Description of an unusually large outbreak of nervous system disorders caused by equine herpesvirus 1 (EHV1) in 2009 in Belgium

Beschrijving van een ongewoon grote uitbraak van zenuwstoornissen veroorzaakt door het equiene herpesvirus 1 (EHV1) in 2009 in België

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ABSTRACT

Neurological diseases caused by neuropathogenic strains of EHV1 are being reported with increasing frequency. Consequently, concern is being voiced within the US horse industry that the neurologic form of EHV1 may be intensifying in prevalence and/or morbidity and mortality. In Belgium, outbreaks of EHV1-induced abortions are an annually recurrent phenomenon, but outbreaks of equine herpes myelitis (EHM) are rare. This report describes an unusually large outbreak of EHV1-induced EHM that involved at least 13 different premises. Seven of them were characterized in more detail and were included in this study. A morbidity of 26% was seen, with an EHM incidence of 43% in the affected horses. The outbreak was characterized by rapid occurrence of ataxia and paralysis immediately after disappearance of the fever. EHV1 was diagnosed by means of virus isolation and/or seroconversion. The isolated virus was classified either as neuropathogenic or as belonging to group 4 after sequencing in the ORF30 and ORF68 regions, respectively.

The extent of this outbreak and the high percentage of neurological disease, along with the fact that EHM is only sporadically seen in Belgium, might indicate that the neurological form of EHV1 is possibly also emerging in Belgium.

SAMENVATTING

Zenuwstoornissen veroorzaakt door neuropathogene stammen van EHV1 worden met stijgende frequentie waargenomen. In de paardenindustrie van de Verenigde Staten is bezorgdheid geuit omtrent het feit dat de prevalentie van de neurologische vorm van EHV1, evenals de morbiditeit en mortaliteit, aan het toenemen zijn. Uitbraken van door EHV1-geïnduceerde abortus worden in België jaarlijks waargenomen, maar uitbraken van equiene herpes myelitis (EHM) zijn zeldzaam. In dit artikel wordt een ongewoon grote uitbraak van EHV1-geïnduceerde EHM beschreven, waarbij tenminste 13 bedrijven betrokken waren. Zeven van hen werden in meer detail onderzocht en werden in voorliggend onderzoek ingesloten. Er werd een morbiditeit gezien van 26%, met een EHM-incidentie van 43% bij de aangetaste paarden. De uitbraak werd gekarakteriseerd door het snel optreden van ataxie en paralyse onmiddellijk na het verdwijnen van de koorts. EHV1 werd gediagnosticeerd door middel van virusisolatie en/of seroconversie. Het geïsoleerde virus werd geclassificeerd als neuropathogeen en behorende tot groep 4 na sequenering in de ORF30- en ORF68-regio respectievelijk.

De omvang van deze uitbraak en het hoge percentage neurologische ziekte, samen met het feit dat EHM slechts sporadisch wordt gezien in België, kunnen erop wijzen dat de neurologische vorm van EHV1 ook in opmars is in België.

INTRODUCTION

Equine herpesvirus type 1 (EHV1), a member of the *Alphaherpesvirinae*, is endemic in the horse population worldwide. After infection, the virus replicates in tissues of the upper respiratory tract. Primary replication is followed by a leukocyte-associated viremia that enables EHV1 to reach internal organs (Gryspeerdt *et al.*, 2009; Kydd *et al.*, 1994a, b). A second replication in endothelial cells at the site of the pregnant uterus may result in abortion and neonatal foal death, and a second replication in the nervous system may result in nervous system disorders (Allen and Bryans, 1986; Gilkerson and Barrett, 2008). The form in the nervous system, which is also designated as equine herpes myelitis (EHM), is a severe condition that is still poorly understood, despite the correlation between a single amino acid variation in the ORF30 region of the viral DNA polymerase and neurological disease (Lunn *et al.*, 2009; Van de Walle *et al.*, 2009).

