

THE PREVALENCE OF ANTIBODIES AGAINST EQUINE INFLUENZA VIRUS, EQUINE HERPESVIRUS 1 AND 4, EQUINE ARTERITIS VIRUS AND EQUINE RHINOVIRUS 1 AND 2 IN DUTCH STANDARD BRED HORSES

Prevalentie van antistoffen tegen equiene influenzavirus, equiene herpesvirus 1 en -4, equiene arteritisvirus en equiene rhinovirus 1 en 2 bij Nederlandse warmbloedpaarden

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ABSTRACT

A random cross-sectional seroprevalence study was conducted in 1996 by taking blood samples from 330 different Standardbred horses on all racetracks in the Netherlands. This blood sampling took place on four consecutive days, with each horse being sampled only once. Samples were investigated for antibodies against several strains of equine influenza virus, equine herpesvirus types 1 and 4, equine arteritis virus, and equine rhinovirus types 1 and 2. A type specific gG ELISA was used to determine specific seroprevalences of equine herpesviruses 1 and 4. Influenza serology, using influenza A/equi/1/Prague/56 as an indicator virus for vaccination, demonstrated that 38% of the horses were either unvaccinated or inadequately vaccinated. Many of these horses appeared to have experienced an influenza A/equi-2 field infection. Neutralizing and complement fixing antibodies against both EHV1 and EHV4 were found in a high percentage of the samples. However, for EHV1 this high seroprevalence was putatively caused by cross-reacting EHV4 antibodies since, in a type-specific gG ELISA, the EHV1 seroprevalence was only 28%, as compared with a 99% seroprevalence for EHV4. High seroprevalences were also found for equine rhinovirus type 1 and for the equine arteritis virus, thus indicating the endemic nature of these viruses. Many of these infections may be subclinical. The seroprevalence of equine rhinovirus type 2 was surprisingly low. The possible relationship between viral infections and upper respiratory tract disease and/or inflammatory airway disease is discussed.

SAMENVATTING

Een gerandomiseerd 'cross-sectional' seroprevalentieonderzoek werd uitgevoerd bij warmbloedpaarden (n=330) op alle draf- en renbanen in Nederland door het eenmalig nemen van een bloedstaal op vier opeenvolgende dagen. De serumstalen werden onderzocht op antistoffen tegen verscheidene equiene influenzastammen, equiene herpesvirus type 1 en 4, equiene arteritisvirus en rhinovirus type 1 en 2. Voor het bepalen van de afzonderlijke seroprevalentie van EHV1 en EHV4 werd een typespecifieke gG ELISA gebruikt. De influenzaserologie op basis van influenza A/equi-1/Praag/56 als indicatorvirus toonde aan dat 38 % van de paarden niet of onvoldoende gevaccineerd was. Veel van deze paarden bleken wel een influenza A/equi-2 veldinfectie te hebben doorgemaakt. In een hoog percentage van de stalen werden neutraliserende en complementbindende antistoffen tegen EHV1/4 aangetoond. Voor EHV1 werd deze hoge seroprevalentie waarschijnlijk veroorzaakt door kruisreagerende anti-

stoffen tegen EHV4, de seroprevalentie van 28 % tegen EHV1 en 99 % tegen EHV4 in een typespecifieke gG ELISA in acht genomen. Gezien de relatief hoge seroprevalenties die bij de Nederlandse warmbloedpopulatie gevonden werden, moeten hier ook equiene rhinovirus type 1 en equiene arteritisvirus als enzoötisch worden beschouwd. Veel van deze infecties lijken subklinisch te verlopen. De seroprevalentie van equiene rhinovirus type 2 was verrassend laag. In de discussie wordt aandacht besteed aan het mogelijke verband tussen virale infecties en ziekteverschijnselen van de bovenste luchtwegen.

INTRODUCTION

Respiratory diseases are among the most common problems of racehorses in training, and sudden failure to perform to expectations is often attributed to "the virus" (Mumford and Rossdale, 1980). If poor performance shows endemic dimensions in one or more of the training establishments, especially when respiratory signs accompany the syndrome, a viral etiology is possible. The effects of these viral infections as predecessors of later occurring bacterial infections of the lower airways, however, have not been explicitly elucidated in longitudinal studies.

In the event that viral infections in the early training period predispose to later occurring inflammatory airway diseases (IAD), it would be helpful to know which viruses are circulating among horses in stable yards or racing grounds. Indirect evidence of this may be obtained by serological testing for specific viruses.

