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Short communication

Epizotiology and phylogeny of equine arteritis virus in hucul horses

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ABSTRACT

The aim of the study was to determine the situation of equine arteritis virus (EAV) infections in hucul horses. A total of 176 horses (154 mares and 22 stallions) from the biggest hucul horse stud in Poland were tested. Antibodies against EAV were detected in 97 (55.1%) horses. The EAV seroprevalence among mares was 53.2% while in stallions – 68.2%. The percentage of positive mares increased with their age, thus amongst the mares of less than 2 years of age the percentage was 32.5%, while in the group of 3–5 years old increased to 59.4% and in the mares in the age of 6–10 years and older than 10 years 89.5% and 95% were seropositive, respectively. Among 11 seropositive stallions five were supposed to be shedders of EAV with their semen. It is likely that those persistently infected stallions were the reservoirs of the virus in the stud. Genetic studies using of ORF5 gene showed high homology between the viruses detected in the semen of those stallions what suggested lateral transmission between the stallions sharing the same stable. Persistent infection in an immature stallion, which has not yet been used for breeding, was established as a result of infection via respiratory route. Phylogenetic analysis confirmed that all hucul viruses shared the same ancestor and as most of EAV strains dominating in Polish horse population belonged to the European origin EAV subgroup (EU-1).

1. Introduction

Equine arteritis virus (EAV) is one of the major viral pathogens of horses. It is the causative agent of equine viral arteritis (EVA), a contagious disease of horses and other equid species. The virus belongs to the genus *Arterivirus* of the family *Arteriviridae* in the *Nidovirales* order (Cavanagh, 1999; Snijder and Meulenberg, 1998). The viral genome consists of a single stranded, positive-sense RNA which includes nine known open reading frames (ORFs). ORF5 encodes viral membrane glycoprotein GP5, which contains epitopes responsible for virus neutralisation (Balasuriya et al., 1997). Due to the ORF5 variability, this gene is used as target for phylogenetic analyses of EAV, whereas the majority of diagnostic RT-PCRs are based on the primers derived from the most conservative ORF7 gene encoding nucleocapsid protein N. EAV was first isolated during an outbreak of respiratory disease and abortion on a Standardbred farm in Bucyrus, Ohio in 1953 (Doll et al., 1957). There is only one recognized serotype of EAV but field strains of the virus vary significantly in their virulence. Exposure to EAV most frequently results in asymptomatic infection but certain strains can cause disease of varying clinical severity characterized by anorexia, depression, fever, dependant limb oedema, ocular and nasal discharge and conjunctivitis. The virus can also cause abortion in pregnant mares. If exposure to virus occurs in late phase of pregnancy the mare may not abort but newborn foal is infected and may die within a few weeks with symptoms of interstitial pneumonia or pneumoenteritis. Most infected horses recover without complications but up to 10–70% of stallions infected with

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EAV can subsequently become carriers and constantly shed the virus in their semen through several weeks, months or years (Timoney et al., 1986, 1987). Such stallions are main reservoirs of EAV in the equine population, spreading the virus to the naïve mares as well as transmitting it laterally to other horses sharing same stables (Guthrie et al., 2003).