EHV1 is a ubiquitous pathogen that has a worldwide distribution and is responsible for widespread annual problems with respiratory disorders and abortion. In contrast, outbreaks of nervous system dis-

orders caused by infection with EHV1 seem to be more rare. However, during the last decade, large outbreaks of EHM have been reported in the USA with increasing frequency, and the rate of fatal outcomes after substantial suffering also appear to be on the rise (Lunn *et al.*, 2009). EHV1 is also endemic in the horse population in Belgium, with EHV1-induced abortion as an annually recurrent event. However, EHM is rare and mostly involves a limited number of horses on a single premise (van der Meulen *et al.*, 2000). To date, only one description of EHV1-induced neurological disorders has been reported in Belgium. No extensive spread of EHM was seen during this episode and severe disease was limited to the affected premise. The signs in neurologically affected horses consisted mainly of ataxia and paralysis, but cerebral dysfunctions were also noted (van der Meulen *et al.*, 2003).

The present report describes a large outbreak of EHV1-induced neurological disease in Flanders in 2009 that started on a riding school and spread to at least 12 premises that had been in direct contact with the riding school. The extent of this outbreak, along with the higher percentage of horses with neurological signs, but without the occurrence of cerebral dysfunction, and the rapid occurrence of severe signs, raises the intriguing question as to whether EHM is also emerging in Belgium.

MATERIALS AND METHODS

Description of the premises

During an outbreak of EHV1-induced nervous system disorders in Antwerp, Belgium (June 2009), fever and neurological disease were observed on at least 13 premises. All these premises had attended a jumping contest on May 30th, 2009, at a riding school (premise no. 1) where the first cases of neurological signs occurred. Although 13 premises reported neurological disease, only the data from 7 of these premises has been included in this report. On these premises, EHV1 infection was confirmed by virus isolation and/or seroconversion (Table 1). The other 6 affected premises had several horses with neurological problems, but refused to give any further information and were therefore not included in this study (Figure 1). Information about vaccination state, management and housing can be found in Figure 1 and Table 2. Each premise was visited to evaluate the clinical signs, and local veterinarians were asked to collect blood samples from the horses suspected of infection with EHV1, both for the purpose of virus isolation from leucocytes and for the detection of seroconversion in paired serum samples.

Serological examination

The presence of EHV-specific antibodies was determined with a complement-dependent seroneutralization (SN) test in acute serum samples and in convalescent serum samples three weeks later. Two-fold dilution series of the sera were prepared in MEM. Fifty µl of each of these serial dilutions was incubated for 23 hours (h) at 37°C with a fixed number of infectious virus (300 $TCID_{50}$ of EHV1 strain Arabica in 50 µl). Thereafter, 25 µl of guinea pig complement was added. After 1 h of incubation, the mixture of serum, virus and complement was added to RK13 cells. The inoculated cultures were further incubated at 37°C in an atmosphere containing 5% CO_2. After 7 days of incubation, the cultures were analyzed for the presence of cytopathic effect. The neutralization titer was calculated as the reciprocal of the highest dilution of serum that was able to completely block EHV1 infection in RK13 cells. Seroconversion was present when the antibody titer of the convalescent sample was at least four times higher than in the acute sample.

Table 1. Results from different diagnostic tools to confirm EHV1 infection in discussed horses.

Premise	Horse	Signs at the moment of sampling	Isolation of EHV1 from PBMC Isolation	Antibody titer in serum	
				Acute sample	Convalescent sample
1	1	fever	+	128	1536
	2	quadriplegia	+	64	NA
2	1	fever	+	48	512
	2	fever	+	16	256
3	1	ataxia	+	192	512
	2	ataxia	+	96	1024
	3	ataxia	-	16	256
4	1	quadriplegia	+	96	NA
5	1	fever	+	4	1536
	2	fever	+	12	768
	3	fever	-	8	1024
	4	fever	+	12	2048
6	1	ataxia	+	32	1536
7	1	ataxia	+	4	1536
	2	ataxia	-	8	1536

NA: not available due to euthanasia

Virological examination

Virus isolation from peripheral blood mononuclear cells (PBMC) was performed on RK13 cells. Unclotted blood samples were taken from horses with clinical signs (fever and/or neurological disorders). PBMC were isolated by means of density centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) and subsequently inoculated on monolayers of RK13 cells. These monolayers were incubated at 37°C in an atmosphere containing 5% CO_2 and examined daily for 7 consecutive days for the presence of cytopathic effects.

