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ORIGINAL ARTICLE

Detection and Sequence Analysis of Equine Gammaherpesviruses from Horses with Respiratory Tract Disease in Turkey

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Summary

The equid herpesvirus 2 (EHV-2) and 5 (EHV-5), identified agents of respiratory infections and keratoconjunctivitis cases in some equids, comprise a high degree of antigenic heterogeneity. Prevalence and genetic characterization of EHV-2 and EHV-5 strains from Turkey were investigated in this study. A total of 73 nasal swabs and 54 blood specimens were sampled from horses with respiratory tract diseases characterized by mucopurulent nasal discharge and occasional coughing. Overall, EHV-2- and EHV-5-specific DNA amplicons were obtained from 19.2% (14/73) and 21.9% (16/73) of horses tested by multiplex nested PCR. Sequences of EHV-2 and EHV-5 glycoprotein B (*gB*) gene were used in a phylogenetic analysis that included six EHV-2 and three EHV-5 isolates, which showed that the Turkish EHV-2 and EHV-5 strains have marked sequence divergence from European strains and from each other. Turkish EHV-2 isolates were divided into two distinct subdivisions, and a few isolates were located on a separate branch. This study provides the first epidemiological and phylogenetical report about EHV-2 and EHV-5 infections in Turkey.

Introduction

The equid herpesvirus 2 (EHV-2) and 5 (EHV-5) belong to the *Gammaherpesvirinae* subfamily. Equid herpesvirus 2 (EHV-2) causes the development of upper respiratory diseases, pneumonia, pharyngitis, immunosuppression, enlarged lymph nodes, ulcerative lesions in the oral mucosa, and keratoconjunctivitis (Kershaw et al., 2001; Allen and Murray, 2004; Vengust et al., 2008). Equid herpesvirus 5 (EHV-5) has recently been associated with pulmonary disease characterized by equine multinodular pulmonary fibrosis (Williams et al., 2007), although the pathogenic role of EHV-5 is apparently unknown (Allen and Murray, 2004; Craig et al., 2005). Both viruses have been identified in peripheral blood leucocytes, tracheal aspirates, nasal swabs, nasopharyngeal secretions, and bronchoalveolar lavage from horses showing respiratory symptoms, from apparently healthy horses, and even from wild horses (Browning and Studdert, 1987a; Borchers et al., 1999; Dunowska et al., 2002; Wang et al., 2007; Torfason et al., 2008; Fortier et al., 2009).

Gammaherpesviruses can establish latency in circulating B and/or T lymphocytes (Gleeson and Coggins, 1985; Browning and Studdert, 1987a; Drummer et al., 1996), and the latent non-productive infection could be induced in lymphocytes because of limited gene expression (Gleeson and Coggins, 1985). EHV-2 is spread through nasal and ocular excretions often in the first few months of life (Fu et al., 1986; Kershaw et al., 2001). Equid herpesvirus 5 (EHV-5) is mainly spread by nasal secretions (Dunowska

et al., 2002; Wang et al., 2007). Indeed, virus-shedding mares transmit the viruses to foals after birth (Fu et al., 1986), and horses that shed the viruses play a major epidemiological role in the dissemination of EHV-2 and EHV-5 infections.

The polymerase chain reaction (PCR) provides important tools for the laboratory diagnosis in the viral infections (Nordengrahn et al., 2002; Wang et al., 2007), and multiplex nested PCR is useful for the diagnosis of mixed infections caused by genetically similar viruses (Ataseven et al., 2009) like EHV-2 and EHV-5. Conventional virus detection techniques have several disadvantages compared to PCR such as increased costs, labour and time (3–7 days for growing the viruses) (Wang et al., 2007; Ataseven et al., 2009).

Isolation and genetic characterization of EHV-2 and EHV-5 viruses have been carried out worldwide (Browning and Studdert, 1987a; Holloway et al., 1999; Sharp et al., 2007; Wang et al., 2007; Diallo et al., 2008; Fortier et al., 2009; Richter et al., 2009). Previous studies (Murray et al., 1996; Borchers et al., 1997; Dunowska et al., 2002) report that EHV-2 was an infectious agent in horses showing clinical signs of respiratory infections, but limited information on the role of EHV-5 in equine respiratory disease exists. Nevertheless, molecular and epidemiological data for EHV-2 and EHV-5 infections in the Turkey have not been described. The objectives of our study were to provide information on the detection of both viruses in respiratory disease of the horses in Turkey and to characterize genetically the detected EHV-2/EHV-5 isolates by the *gB* sequencing.