The virus is distributed worldwide, but seroprevalence of EAV infection of horses varies between countries and different breeds. Since the first EVA cases described in Ohio in 1953, the disease spread over the world. The only countries that are considered to be free of EAV infections are Japan and Island. First EVA cases in Poland were confirmed in 1976–1977 in the thoroughbred horse stud on the south of the country and the first EAV strain was named Wroclaw-2 (Golnik and Michalak, 1978). In the recent years, the intensified horse trade, poor execution of biosecurity rules and lack of unified control regulations caused increase in dissemination of EAV. Eichhorn et al. (1995) observed significant elevation of the percentage of EAV seropositive horses in the horse population in Germany from 1.8% in late 1980s to almost 20% in 1995. Increase of EAV infection in horses was also described in Sweden and Italy (Glaser et al., 1997). In many of the EVA serologic studies carried out in Europe more than 20% of animals were infected with the virus (Glaser et al., 1997), except Great Britain where the percentage was not exceeding 2%, however the abortion storms and the mortality in the young foals caused by EAV are still a problem there (Newton et al., 1999). The significance of EAV infections is often underestimated by the horse breeders in Poland, however the incidence of abortions in mares caused by EAV infection could be estimated as one third of all the abortion cases in our country (Golnik and Sordyl, 2004). In Hungary, Szeredi et al. (2005) found that 10% of the abortions were due to EVA. Generally the percentage of EAV seropositive animals is considerably higher in Standardbreds then in other breeds. Very little is known about epidemiology of EAV infection in hucul horses. Huculs are a primitive breed of small mountain horses. It is one of the oldest primitive breeds described in Poland. The breed originates from the Hucul region in the East Carpathian Mountains. It is a region situated in the mountainous basin of the Prut and Cheremosh rivers, in the East Carpathians. At present it is located in the borderland between Ukraine and Romania. The breed was formed under the influence of harsh mountain climate in areas poor in feed and offering very primitive habitat conditions. Therefore hucul horses are known for their physical endurance, low feed requirement, fertility, longevity and resistance to illnesses. In the past they were used as saddle and pack horses but today mostly for mountain recreation and hippotherapy. Hucul are included in the protected gene fund of original and primitive animal breeds of FAO. To protect breed purity, coordination of the breeding goals and plans as well as the origin control, the hucul international federation (HIF) was founded in 1994 at Balice near Cracow in Poland as a result of cooperation between breeders from Austria, Hungary, Czech, Slovakia, Romania, Ukraine and Poland.

The purpose of the study was to investigate prevalence of EAV infection, the frequency of carrier state in stallions and the genetic variability among strains of EAV isolated from hucul horses in Poland.

2. Materials and methods

2.1. Sample collection

Most of the samples for the study were collected in the period of 2006–2008 from the biggest hucul horse stud in Poland. Mares with foals and stallions used for breeding were kept in the main stables whereas yearlings and immature stallions in other farm buildings located in the walking distance from the previous ones. No history of respiratory disorders, abortions or decrease in the pregnancy rate was notified previously and any clinical signs of disease in the stud horses were unapparent in the time of sampling. None of the horses was vaccinated against EAV.

For serological tests 176 blood samples were collected including 154 samples from mares and 22 from stallions. Blood was collected in vacutainers, allowed to clot and centrifuged at $2000 \times g$ for 10 min and serum was decanted to the fresh tubes. Subsequently all samples were tested with virus neutralization test. After that 11 serologically positive stallions were checked for the presence of EAV in their semen. Altogether 11 semen samples from 10 adult stallions sharing the same stable and one young male standing in the separate building of the same holding were collected.

2.2. Virus neutralisation test (VNT)

VNT was performed according to the procedure described in the OIE Manual (Timoney, 2004). All sera were inactivated for 30 min at 56 °C. Subsequently, sera samples were added to the 96-well microtitre plate and serial twofold dilutions in serum-free cell culture medium (Eagle MEM) were made starting from a 1:2 dilution. To prepare working dilution of the stock virus containing from 100 to 300 $TCID_{50}$ per 25 μl, the reference Bucyrus strain (ATCC VR-796) was diluted in Eagle MEM with guinea-pig complement at a final concentration of 10%. Such dose of the virus was added to every well containing relevant serum dilutions, except the test serum control wells. The plates were shaken gently and incubated for 1 h at 37 °C. After that time 50 μl of cells suspension RK13 (ATCC CCL-37) with concentration approximately 10^5 cells/ml were added to each well. The plates were sealed with parafilm and incubated for 3–5 d at 37 °C in a humid atmosphere of 5% CO_2 and read under microscope for the presence of cytopathic effect (CPE). A titre of 1:4 or greater was considered as positive.

2.3. Viral RNA extraction and RT-PCR

Total RNA was extracted directly from seminal plasma using TRI Reagent (Sigma–Aldrich). The semen samples were first tested in diagnostic RT-PCR using set of primers flanking 395 bp fragment of ORF7, previously described by Belák et al. (1994). For this purpose single tube kit Access RT-PCR System (Promega) was used. RT-PCR reaction mix consisted of 10 μl of AMV/Tfl 5× reaction buffer, 1 μl of