PCR amplification and sequencing of ORF30 and ORF68

DNA was extracted from EHV1 isolates using a QiaAmp DNA extraction kit (Qiagen Benelux, Venlo, The Netherlands). Amplification of ORF30 was performed using the primers ORF30fw (gctacttctgaaaacggaggc) and ORF30rev (ctatcctcagacacggcaaca). Amplification of ORF68 was performed using either the primer ORF68atg1 (atgggtgtggtcttaattac) or the primer ORF68fw (aagcattgccaaacagttcc), in either case in combination with ORF68end1 (aacgtgtggatgtccggcc).

The PCR products were treated with Exonuclease I and Antarctic Phosphatase (New England Biolabs, Ipswich, USA) and used directly for cycle sequencing with a Big Dye Terminator Cycle sequencing kit V1.1 (Applied Biosystems, Foster City, USA). Additional internal oligonucleotides were used for the sequencing of ORF68. The cycle sequencing reaction products were purified using ethanol precipitation and separated on an ABI Genetic Analyzer 310 (Applied Biosystems, Foster City, USA). The sequences were analyzed and compiled using Align, ClustalW and Sixframe in the workbench (workbench.sdsc.edu) and BlastN and BlastP at www.ncbi.nlm.nih.gov. To validate the sequencing, previously characterized strains 97P70 (Genbank accession number: GU271940), 03P37 (# GU271941) and Ab4 (# AY665713) were included (Garré *et al.*, 2007; Nugent *et al.*, 2006).

RESULTS

Case history (Figure 1 and Table 2)

Initial outbreak

In May and June of 2009 an outbreak of EHV1 occurred in a riding school with 96 horses. This premise (premise no. 1) was a riding school where lessons and contests took place frequently and from where horses