The aim of this study was to determine the seroprevalence of antibodies against equine influenza viruses, equine herpes virus 1 and 4 (EHV1 and 4), equine arteritis virus (EAV) and equine rhinovirus types 1 and 2 (ERV1 and 2) in a population of Dutch Standardbreds and to make inferences concerning the epidemiological situation. However, since reliable estimates of sensitivity and specificity of the assays used were not available, we were not able to calculate true prevalence data from the seroprevalence data. As the population was only vaccinated against influenza A/equi/1 and A/equi/2 (and a minority of the population against EHV1/4), an antibody titer against a specific virus other than influenza or EHV1/4 (where there was a history of vaccination) was considered to be indicative of exposure to that virus by natural infection.

MATERIALS AND METHODS

Horses

A total of 330 different Dutch horses were blood sampled in the Netherlands. This blood sampling took place over four consecutive days, with each horse being sampled only once. The horses were either

brought in for racing or were stabled on the tracks. Horses from various stables and tracks also competed on other tracks. Three racetracks – Alkmaar, Hilversum and Wolvega – were used only for the training and racing of Standardbred trotters. One track, Duindigt, had a mixed population of Standardbreds and Thoroughbred horses.

Samples

Blood was collected from the jugular vein with the venoject system in vacuum plain silicone coated tubes (Venoject Therumo). After collection, the samples were kept at 4°-8°C overnight and transported to the laboratory, where the serum was harvested after centrifugation. The serum was stored at -20°C until analysis.

There was a poor serum yield from a small number of samples and not all the serological tests could be performed on the serum of each horse.

Serology

The Single Radial Hemolysis (SRH) titers against influenza A/equi/1/Prague/56, A/equi/2/Miami /63, A/equi/2/Kentucky/91 and A/equi/2/Suffolk/89 were determined as described by Wood *et al.* (1983). The sera were tested undiluted.

Complement Fixation (CF) tests for EHV 1 and 4 and ERV 1 and 2 and Virus Neutralization (VN) tests for EHV1 and EHV4 were carried out according to standard techniques. For the CF test, the sera were diluted five times in the first well, followed by 2-fold serial dilution, whereas for the VN test, the sera were diluted 2 times in the first well, followed by 2-fold serial dilution. The appropriate homologous antigens were used to establish the antibody responses induced by the different virus types. The CF titers were expressed as the reciprocal of the highest dilution of serum displaying 50% hemolysis, whereas the VN titers were expressed as the reciprocal of the highest serum dilution displaying the cytopathogenic effect in 50% of the wells. A VN titer of $\geq 1:2$ was considered a significant antibody level for EHV1 and EHV4.

Type-specific antibodies against EHV1 and EHV4 were identified using a gG ELISA (Crabb *et al.*, 1995). Type-specific fusion proteins and control antigen (pEG1var, pEG4var and GST-only) were generously provided by Dr. Crabb and Dr. Studdert of the Centre for Equine Virology, Melbourne, Australia. This ELISA was performed as a single dilution test. All sera were tested in the same test run.

EAV serology was carried out using a standard micro VN assay. Briefly, neutralizing antibodies to EAV were determined in Rabbit Kidney (RK-13) cells using 100 (accepted range 30-300) TCID₅₀ of the modified Bucyrus strain by a complement-dependent micro-neutralization assay. The sera were heat-inactivated (56°C, 30 min) before use and analyzed in duplicate. The tests were read after five days incubation at 37°C, 5% CO₂. A VN titer of $\geq 1:4$ (i.e. final serum dilution after addition of an equal volume of the appropriate dilution of stock virus to each well) was considered positive for EAV infection.

Statistics

Routine descriptive statistics were used for characterization of the different seroprevalences.

RESULTS

Influenza serology

SRH titers against the various influenza types varied from as low as 7 mm² to as high as 308 mm² (Table 1). Titers higher than 10 mm² were considered significant for data analysis and biologically relevant. Over the entire population, the mean SRH titers were 140 mm² for A/equi-1 virus and higher than 160 mm² for the different A/equi-2 viruses. The median antibody levels for A/equi-1 virus and A/equi-2 viruses were 162 mm² and > 172 mm², respectively. Twenty-five per cent of the horses had antibody levels <76 mm² against A/equi-1/Prague/56 antigen, whereas 25% of the horses had antibody levels <137 mm² against A/equi-2/Miami/63. Seventy-five per cent of the horses had antibody titers < 207 mm² against Prague/56, whereas 75% of the horses had titers < 206 mm² against the A/equi-2/Suffolk/89 or even higher against the other A/equi-2 strains. One horse (0.3% of the population) had no detectable antibodies against any of the influenza strains tested. A relatively large fraction of the horses (12.7%) were seronegative (SRH < 7 mm²) for A-equi-1/Prague/56, while only 2.3%, 1.5% and 1% of the horses were seronegative

Table 1. Viral antibody titers/levels in Standardbred trotters.