Materials and Methods

Horse population

Fifty-four heparinized blood samples and 73 nasal swabs from horses up to 13 years old in Turkey showing clinical signs of respiratory infections, such as upper respiratory disease or severe pneumonia, were obtained.

Heparinized bloods were centrifuged at 800 ***g*** for 10 min; the leucocyte fraction was removed by capillary pipette. The nasal swab samples were centrifuged at 800 ***g*** for 10 min and the supernatants were collected (Ataseven et al., 2009). These samples were used for virus isolation in cell culture and for EHV-2 and EHV-5 DNA detection using multiplex nested PCR as described elsewere (Wang et al., 2007).

Cell cultures

A total of 40 nasal swabs and 21 leucocyte samples were inoculated into rabbit kidney-13 (RK-13) cell cultures, and 33 nasal swabs and 33 leucocyte samples were also inoculated into the primary equine embryonic lung (EEL) cell cultures. The EEL and RK-13 cell cultures were grown in Dulbecco's Minimal Essential Medium (DMEM) (Biochrom, Germany) supplemented with 1% v/v foetal bovine serum (Biochrom, Germany), 50 units/ml of combined penicillin to streptomycin and 2.5 μg/ml amphotericin B at 37°C in a 5% CO_2 atmosphere and subsequently it was used for virus isolation. The cell cultures were kindly provided by the Animal Health Trust, UK.

Virus isolation

Briefly, 0.2 ml of each nasal swab and leucocyte extract was inoculated separately onto a confluent monolayers grown in cell culture tubes (Greiner, Germany). The infected monolayers were incubated at 37°C in a 5% CO_2 atmosphere for 7 days with daily microscopical examination for the presence of cytopathic effect (CPE). All cultures were passaged at weekly intervals for a total of three passages. The samples were considered as positive if CPE was shown. Uninoculated monolayers were used as cell controls.

Multiplex nested polymerase chain reaction (Mn-PCR)

Total DNA was extracted from 300-μl volumes of the leucocyte and nasal swab samples and from infected cell cultures, using a High Pure Viral Nucleic Acid kit (Roche Diagnostic, Germany). An 817-bp conserved region of the glycoprotein B (*gB*) gene of EHV-2 and the 410-bp conserved region of *gB* gene of EHV-5 were amplified by PCR, using first and second rounds primers as described by Wang et al. (2007). Sterile molecular biology grade water was used as a negative control. The final specific PCR products were visualized using 1.5% agarose gel electrophoresis. The gels were examined for specific size bands using a UV transilluminator (Kodak, Germany).

Sequence analysis

EHV-2-and EHV-5-specific primers obtained from the 817-bp and the 410-bp fragments were used for sequence analysis. PCR products were purified by using High Pure PCR Product Purification Kit (Roche Diagnostic, Germany) and sequenced by using the EHV-2- and EHV-5-specific forward and reverse primers on ABI 3100 automated sequencer. The sequences from both directions were assembled together for each isolate to obtain consensus sequences, which were used in multiple alignments of sequences from different isolates using BioEdit v.7.0.9.0 (Hall, 1999). A phylogenetic tree was created using the Mega 4.0 software program (Tamura et al.,

2007). Bootstrap values were calculated from 1000 replicates with 111 random seeds.

Results

Prevalence of EHV-2 and EHV-5 in horses showing respiratory symptoms

A total of 73 nasal swabs and 54 leucocytes were inoculated into cell cultures. Cytopathic effect (CPE) was presented in 29 of the leucocyte and 35 of the nasal swab samples. For direct detection on extracts, EHV-2-specific DNA was identified in six (8.2%) of the nasal swab extracts and in two (3.7%) of the leucocyte extracts tested. EHV-5-specific DNA was detected directly from five (6.8%) of the nasal swab samples and from seven (12.9%) of the leucocyte samples tested. Following virus isolation, EHV-2-specific DNA was detected in 11 (15.0%) of the nasal swab samples. EHV-5-specific DNA was detected in 8 (10.9%) of the nasal swab extracts and in seven (12.9%) of the leucocyte samples following virus isolation. Three horses were also coinfected with both EHV-2 and EHV-5. Overall, EHV-2- and EHV-5-specific DNA amplicons were obtained in 14 (19.2%) and 16 (21.9%), respectively, of all horses sampled by multiplex nested PCR.