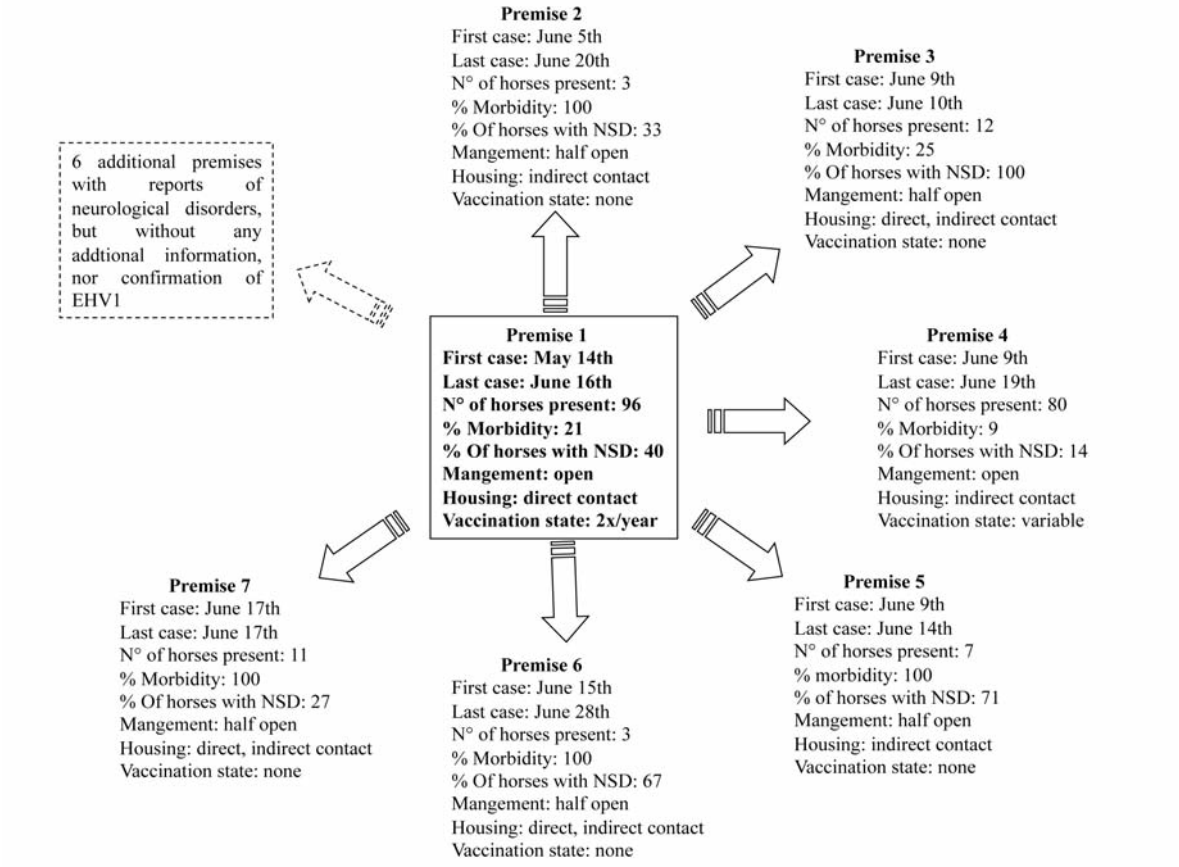

Figure 1. Schematic overview of the course of the outbreak of EHV1-induced neurological disorders on the different premises. NSD: nervous system disorders; open management: public riding school with incoming and outgoing horses; half-open management: private premise, but where horses attend competitions at other premises; closed management: private premise, no contact with other horses or premises; housing with direct contact: stables separated from each other by bars; housing with indirect contact: stables separated from each other by full walls.

were regularly transported to contests outside the premise.

On May 14, the local practitioner was consulted for a 5-year-old mare with high fever. The mare was treated with antibiotics and the fever lasted for 4 days without any further signs. On May 26, another mare (age 20) was presented with high fever. She was also treated with antibiotics. On the fifth day, the fever ceased but the mare was found recumbent in her stable. Since at that point in time there was no suspicion of EHV1-induced neurological disease, a large contest was organized at this riding school, with horses from different premises. On June 1st, two additional mares (9 and 12 years) on premise no. 1 showed fever as well as edema of the hind limbs. The fever lasted for 5 and 3 days, respectively, and no other clinical signs were noted. On June 5, a 9-year-old mare showed fever lasting for 4 days, after which she became severely ataxic and then, shortly thereafter, completely paralyzed. The next day, eight more horses showed high fever, five of which (one mare and four geldings between 4 and 14 years) became clinically normal after a 2-3 day period of fever. One mare (12 years), however, showed paralysis after 3 days of fever. Two other horses showed neurological signs after 5 days of fever. The first horse was a 12-year-old mare that was found with hind leg paralysis. The second horse, a gelding of 12 years, was severely ataxic, being barely able to stand up and showedsevere muscle trembling. On June 7, two other mares (8 and 12 years) developed signs. The first mare developed severe ataxia without preceding fever, and the second mare developed a fever that lasted for three days without further signs. On June 8, a 12-year-old gelding became febrile for 7 days, but no further signs developed. All the horses with severe neurological disorders were euthanized and, since EHV1 was suspected as the etiological agent, the riding school was completely shut down at this time. On June 9, a mare (11 years) showed an elevated body temperature for 4 days and a 13-year-old gelding was found to be slightly ataxic without preceding fever. On June 11, an 8-year-old mare suddenly became severely ataxic, without a preceding febrile period, and this condition quickly evolved to complete paralysis. At that time, the Faculty of Veterinary Medicine was contacted for an etiological diagnosis, whereby EHV1 was isolated from the PBMC from this horse, thus confirming the suspicion of a severe outbreak of EHV1. The last affected horse, presented on June 16, was a 4-year-old gelding with fever for 5 days, followed by urticaria and muscle tremors. EHV1 was confirmed by virus isolation and the horse was treated with the antiviral drug valacyclovir (Garré *et al.*, 2009). As the muscle tremors stopped the next day and no other signs of EHV1 infection were observed, the therapy was stopped after 2 days.