Test/Virus	N=	Range	Mean	Median*	25% **	75% ***
SRH A/1/Prague	300	7–302	140	162	76	207
SRH A/2/Miami	308	7–293	167	173	137	211
SRH A/2/Kent	329	7–308	181	189	156	219
SRH A/2/Suffolk	306	7–284	168	172	141	206
SNT EHV1	333	2-525	35	11	6	32
CFT EHV1	312	2-720	34	10	<2	30
SNT EHV4	333	2-525	356	525	178	525
CFT EHV4	310	2-320	16	7	<2	15
CFT ERV1	305	2-640	52	10	<5	40
CFT ERV2	305	2-160	2	<2	<2	<2
SNT EAV	310	4-2048	111	<4	<4	48

*: 50% of the horses have a titer lower than

** : 25% of the horses have a titer lower than

***: 75% of the horses have a titer lower than

tive for A/equi-2/Miami/63, A/equi-2/Kentucky/81 and A/equi-2/Suffolk/89, respectively.

Twenty-five percent of the horses did not have protective antibody levels against A/equi-1/Prague/56, (i.e. SRH antibody levels greater than 85 mm²) (Mumford *et al.* 1998), compared with 8.4%, 5.5% and 6.9% for A/equi-2/Miami/63, A/equi-2/Kentucky/81 and A/equi-2/Suffolk/89, respectively.

In the population of horses that had no SRH antibody levels against A/equi-1/Prague/56, 60.5% of the horses had high titers (>100 mm²) against all the A/equi-2 strains. A breakdown of figures for the individual A/equi-2 strains showed that 60.5 % of these horses had high levels against A/equi-2/Miami/63, 79% against A/equi-2/Kentucky/81 and 73.7 % against A/equi-2/Suffolk/89.

EHV1/4 serology

The serum samples were also tested for the presence of EHV1/EHV4 antibodies by VN, CF, and a type-specific gG-ELISA (Crabb *et al.*, 1995). The results are presented in Table 1. Overall, 93% of the horses had VN titers against EHV1, whereas all the horses had VN titers against EHV4. The VN titers for both EHV1 and EHV4 ranged from <2 to 525, whereas the mean titer against EHV4 was higher than that against EHV1.

CF titers to EHV1 and EHV4 were found in 74% and 72% of the horses, respectively. These titers ranged from <2 to 720 for EHV1 and from <2 to 320 for EHV4 (Table 1). High CF titers were found only in a few animals, as 75% of the animals had CF titers lower than 30 for EHV1 and lower than 15 for EHV4. Since both VN and CF antibodies against EHV1 and EHV4 are cross-reactive (Allen and Bryans, 1986), type-specific gG antibodies against EHV1 and EHV4 were determined. Twenty-eight percent of the horses had specific EHV1 gG antibodies and 99% of the horses had specific EHV4 gG antibodies. All horses that were seropositive for EHV1 were also seropositive for EHV4. Seventy-one percent of the horses that were seronegative for EHV1 were seropositive for EHV4. Less than 1% of the horses were seronegative for EHV1 and EHV4.

EAV serology

The serum samples were tested for the presence of EAV antibodies, the results of which are shown in Table 1. The titers ranged from as low as <4 to as high as 2048. The population mean was calculated at 111 and

the median antibody level at <4. Since 75% of the population had antibodies lower than 48, the high population mean is attributable to a few individuals with very high titers, which are indicative of recent exposure. Twenty-five percent of the horses had no detectable antibodies, 46.4% had antibody titres 1 : 4, and 33.9% had antibody titers 1 : 8. Twenty-five percent of the horses had titers above 1:48, reaching maxima of 1:2048.

ERV1/2 serology

Serum antibody levels against ERV1 and ERV-2 are shown in Table 1. Seventy-four percent of the horses had detectable antibody titers against ERV1, 8% had antibody titers against ERV2 and 7% had antibody titers against both viruses. As shown in Table 1, titers against ERV1 ranged from <2 to 640, whereas titers against ERV2 did not exceed 160. Thirty-five percent of the animals had titers higher than 20 against ERV1, compared to 1% against ERV2. The mean titer against ERV1 was 20 and the median was 10. Horses that were seropositive for ERV2 were also seropositive for ERV1, whereas only a few animals with titers against ERV1 were seropositive for ERV2.

DISCUSSION

At the time the trial was executed it was mandatory for competition horses to be vaccinated against equine influenza. About 25% of the competing horses had Prague/56 antibody levels lower than 75-85 mm², which is the serological threshold level for clinical protection (Mumford *et al.*, 1998), with 13% of the animals having no detectable antibody levels at all. As vaccination is mandatory for sport horses in the Netherlands as well as in many other countries, and as all commercial vaccines available at the time did contain antigen Prague/56, it can be concluded that these animals were not protected due either to the use of non-efficacious vaccines or to inadequate vaccination or to the failure to vaccinate at the recommended time. As A/Equi-1/Prague/56 is extinct (Webster, 1993), the presence of antibodies against this antigen is a demonstration of the efficacy of vaccination. An investigation of the efficacy of several commercially available vaccines in the Netherlands (Mumford *et al.*, 1998; Heldens *et al.*, 2002; Heldens *et al.*, 2003) showed a long duration of immunity against the clinical signs of H3N8 and H7N7 equine influenza virus infection. Moreover, these studies showed that antibodies against H7N7 and H3N8 antigens can be induced

at comparable and protective levels after adequate vaccination and have similar half-lives. Therefore, on the basis of these studies, it is highly unlikely that all of these animals were vaccinated with an inefficacious vaccine.