Phylogenetic analysis of the *gB* sequences of EHV-2 and EHV-5

The phylogenetic tree was inferred using neighbour-joining method as implemented in Clustal X algorithm in Mega 4.0. Partial sequences of the *gB* gene of six EHV-2 and three EHV-5 strains obtained from Turkish horses were compared with the previous European (EU) strains and to each other (Fig. 1).

The sequenced isolates were obtained from three different regions in the same year. EHV-5-TR-NS26, EHV-5-TR-NS31, and EHV-2-TR-NS28 belonged to the first region. EHV-5-TR-L2 was obtained from the second region together with EHV-2-TR-NS3, EHV-2-TR-L1-1, and EHV-2-TR-L1-3. Also, both EHV-2-TR-NS41 and EHV-2-TR-NS46 belonged to the third region.

The phylogenetic analysis of the EHV-2 isolates revealed that there was a sequence divergence between Turkish (TR) isolates and EU strains (Fig. 1). There was not a close relation between EHV-2-TR-NS3 and EHV-2-TR-NS28, unlike the EHV-2-TR-NS41 and EHV-2-TR-NS46 isolates. The majority of EHV-2 TR isolates (EHV-2-TR-NS3, EHV-2-TR-NS28, EHV-2-TR-NS41, EHV-2-TR-NS46) were closely related and formed a separate branch of the EHV-2 *gB* lineage; however, two of the EHV-2 isolates (EHV-2-TR-L1-1, EHV-2-TR-L1-3) were divergent from the formers and clustered in a separate branch along with isolates from England. Although EHV-2-TR-NS3, EHV-2-TR-L1-1, and EHV-2-TR-L1-3 were obtained from the same region, EHV-2-TR-NS3 was located on a different branch from EHV-2-TR-L1-1 and EHV-2-TR-L1-3. Sequence analysis demonstrated that the TR EHV-2 isolates shared 34.6–97.8% nucleotide sequence identities with each other. A comparison with EU EHV-2 strain sequences available in GenBank indicated that none of the TR EHV-2 isolates studied here were 100% identical EU strains.

Equid herpesvirus 5 (EHV-5) isolates from Turkey were also quite divergent from their European counterparts and they were located on a different branch in phylogenetic tree. EHV-5-TR-NS26 and EHV-5-TR-NS31 belonged to the same region, while EHV-5-TR-L2 was sampled from a different region. However, sequencing comparison of the *gB* gene of TR EHV-5 isolates showed marked heterogeneity. These isolates shared 77.3–90.2% nucleotide (nt) sequence identities with each other. TR EHV-5 isolates were compared to sequences of EU EHV-5 strains available in GenBank and showed a high degree of heterogeneity (29.5–32.4% nt identity).

Discussion

EHV-2 and EHV-5 have been associated with respiratory diseases in foals and adult horses (Kershaw et al., 2001; Torfason et al., 2008). Most studies report EHV-5 as having a lower prevalence than EHV-2 (Borchers et al., 1999; Dunowska et al., 1999; Nordengrahn et al., 2002; Bell et al., 2006; Torfason et al., 2008), except in some reports (Wang et al., 2007; Diallo et al., 2008; Richter et al., 2009). In this study, EHV-2 and EHV-5 infections of horses in Turkey have high prevalence in the clinically infected adult horses, and the prevalence of EHV-5 (21.9%) was relatively higher than EHV-2 (19.2%). Moreover, virus isolation in conjunction with nested multiplex PCR assay would lead to a higher virus identification rate in multiple respiratory infections caused by EHV-2 and EHV-5.