Secondary outbreaks

From June 5 till June 28, several premises reported neurological disease after attending the jumping contest on premise no. 1. On premise no. 2, a 6-year-old mare developed fever on June 5 and showed severe paralysis after 3 days of fever. Fifteen days later, two other horses (a 13-year-old mare and an 8-year-old gelding) showed fever for 3 consecutive days, without developing any other signs.

On premise no. 3, two mares (8 and 5 years) that had attended the competition became slightly ataxic without preceding fever, on June 9. One day later, a third mare (10 years) showed weakness for 1 day but became clinically normal afterwards. On premise n° 4, two mares showed a slightly elevated body temperature on June 9. Seven days later, two mares and two geldings showed fever for 2 or 3 days. On June 19, a 7-year-old mare was found quadriplegic after 3 days of fever and was treated with valacyclovir 1.5 days later. The first 2 days after the treatment was initiated, the mare showed good clinical improvement. She was able to stand and to move around with the support of a sling. After 3 days, however, treatment was stopped due to a lack of available valacyclovir. The mare remained clinically stable for several days, but no further improvement of clinical signs was observed. Two weeks after the onset of neurological signs, she was able to stand in the sling and started to regain bladder control, but was not able to lie in sternal position or to stand up on her own. Because this caused severe anxiety for the horse, she was eventually euthanized. On premise n° 5, a 9-year-old mare became slightly ataxic on June 9. On the same day, another horse (10 years) showed weakness in the limbs. Also starting on June 9, a 5-year-old gelding had elevated temperatures for 3 days and was weak. Three days later, the remaining four horses of premise no. 5 showed high fever for several days. Two of these horses (both mares, 7 and 5 years old) became quadriplegic after 2 days. These two mares were treated with valacyclovir at the onset of nervous system disorders. The first mare was hung in a sling and showed rapid improvement, so the treatment was stopped 5 days later. The second mare was treated for 3 consecutive days, with rapid improvement during the treatment, but with no further improvement once the treatment was stopped.

On June 15, premise no. 6 reported affected horses, starting with a 10-year-old mare that had attended the jumping contest. She became anorectic and febrile for 3 days, after which she became slightly ataxic. An 11-year-old gelding became severely ataxic after 3 days of fever. Four days later, a gelding (6 years) showed fever for 2 days, but did not develop any other signs.

Finally, on June 17 on premise no. 7, two horses (a gelding of 16 years and a mare of 11 years) that had attended the contest developed a high fever for 3 days. After the fever had subsided, the two horses became severely ataxic. Two days later, a 5-year-old mare developed slight ataxia. The other eight horses on the premise developed high temperatures over the course of several days, but no neurological disorders were noted.

Virology and serology

Blood samples were collected from seven horses with high fever, six horses with ataxia and two horses with quadriplegia at the moment of sampling (Table 1). EHV1 was isolated by means of PBMC co-cultivation from six of the seven horses with high fever (86%), from four of the six horses with ataxia (67%) and from the two horses with quadriplegia (100%) (Table 1). Seroconversion of EHV-specific antibodies was evaluated for thirteen of the fifteen horses, both at the time when the clinical signs appeared (acute sample) and three weeks later (convalescent sample). Twelve horses seroconverted for EHV1 (Table 1).

All EHV1 isolates were typed as neuropathogenic by sequencing of the D_{752} allele in the ORF30 region (e.g. 09P240, # GU271939). In addition, all the viruses were sequenced in their ORF68 region, a genetic marker for grouping isolates into six major geographically restricted groups (Nugent *et al.*, 2006). All the isolates in this outbreak of neurological disease belonged to group 4. These genetic analyses, along with the direct link between the confirmed premises and premise no. 1, further confirmed the assumption that all EHV1 infections on the different premises were caused by the same virus spread from premise no. 1.