About two-third of these unvaccinated or inadequately vaccinated horses appeared to have been recently exposed to an A/equi-2 influenza virus, as SRH titers were high against Miami/63, Kentucky/81 and Suffolk/89. The circulating A/equi-2 virus had probably induced sufficient immunity in the majority of the poorly or non-vaccinated horses. Due to cross-reacting immunity, the exact strain of the circulating virus could not be deduced from serology, but the relative number of animals with high Kentucky/81 and Suffolk/89 antibodies suggested that a strain related to Suffolk/89 (an Eurasian-like H3N8 virus) had been circulating not long before the samples were taken.

Unvaccinated and seronegative animals constitute a risk to vaccinated or seropositive cohorts as they are more susceptible to infection and shed greater quantities of virus (Mumford *et al.*, 1998; Newton *et al.*, 2000). Suffolk/89, Newmarket/93 and Moulton/98 viruses were responsible for influenza epidemics in the United Kingdom causing clinical disease both in vaccinated and unvaccinated horses (Mumford, 1998).

Although EHV1/4 vaccination was not widely practiced in the Netherlands at the time of the trial, 25% of these Standardbreds had an EHV1/4 vaccination history. This relatively high percentage was attributed to growing public awareness of recent outbreaks of EHV 1-associated paralysis and abortion (van Maanen *et al.*, 2000; van Maanen *et al.*, 2001). However, only one-third of the seropositive horses had been vaccinated, which indicated a high number of recent natural infections. With the aid of the specific qualitative gG antibody tests, it was found that nearly all horses had been infected with EHV4, whereas less than 30 % had experienced an EHV1 infection. Hence it was concluded that EHV4 is more prevalent than EHV1 in the Netherlands. It should be realized that the seronegative horses may have experienced an EHV infection earlier in life, resulting in a possible decrease in anti-EHV antibodies.

Nearly half of the competing Dutch Standardbreds were seropositive for EAV, with 33.9 % of the horses showing antibody titers 1:8, suggesting that this virus has been present for a much longer time in the Netherlands than was realized and that many training yards frequently obtain horses directly or indirectly

from the USA or other countries where the virus is endemic. Despite preventive steps such as vaccination and/or elimination of seropositive stallions, between 12.5% and 70% of the stallions are persistently infected in the USA (Timoney *et al.*, 1987; Timoney *et al.*, 2000). Our data strongly suggest that the EAV status of Dutch Standardbreds should be assessed prior to their use as breeding stallions.

ERV1 is associated with inapparent infection and mild respiratory disease with subsequent loss of performance in young horses, particularly during their first training season. Serological studies in the UK indicate that approximately 87% of susceptible horses in the Newmarket area become infected each racing season (Burrows, 1981). Our results suggest that ERV1 is equally prevalent in Dutch Standardbreds. The prevalence and pathogenic significance of ERV2 is less well evaluated, but there is evidence that many young horses are exposed prior to entering the training yards (Burrows, 1981). Only 8% of the horses in this survey were seropositive for ERV2. Studies conducted in other countries indicated a higher prevalence of antibodies to ERV2 using the SNT. We also might have found a higher seroprevalence if we had used the SNT.

Studies in the UK have indicated that, between epizootics, approximately 7% of the horses per month became infected with a known respiratory virus with the potential to cause upper respiratory tract disease (URT) and to predispose to inflammatory airway disease (IAD), (Wood *et al.*, 1999). The equine influenza viruses, EHV1, EHV4 and ERV1 are important causes of URT disease and it has been suggested that acute respiratory virus infections contribute to IAD (Allen and Bryans, 1986; Li *et al.*, 1997; Willoughby *et al.*, 1992). Horses infected with influenza or EHV4 may suffer from a decreased tracheal clearance rate for up to 32 days after infection and EHV1 causes non-specific immunosuppression for at least 40 days, a condition which may increase the horse's susceptibility to other viral and bacterial infections (Hannant *et al.*, 1991; Willoughby *et al.*, 1992).

We have demonstrated the seroprevalence of respiratory tract virus pathogens in the Dutch racehorse population. Further research should now be instigated into the relative importance of these viral pathogens as a cause of respiratory disease.

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