The shedding characteristics of EHV-2 and EHV-5 in nasal swabs have interestingly significant differences; EHV-2 is shed for a longer time than EHV-5 in nasal secretions, especially in foals (Murray et al., 1996; Dunowska et al., 2002; Nordengrahn et al., 2002; Bell et al., 2006). When tested by direct PCR, 8.2% of the nasal swab samples tested positive for EHV-2-specific DNA and 6.8% tested positive for EHV-5-specific DNA. However, EHV-2-and EHV-5-specific DNA were detected in 15.0% and 10.9% of the nasal swab extracts, respectively, when using multiplex PCR following cell culture. Several reports (Borchers et al., 1997; Franchini et al., 1997; Wang et al., 2007) indicate that cell culture passages increase at a low level the amount of virus. However,

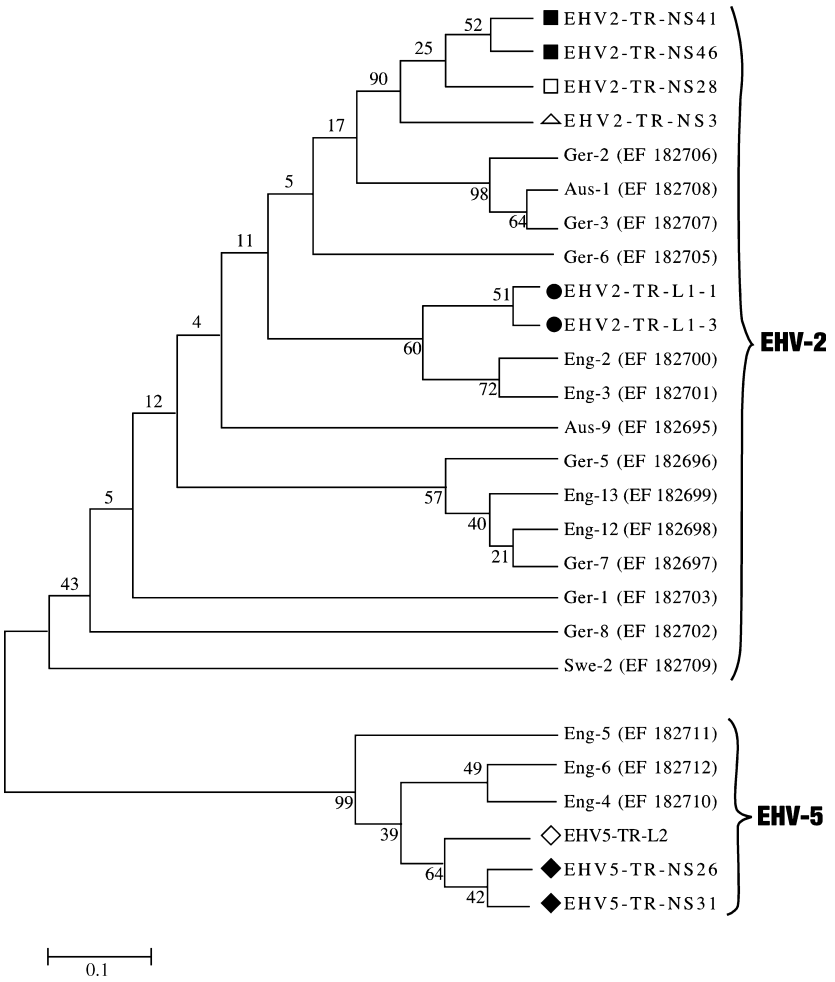

Fig. 1. The phylogenetic tree for *gB* gene after sequences alignment was constructed in Bioedit. A total of 578 nucleotide positions was analyzed. The accession number of sequences of reference viruses downloaded from the GenBank database were shown on the figure. Bar: number of base substitutions per site.The sequences identified in this study were assigned in GenBank under accession numbers of TR-EHV-2: EHV2-TR-L1-1 (GU065277), EHV2-TR-L1-3 (GU065278), EHV2-TR-NS28 (GU065279), EHV2-TR-NS3 (GU065280), EHV2-TR-NS41 (GU065281), EHV2-TR-NS46 (GU065282); TR-EHV-5: EHV5-TR-L2 (GU065283), EHV5-TR-NS26 (GU065284), EHV5-TR-NS31(GU065285).