DISCUSSION

The present paper reports a large outbreak of EHV1-induced neurological disease in Antwerp, Belgium in June 2009. How this epidemic started is not known. However, two weeks before the first horse became recumbent, a 5-year-old mare was presented with fever, and it could be hypothesized that this mare either reactivated latent EHV1 or got infected with EHV1 due to the many contacts with visiting horses on the premise. Secondary outbreaks on other premises occurred as a consequence of direct contact with infected horses from the first premise. Both the first premise and the secondarily affected premises were quarantined for three weeks and all the horses in the neighborhood that were without clinical signs were vaccinated. The outbreak was successfully controlled due to management, quarantine and vaccination measures, and one month after the onset of the outbreak, no more cases of EHV1 infection were diagnosed. During this large outbreak, several interesting observations were made.

Firstly, the morbidity rates were lower (7 to 21%) on the vaccinated premises (no. 1 and 4) compared to the non-vaccinated premises (25 to 100%) (Figure 1). On premise no. 4, where both vaccinated and unvaccinated horses were present, fever and neurological disease were only reported in the unvaccinated horses, whereas the vaccinated horses showed no clinical signs of EHV1 infection. A similar observation was made during other outbreaks of EHM, most of which occurred on premises with unvaccinated horses (Goehring *et al.*, 2006; van der Meulen *et al.*, 2003). This could indicate that vaccinated horses have clinical protection against severe symptoms of EHV1. Whether this protection is the result of the reduced spread of the virus or of a virological effect is not clear. Interestingly, although lower morbidity rates were observed on vaccinated premises no. 1 and no. 4, no difference could be seen in the number of horses that eventually developed nervous system disorders compared to the non-vaccinated premises (Table 2). In Belgium, only inactivated vaccines are registered for use in horses. These vaccines mainly stimulate humoral immunity, whereas it has been suggested that cell-mediated immunity is of paramount importance for limiting EHV1 infection (Kydd *et al.*, 2006; Kydd *et al.*, 2003).

Secondly, four horses were treated orally with the antiviral drug valacyclovir during this outbreak. Experimental *in vivo* studies to evaluate the efficacy of this drug in reducing clinical signs, viral shedding, and viremia have been performed, but with contradictory results (Garré *et al.*, 2009; Maxwell *et al.*, 2008). Although during the present outbreak the treated horses tended to recover more rapid as compared to untreated horses with similar clinical features, it remains difficult to evaluate the benefits of valacyclovir treatment during this outbreak due to the limited number of treated horses versus non-treated horses and the variable treatment periods.

Thirdly, during the current outbreak, equal numbers of male and female horses were exposed to EHV1 and became infected (Table 2), thus indicating no influence of gender on EHV1 susceptibility. In contrast, however,

Table 2. Description of the animals on the premises with EHV1 associated neurological disorders.

Premise N°	Total n° of horses present			Total n° of affected horses			N° of horses with neurological disorders		
	Male	Female	Total	Male	Female	Total (%)	Male	Female	Total (%)
1	53	43	96	8	12	20 (21%)	2	6	8 (40%)
2	1	2	3	1	2	3 (100%)	0	1	1 (30%)
3	2	10	12	0	3	3 (25%)	0	3	3 (100%)
4	42	38	80	2	5	7 (9%)	0	1	1 (14%)
5	3	4	7	3	4	7 (100%)	1	4	5 (71%)
6	2	1	3	2	1	3 (100%)	1	1	2 (67%)
7	5	6	11	5	6	11 (100%)	1	2	3 (27%)
	108	104	212	21	33	54 (26%)	5	18	23 (43%)

we did observe that once infected, the mares appeared to be predisposed to developing severe ataxia and paralysis. Indeed, 55% (18/33) of the infected mares developed nervous system disorders, whereas this was only 24% (5/21) for the infected male horses (Table 2). The higher sensitivity of mares to developing severe neurological signs during EHV1 infection has previously been described (Goehring *et al.*, 2006; McCartan *et al.*, 1995), but so far, no explanation for this observation has been found.

