|---|---|
| Group 1: | | | | | | |
| V09-3-2 | mare I | abortion and CNS | 1:40 | 1:40 | 1:20 | 1:40 |
| V09-3-3 | mare II | abortion and CNS | 1:20 | 1:40 | 1:10 | 1:40 |
| V09-3-4 | gelding | CNS | 1:10 | 1:40 | 1:10 | 1:80 |
| V09-4-3 | mare | CNS | no serum | 1:20 | no serum | 1:20 |
| Group 2: | | | | | | |
| V09-3-5 | stallion | fever | ⟨1:10 | 1:20 | ⟨1:10 | 1:20 |
| V09-3-6 | stallion | fever | ⟨1:10 | 1:10 | ⟨1:10 | 1:10 |
| V09-3-7 | stallion | fever | ⟨1:10 | 1:10 | ⟨1:10 | 1:10 |
| V09-3-11 | pregnant mare | fever | ⟨1:10 | 1:80 | ⟨1:10 | 1:40 |
| Group 3: | | | | | | |
| V09-3-8 | gelding | fever | 1:10 | 1:10 | 1:10 | 1:10 |
| V09-3-9 | mare | fever | 1:10 | 1:40 | ⟨1:10 | 1:20 |
| V09-3-10 | mare | fever | 1:20 | 1:40 | 1:40 | 1:80 |

EHV-1/-4 immunofluorescence assay from paired blood samples of horses of stud farm 3 with abortions and neurological signs:

| Horse | Gender | Signs | EHV-1 08.03.09 | IFA 03.04.09 | EHV-4 08.03.09 | IFA 03.04.09 |
|---|---|---|---|---|---|---|
| Group 1: | | | | | | |
| V09-3-2 | mare I | abortion and CNS | 1:10240 | 1:20480 | 1:10240 | 1:20480 |
| V09-3-3 | mare II | abortion and CNS | 1:20480 | 1:20480 | 1:20480 | 1:20480 |
| V09-3-4 | gelding | CNS | 1:2560 | 1:20480 | 1:2560 | 1:20480 |
| V09-4-3 | mare | CNS | no serum | 1:20480 | no serum | 1:20480 |
| Group 2: | | | | | | |
| V09-3-5 | stallion | fever | 1:5120 | 1:20480 | 1:5120 | 1:20480 |
| V09-3-6 | stallion | fever | 1:2560 | 1:20480 | 1:2560 | 1:20480 |
| V09-3-7 | stallion | fever | 1:1280 | 1:10240 | 1:1280 | 1:10240 |
| V09-3-11 | pregnant mare | fever | 1:5120 | 1:20480 | 1:5120 | 1:2048 |
| Group 3: | | | | | | |
| V09-3-8 | gelding | fever | 1:5120 | 1:20480 | 1:5120 | 1:20480 |
| V09-3-9 | mare | fever | 1:5120 | 1:20480 | 1:5120 | 1:20480 |
| V09-3-10 | mare | fever | 1:10240 | 1:20480 | 1:10240 | 1:20480 |

### 10.5.2 Stud farm 4: EHV-1/-4 neutralization test

| Mare | EHV-1 | NT | EHV-4 | NT |
|---|---|---|---|---|
| | 13.07.09 | 21.08.09 | 13.07.09 | 21.08.09 |
| Barn 1: abort | | | | |
| A | 1:10 | 1:20 | ⟨1:10 | 1:40 |
| B | 1:10 | 1:10 | ⟨1:10 | 1:20 |
| C | 1:20 | 1:20 | 1:40 | 1:80 |
| D | 1:10 | 1:10 | 1:10 | 1:20 |
| E | 1:20 | 1:20 | 1:20 | 1:40 |
| F | 1:10 | 1:20 | 1:10 | 1:40 |
| Barn 2: normal delivery | | | | |
| G | ⟨1:10 | 1:20 | ⟨1:10 | 1:40 |
| H | 1:10 | 1:20 | 1:10 | 1:40 |
| I | 1:10 | 1:20 | 1:10 | 1:40 |
| J | 1:20 | 1:40 | 1:10 | 1:40 |
| K | ⟨1:10 | 1:20 | ⟨1:10 | 1:20 |
| L | 1:10 | 1:20 | 1:10 | 1:40 |
| M | 1:10 | 1:40 | ⟨1:10 | 1:40 |

## 10.6 List of own publications

Selected results of this work have been presented elsewhere or published:

Fritsche, A.-K., Borchers, K.
Detection of neuropathogenic strains of Equid Herpesvirus 1 (EHV-1) associated with abortion in Germany.
European Society for Veterinary Virology, 8.th International Congress of Veterinary Virology, Budapest, 23.-26. august 2009 (poster)

Fritsche, A.-K..
Untersuchung zum Vorkommen neuropathogener Stämme des equinen Herpesvirus 1 (EHV-1) bei Aborten in Deutschland.
4. Doktorandensymposium, Freie Universität Berlin, 2009 (talk)

Fritsche, A.-K., Borchers, K.
Detection of neuropathogenic strains of Equid Herpesvirus 1 (EHV-1) associated with abortion in Germany.
Veterinary Microbiology 147 (2011) 176-180

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## 10.8 Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 24.07.2015

Ann-Kathrin Fritsche