Characterization of high virulent strains of equine rhinopneumonitis virus

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Abstract. This article presents results of screening samples collected from horses during acute outbreaks of abortion and neurological disorders recorded in 2019-2020 in Russia. The signs of disease were characterized by severe hemorrhagic vasculitis. Massive hemorrhages were observed in organs of aborted fetuses and dead horses. Equine herpesvirus type one was isolated in cell culture. Testing to identify genes encoding glycoprotein B and polymerase catalytic subunit of certain viral isolates were carried out. Virus isolated from horses with signs ofencephalomyelitis from the Northwest region had a high degree of phylogenetic relationship with the strain “89c25” of equine herpesvirus type one from Japan, 1989. The same virus was found in horses with signs of rhinopneumonia from the Ural region. The virus isolated after equine abortion from the North Caucasus region had a high degree of phylogenetic relationship with the strain “IT19m10” of equine herpesvirus type one from Italy, 2019. It was established that all viral isolates belong to non-neuropathogenic genotype - A2254.

1. Introduction
Equine rhinopneumonitis (EHV-1 infection) is one of the most dangerous equine viral diseases. It’s characterized by a significant variety of clinical signs in horses - rhinopneumonia, abortion, neonatal death and myeloencephalopathy. The severity of the EHV-1 infection depends on factors that include sex, age, physiological status (pregnancy), host immunity and properties of virus strains. Some strains of EHV-1 are high virulent and cause disease with increasingly severe symptoms [1].

In the USSR, isolation and identification of EHV-1 was first carried out by K.P. Yurov and N.N. Kryukov in 1969 during outbreaks in two farms located in different geographical areas of the country [2]. The challenge of horse-herds by EHV-1, which had not previously been in contact with, usually leads to mass abortions in mares, regardless of breed, constitution and environmental conditions. During an outbreak, up to 70 - 80% of mares are aborted; abortions are observed in the second half of pregnancy (7-11 months) such as when it was in 1974 among free-roaming horses located on high mountain pastures in Kyrgyzstan [3]. Encephalomyelitis may occur alone or in combination with respiratory disease; in most cases, the neurological form is described after an abortion or precedes it. It is presumed that EHV-1 neurological disorders are more likely to be remission than primary challenge [4].

K.P. Yurov in 1984 described a neurological disease in the first days after an abortion in 1 - 2% mares. The disease was characterized by facial nerve dysfunction, paralysis and paresis of the hind limbs. Decreased sensitivity of skin in the area of croup, lameness expressed to a varying degree, stiffness, slowness of movements, or complete lack of balance were observed. With timely treatment,
the acute signs underwent a regress within 1 - 2 weeks; however, residual effects (dynamic ataxia, curvature of the neck, etc.) remained for several years. With a favorable outcome, the described phenomena disappeared within a few days or weeks. In severe cases, the horses died with general paralysis. During outbreaks the stallions usually do not have the typical fever and respiratory distress and disease was starting at once with damage of central nervous system - paralysis, paresis [3] - [5].

In our country a case of neurological disease was noted in trotter horses after immunization with vaccine against equine rhinopneumonitis (“RPK”, Czechoslovakia). One stallion and two mares became sick 70-72 hours after. It started as ataxia and muscle tremors. When a stallion was taken out of the horsebox, he fell and was in a coma for 5-7 minutes. Then, he was able to get up with the help of the groom, but got into the wrong position - he stood swaying with his hind limbs crossed. Trying to move, the stallion lost his balance and fell. Body temperature was increased to 39.2°C, pulse and respiration were rapid. Defecation and urination were impaired. After treatment in the next 2 to 5 weeks, symptoms underwent a regress. However, limp on the hind limbs and some stiffness were remained in subsequent years. At the same time, a milder form of disease as lameness, ataxia, and depression was observed in two vaccinated mares. They returned to normal 2-3 days later. Subsequently, the mares gave birth to healthy foals [3] - [5].

A significant increase in the incidence of equine myeloencephalopathy (EME) has been noted in recent years in the countries with advanced horse breeding (USA, Canada, and EU). EHV-1 outbreaks also can disrupt important economic industry such as horse racing. Manifestation of EHV-1 infection varies from single sporadic cases to the challenge of 90% of the herd. Mortality also varies during outbreaks and may be reaching 40–50% [6] - [7].

Most researchers attribute the spread of EME to the emergence of new mutant EHV-1 strains of genotype G224, as well as to the high frequency and longer duration of leukocyte-associated viremia [8] - [9]. This is to determine the need for new effective means and efforts to combat infection.

Therefore, study of genomic structure of EHV-1 strains which were a cause of abortions and respiratory distress; identification of neuropathogenic variants are particularly relevant. In 2019-2020 local outbreaks of EHV-1 infection in horses with signs of encephalomyelitis and atypical abortions were recorded on the territory of the Russian Federation. This article presents the results of a study viruses isolated during these outbreaks.

2. Materials and methods
In the study, we tested the clinical and pathological material from sick horses: blood, nasal and genital swabs, parenchymal organs from aborted fetuses and dead horses. Virus isolation was carried out in pig kidney cell culture. Viral DNA was isolated on spin columns with the components of the K-Sorb commercial kit manufactured by Synthol Co. (Russia). DNA with a purity of 1.8 - 1.9 was taken according to ratio of A260/A280 at concentration of 500 ng. We used PCR primers complementary to gpB gene of EHV-1 according to recommendation by the World Organization for Animal Health [10]. Determination of the point mutation in DNA polymerase gene (ORF 30) of EHV-1; PCR for the diagnosis of EHV-3 infection and equine viral arteritis were carried out as described previously [11] - [12]. Synthesis of primers and determination of nucleotide sequences were performed by Syntol Co. Multiple alignment was conducted using the computer programme ClustalW2 made by the European Institute for Bioinformatics. For comparison, the nucleotide sequences of gpB gene and ORF30 of the reference EHV-1 strains from GenBank database (INSDC) were used. NT with EHV-1, NT with equine arterivirus and samples of sera from horses were carried out in 96-well culture plates using conventional methods. The results were neutralization of EHV-1 at sera dilution of 1:16 or more and neutralization of equine arterivirus at sera dilution of 1: 4 or more.