Finally, both virus isolation from peripheral blood mononuclear cells (PBMC) and seroconversion were used to diagnose EHV1 during this outbreak. Moreover, EHV1 was successfully isolated from PBMC during this outbreak. This is in contrast to previous published studies, which mention that virus isolation from PBMC in outbreak situations can be problematic (Lunn *et al.*, 2009; van Maanen *et al.*, 2001). In addition, seroconversion has also been reported to have limitations in confirming a diagnosis of EHV1, especially in horses with neurological signs (Lunn *et al.*, 2009). Interestingly, during the outbreak in the present study, all the horses with neurological disorders seroconverted, with the exception of one horse. One explanation for the successful diagnosis during this outbreak could be the rapid occurrence of severe signs during the outbreak, which increased the chances of detecting infectious virus and seroconversion. The low initial levels of EHV1 antibodies in a largely unvaccinated population could also have contributed to the easy detection of seroconversion.

During the only other reported outbreak of EHV1-induced neurological disease in Belgium, which occurred six years ago, a morbidity of 42% was seen, with 15% of the horses showing neurological disorders. Ataxia and paralysis were observed, but also cerebral disorders such as blindness, torticollis and severe apathy (van der Meulen *et al.*, 2003). During the recent outbreak, cerebral disorders were not observed, but interestingly, the outbreak was characterized by the rapid onset of ataxia and paralysis immediately after the disappearance of fever. In addition, the incidence of neurological signs in the EHV1-affected horses was much higher in the recent outbreak (43%) compared to the outbreak in 2003 (15%) (van der Meulen *et al.*, 2003). These observations might possibly point towards a change in neuropathogenic potential of naturally circulating EHV1 strains.

During the preparation of this manuscript, two new unrelated outbreaks of EHV1-induced EHM were reported and diagnosed. These outbreaks occurred in June 2010 and will be summarized briefly. The first affected premise had a half-open management system without any vaccination protocol. One mare had been transported from the premise to a breeding facility where problems of high fever and edema of the limbs were then being observed. When the mare was brought back, she was showing the same symptoms, and the virus then spread to the 5 other horses on the home premise (6 in total). Two of these horses were euthanized due to severe paralysis, three showed severe ataxia, which stabilized upon valacyclovir treatment, and one of them aborted at 10.5 months of gestation. EHV1 was isolated from PBMC from all 6 of the horses and was typed neuropathogenic by sequencing in the ORF30 region. The second outbreak occurred on a premise with an open management system and a once-a-year vaccination protocol. Here too, several horses showed high fever and edema of the limbs. One mare on this premise, which had not been given the yearly vaccination, showed severe ataxia and EHV1 was isolated and subsequently typed as non-neuropathogenic.

In summary, concern has been voiced within the U.S. horse industry that the neurologic form of EHV1 might be emerging, based on the increasing numbers of EHM outbreaks and the growing numbers of seriously affected horses throughout the United States over the last decade (USDA, 2007). Up until the recent outbreak, no such evolution had been seen in Belgium and no reports of EHM outbreaks had been reported, with the exception of the single limited outbreak in 2003. The present report describes this recent, large outbreak of EHV1, with its rapid spread and the remarkably high numbers of animals suffering from EHM. This, together with the fact that in 2010 two other small outbreaks were also reported, raises the question as to whether the neurological form of EHV1 is also emerging in Belgium and hence Europe. However, it is not clear whether these observations are related to the possible increasing occurrence and virulence of the disease itself, or to the increasing awareness of the disease and the development of increasingly effective diagnostic methods (USDA, 2007). Many data gaps exist, and more investigations need to be conducted to better understand this evolving situation and to identify other possible factors that may be playing a role. It is for this reason that a good surveillance system in Belgium and Europe is needed to monitor outbreaks of EHM, to acquire better understanding of the virulence of naturally circulating EHV1 strains and to determine whether EHM is in fact an emerging disease.

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