EHV-2 in nasal swabs was more prevalent than EHV-5, and some samples that were positive by direct PCR were not confirmed by PCR following virus isolation. EHV-2 and EHV-5 grow very slowly in cells (Dunowska et al., 2002; Ackermann, 2006) and may have different growth properties in different cell types because of differences in virus cell entry and replication (Dunowska et al., 2000; Diallo et al., 2008). It is likely that different genotypes among the isolates might have also influenced the growth characteristics in cell cultures. Therefore, depending on presence of CPE in cell cultures, there might have been coinfections of horses related to other viral respiratory tract agents such as EHV1 and EHV4 in the same horses which may have interfered with the replication of EHV-2 and EHV-5 in cell cultures. In this study, the horses in which EHV-specific DNA was detected in leucocytes only may be indicative of a latent state infection; however, the infection status (reinfection, latent, or acute) of horses having EHV-2 or EHV-5-specific DNA in both nasal swab samples and leucocytes was difficult to determine. Contrary to our findings for the nasal swabs, the detection rate of EHV-5 in leucocytes was greater than that for EHV-2, unlike the results described by Nordengrahn et al. (2002). Latent viruses present in cells might be reactivated by cocultivation (direct cell to cell contact) (Drummer et al., 1996), and this amplification step enhances the chance of virus detection (Borchers et al., 1997; Wang et al., 2007). IL-2 might also stimulate the reactivation of latent virus, especially EHV-2 (Wang et al., 2007). In this study, we did not add IL-2 to the cell cultures. For this

reason, the isolation rate, especially in leucocytes, might be lower. Furthermore, EHV-2 infects B lymphocytes and/or macrophages (Drummer et al., 1996; Borchers et al., 1997), but target cells for EHV-5 are not known. Further studies are also needed to confirm the specific cell types involved, and to understand the factors affecting the isolation rates for these viruses in cell cultures.

Equid gammaherpesviruses exhibit a high degree of genetic heterogeneity (Browning and Studdert, 1987b; Dunowska et al., 2000; Bell et al., 2006). Based on the *gB* gene sequences, EU EHV-2 strains isolated could be divided in three distinct subgroups by Sharp et al. (2007). In our study, the sequence comparison of the EHV-2 and EHV-5 *gB* gene demonstrated heterogeneity amongst our isolates and EU strains as described elsewhere (Sharp et al., 2007). Moreover, two TR EHV-2 isolates (EHV-2-TR-L1-1 and EHV-2-TR-L1-3) were grouped as subgroup 1 by Sharp et al. (2007). The other four sequenced isolates (EHV-2-TR-NS28, EHV-2-TR-NS3, EHV-2-TR-NS41, and EHV-2-TR-NS46) were clearly different from the EU strains and located on a separate branch, which were grouped within subgroup 2. EHV-2-TR-NS41 and EHV-2-TR-NS46 were closer genetically to EHV-2-TR-NS28 and EHV-2-TR-NS3. It is possible that all four of these field isolates may represent a phylogenetically distinct subgroup of EHV-2, but further studies by phylogenetic analysis of multi locus gene sequences will help us to better confirm this speculation. None of our isolates were located in subgroup 3. Only, EHV-2 was sequenced from the mixed infections with EHV-2 and EHV-5 because the specific bands for EHV-5 were not resolved well enough for further sequencing reactions. Furthermore, EHV-2 and EHV-5 isolates based on the *gB* gene region did not show a close relationship. Our results demonstrated that genetic diversity was higher amongst TR-EHV-2 isolates than that of TR-EHV-5 isolates.

In conclusion, the findings of this study indicate that EHV-2 and EHV-5 infections are prevalent in respiratory tract disease of adult horses, and our finding supports some works (Fu et al., 1986; Diallo et al., 2008) that suggested both viruses might have played a pre-disposing role in the occurrence of respiratory diseases. Furthermore, the present data indicate that based on phylogenetic analysis of *gB* gene sequences, Turkish EHV-2 and EHV-5 strains appeared to have a marked sequence divergence from each other and also from the European strains. This study provides general epidemiological and phylogenetical information about EHV-2 and EHV-5 infections in Turkey, which will be highly useful for guiding future studies on the prevalence, antigenic and genetic characterization of these viruses at a larger scale for a deeper understanding of equine gammaherpesviruses.

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