Prevalence of Equine Herpesvirus Type 1 Strains of Neuropathogenic Genotype in a Major Breeding Area of Japan

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Abstract. A single non-synonymous nucleotide substitution of guanine (G) for adenine (A) at position 2254 in the viral DNA polymerase gene (encoded by open reading frame [ORF] 30) of equine herpesvirus type 1 (EHV-1) has been significantly associated with neuropathogenic potential in strains of this virus. To estimate the prevalence of EHV-1 strains with the neuropathogenic genotype (ORF30 G2254) in the Hidaka district—a major horse breeding area in Japan—we analyzed the ORF30 genomic region in cases of EHV-1 infection in this area during the years 2001–2010. Of the 113 cases analyzed, 3 (2.7%) were induced by ORF30 G2254 strains. This prevalence is lower than those observed in the U.S.A. (10.8–19.4%), Argentina (7.4%), France (24%), and Germany (10.6%).

Key words: EHV-1, neuropathogenic genotype, prevalence in Japan.

Equine herpesvirus type 1 (EHV-1), a member of the family Herpesviridae, the subfamily Alphaherpesvirinae, and the genus Varicellovirus, is ubiquitous in most horse populations throughout the world. It causes respiratory disease in young horses, abortion in pregnant mares, neonatal death in foals, and myeloencephalopathy (equine herpesvirus myeloencephalopathy: EHM), resulting in a serious economic impact on the horse industry [3, 4, 25]. The recent increase in EHM outbreaks among horses in the U.S.A. and Europe is of major concern to the equine veterinary community, since affected animals often have a poor prognosis [6, 8, 12–14, 16]. The molecular epidemiological study conducted by Nugent et al. revealed that a single non-synonymous nucleotide (nt) substitution of guanine (G) for adenine (A) at position 2254 in the viral DNA polymerase gene (encoded by open reading frame [ORF] 30), which results in a change of asparagine to aspartic acid at amino acid position 752, is significantly associated with outbreaks in which neurologic signs have been recorded [22]. Reverse-genetics studies have further demonstrated that site-directed mutagenesis of this single nucleotide in the genome of a neuropathogenic ORF30 G2254 strain or a non-neuropathogenic ORF30 A2254 strain alters the neuropathogenic potential of EHV-1 in experimentally infected horses [15, 27]. ORF30 G2254 strains replicate more efficiently in peripheral blood mononuclear cells in vivo than do A2254 strains, and this is considered to be a key factor in their neuropathogenicity [1, 2, 15, 27]. This is because EHV-1 is transported to the vascular endothelia in the central nervous system via cell-associated viremia after primary replication in the upper respiratory tract [3]. It is generally believed that the neurological deficits associated with EHM are the result of ischemic death of nervous tissue consequent to viral infection of endothelial cells and the accompanying leaky and thrombotic cerebrospinal vasculitis [10, 17, 29].

The Hidaka district of Hokkaido is a major horse breeding area in Japan (As of January 2010, approximately 19,000 horses were being reared in this area). In 2001, a severe EHM outbreak occurred on one Thoroughbred breeding farm located there (outbreak A). In outbreak A, 3 mares that had become recumbent because of severe paralysis were euthanized, and 2 mares aborted. Sequence analysis indicated that the EHV-1 isolate from the peripheral blood mononuclear cells of one of the euthanized mares possessed the ORF30 G2254 genotype [30]. This was the first reported isolation of an EHV-1 ORF30 G2254 strain in Japan. Since then, no epizootic outbreak of EHM has been reported in this area, although there were 3 sporadic EHM cases in 2005, 2007, and 2009, and it is unknown whether the ORF30 G2254 strain has spread in the horse population in the Hidaka district.

Therefore, to estimate the prevalence of ORF30 G2254 strains in the Hidaka district, we analyzed the ORF30 genomic region of the EHV-1 collected from horses in this area. Additionally, we determined the partial nt sequences of ORF68, which has been proposed as the strain grouping marker for EHV-1 field isolates [22], in the ORF30 G2254 strains identified in this study in order to investigate the genetic relatedness among them.

A total of 187 EHV-1-positive samples submitted to the Hokkaido Hidaka Livestock Hygiene Service Center for virological diagnosis from 2001 to 2010 were analyzed (Table 1). The samples were diagnosed as EHV-1 infected by restriction fragment length polymorphism of viral DNA [5], PCR for detection of the EHV-1 gC gene [18], or immunohistochemical staining. One hundred eighty-one samples were homogenates of lung or thymus collected from equine...
fetuses from 109 cases of sporadic abortions or epizootic abortion outbreaks in which no simultaneous neurological disease was registered. Six samples came from 4 EHM-associated cases. Three out of these 6 samples were homogenates of organs collected from 1 horse with EHM (01-I-15 [pulmonary lymph node]) and 2 aborted equine fetuses (01-I-240 [thymus] and 01-I-247 [lung]) involved in the above mentioned outbreak A, where both EHM and abortions were observed. The remaining 3 samples—homogenate of cerebrum (05-I-310), paraffin-embedded cerebrum (TPD111), and cerebral spinal fluid (CSF) (TPD240)—came from individual horses with EHM in 2005, 2007, and 2009, respectively. DNA was extracted from 200 μl of homogenates or CSF by using a QIAamp DNA blood mini kit (Qiagen Sciences, Germantown, MD, U.S.A.) in accordance with the manufacturer’s instructions. DNA was extracted from the paraffin-embedded cerebrum by using a QIAamp DNA mini kit (Qiagen Sciences) in accordance with the manufacturer’s instructions. The final elution volume for all samples was 200 μl.