3. Results and discussion
During outbreaks of equine diseases, hemorrhagic pneumonia, myeloencephalopathy, abortion, and coital exanthema were observed. We tested samples of clinical and pathological material of sick horses and sera samples of convalescent horses. In nasal swabs, parenchymal organs of aborted fetuses, and in
the lungs, brain, and spinal cord of dead horses, EHV-1 was detected by molecular genetic analysis. EHV-1 was also detected in genital swabs of horses challenged by EHV-3. The results were confirmed by a retrospective serological testing in NT. Viral isolates were replicated in cell culture. A description of isolates is shown in table 1.

Table 1. EHV-1 isolates.

| №  | Isolate code | Year of isolation | Type of sample | Clinical signs in horses | Origin (Federal district) |
|----|--------------|------------------|----------------|--------------------------|--------------------------|
| 1  | KGM          | 2019             | Brain          | Encephalomyelitis        | Northwest                |
| 2  | KSM          | 2019             | Spinal cord    | Encephalomyelitis        | Northwest                |
| 3  | KA           | 2019             | Embryonic lung | Abortion                 | Northwest                |
| 4  | TN           | 2019             | Nasal swab     | Rhinopneumonitis         | Urals                    |
| 5  | KBA          | 2020             | Embryonic lung | Abortion                 | North Caucasus           |
| 6  | VGV          | 2018             | Genital swab   | Coital exanthema         | Southern                 |

Our main interest was the “KGM” and “KSM” of EHV-1 isolated in 2019 from horses with signs of encephalomyelitis (Figure 1). Early 2006s a new classification into neuropathogenic G2254 and non-neuropathogenic (“wild”) A2254 types of EHV-1 was based on the point mutation presence or absence in conserved sequence encoded by ORF30. The point mutation leads to amino acid replacement of asparagine with aspartic acid at position 752 of viral DNA polymerase, resulting in mutant EHV-1 strains caused as mainly a myeloencephalopathy [8]. The results of nucleotide sequencing gene DNA polymerase (ORF30) of “KGM”, “KSM”, “KA”, and “TN isolates showed a high degree of their phylogenetic relationship with EHV-1 strain “89c25” from Japan, 1989 [13]. The identity of nucleotide sequences ranged from 99.51 to 99.83%. Despite the fact that the “KGM” and “KSM” of EHV-1 were isolated from the horses with signs of myeloencephalopathy, they are not neuropathogenic mutants and belong, according to genomic structure, to “wild” type of EHV-1 - A2254. Additionally, the samples were tested by PCR for gene gB EHV-1 as ordered in Terrestrial Manual of the World Organization for Animal Health. The results received are in tables 2 and 3.

Table 2. Nucleotide sequences of DNA polymerase catalytic subunit gene of EHV-1 isolates.

| №  | Isolate code | Size (bp) | Homolog in GenBank (accession number) | Identity (%) | Genotype |
|----|--------------|-----------|--------------------------------------|--------------|----------|
| 1  | KGM          | 592       | “89c25” (AB363614.1)                 | 99,83        | A2254, N752 |
| 2  | KSM          | 592       | “89c25”                              | 99,83        | A2254, N752 |
| 3  | KA           | 590       | “89c25”                              | 99,83        | A2254, N752 |
| 4  | TN           | 612       | “89c25”                              | 99,51        | A2254, N752 |
| 5  | KBA          | 587       | “IT19m10”(MN226968.1)                | 99,72        | A2254, N752 |
| 6  | VGV          | 608       | “Hisar-14/2014”                      | 99,67        | A2254, N752 |

Table 3. Nucleotide sequences of gpB gene of EHV-1 isolates.

| №  | Isolate code | Size (bp) | Homolog in GenBank (accession number) | Identity (%) |
|----|--------------|-----------|--------------------------------------|--------------|
| 1  | KGM          | 520       | “Hertfordshire/150/2016” (KY852346.1) | 97,13        |
| 2  | KSM          | 556       | “Hertfordshire/150/2016”              | 98,61        |
| 3  | KA           | 687       | “Hertfordshire/150/2016”              | 98,99        |
| 4  | TN           | 788       | “Oxfordshire/207/2013” (KY206471.1)   | 98,55        |
The “KBA” of EHV-1 was isolated during outbreak of abortion in 2020. Mares were transported to a farm from France. Abortions of 4-6 months pregnancy were observed. At autopsy, typical cases were characterized by massive hemorrhages in organs and tissues of fetuses. An aborted fetus is shown in Figure 4. We saw signs of unusual extensive hematomas on umbilical cord and its incorrect branching (Figure 2). In other cases, foals were born without hairline (Figure 3) and died in the first days. Equine viral arteritis and bacterial placentitis as a cause of disease were excluded. The results of molecular genetic testing of “KBA” isolate showed its similarity with EHV-1 strain “IT19m10” from Italy and its relation to “wild” type — A2254.

4. Conclusion
In recent years, in the territory of the Russian Federation, a high virulent strain, similar to Japanese strain “89c25” belongs to the non-neuropathogenic type of EHV-1 - A2254, but under certain conditions, it may be a cause of equine neurological diseases.
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