A 466-bp fragment of gene ORF30 was amplified by PCR using primers (ORF30: F: GCTACTTCTGAAAACGGAGGC and ORF30_R: CTATCCTACGACACGGCAACA) previously described by Goodman et al. [15]. The reaction mixture (50 μl) consisted of 1 × PCR buffer, 200 μM of dNTP mix, 0.4 μM of each primer, 1 unit of HotStarTaq DNA polymerase (Qiagen GmbH, Hilden, Germany), and 5 μl of extracted DNA sample. The thermal cycling program consisted of 1 cycle of 95°C for 15 min, 35 to 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 10 min. An 831-bp gene fragment of ORF68 was amplified by PCR using the ORF68_F and ORF68_R primers (Table 2). The reaction mixture (50 μl) consisted of 1 × GC 2 PCR buffer, 1 M of GC Melt, 200 μM of dNTP mix, 0.4 μM of each primer, 1 × Advantage-GC 2 polymerase Mix (Clontech Laboratories, Inc., Mountain View, CA, U.S.A.), and 5 μl of extracted DNA sample. The thermal cycling program consisted of 1 cycle of 94°C for 3 min, 35 to 40 cycles of 94°C for 30 sec, and 68°C for 1 min, and a final extension step of 68°C for 3 min. The PCR products were analyzed by electrophoresis in FlashGel Cassettes (Lonza, Rockland, ME, U.S.A.). Following product identification, the PCR products were purified with a QIAquick PCR purification kit (Qiagen GmbH). The purified products were submitted to the sequencing laboratories (Dragon Genomics, Mie, Japan and Bio Matrix Research, Inc., Chiba, Japan) and directly sequenced with PCR primers (for ORF30) or sequencing primers (for ORF68) and Bio Matrix Research, Inc.) and directly sequenced with PCR primers (for ORF30) or sequencing primers (for ORF68) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) on an Applied Biosystems 3730xl DNA Analyzer. VectorNTI (Invitrogen, Carlsbad, CA, U.S.A.) was used for sequence assembly and analysis.

Of the 187 samples analyzed, 5 samples, which originated from 3 EHV-1 cases, contained ORF30 G2254 strains (Table 3). Three out of 5 samples with G2254 strains came from the outbreak A occurred in 2001 (01-I-15, 01-I-240 and 01-I-247), and the other 2 came from the EHM case in 2009 (TPD240) and the abortion outbreak in 2006 (06-I-80) (Table 4). TPD240 (CSF) was collected from a pregnant mare that had been euthanized due to severe paralysis. None of the other horses reared in the farm was reported to exhibit any signs of neurologic disease. 06-I-80 was a
homogenate of lung collected from fetus aborted in a non-neurological abortion outbreak in which the other 2 abortions were observed. The samples collected from these 2 abortions (06-I-68 and 06-I-73) contained ORF30 A2254 strains. All partial ORF30 nt sequences (from nt 2200 to nt 2450) obtained in this study were identical to either the Ab4 (ORF30 G2254) or V592 (ORF30 A2254) strain sequence published in GenBank (accession numbers: for Ab4, DQ180669; for V592, DQ180679), with the exception of 4 A2254 strains from aborted fetuses. One of these 4 strains, which came from an individual sporadic abortion in 2001, showed a change of thymine (T) to C at nt position 2225, corresponding to an amino acid coding change of valine to alanine at position 742. The remaining 3 showed a change of cytosine (C) to A at nt position 2305, corresponding to an amino acid coding change from histidine to asparagine at amino acid position 224 (nt position 672) [21], the mutations observed in the ORF68 sequences from samples 01-I-15, 01-I-240, and 01-I-247) contained unique ORF68 sequence that caused the EHM-associated outbreak in 2001 has not spread widely in the horse population of the Hidaka district. Because the functional domains of EHV-1 ORF68 are considered to be located upstream of amino acid position 224 (nt position 672) [21], the mutations observed in the ORF68 sequences from samples 01-I-15, 01-I-240, and 01-I-247 may not alter any functions of the protein encoded by the gene. Co-circulation of 2 genotypes of EHV-1 existed on one farm in 2006, where 1 abortion (sample 06-I-80) due to G2254 strain and 2 abortions (samples 06-I-68 and 06-I-73) due to A2254 strains were observed. In the present study, the regions from nt positions 724 to 739 of their ORF68 nt sequences, which appeared to contain long poly-G repeats, were not able to be determined (Fig. 1: Data not shown for samples 06-I-68 and 06-I-73). However, the chromatograms of DNA sequencing of them suggested that the numbers of G residues in that region varied among the 3 strains. On the other hand, interestingly, the ORF68 nt sequences from these 3 aborted fetuses possessed a unique change of T to C at nt position 710 that was identified by Nugent et al. in 1 of the 108 EHV-1 strains they analyzed [22]. To reveal the genetic relatedness among these strains precisely, further sequence analysis is required. From the results obtained here, we assume that the current risk of neurological EHV-1 outbreaks induced by ORF30 G2254 strains in the Hidaka district is not high. However, the prevalence of ORF30 G2254 strains in equine abortions in the U.S.A. increased from 3.3% in the 1960s to

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of samples</th>
<th>Sample ID</th>
<th>Clinical sign</th>
<th>Description</th>
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<tr>
<td></td>
<td></td>
<td>01-I-3-240</td>
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<td>Abortion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>01-I-2-247</td>
<td>lung</td>
<td>Abortion</td>
</tr>
<tr>
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<td>3</td>
<td>06-I-80</td>
<td>lung</td>
<td>Abortion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>06-I-68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(lung)</td>
<td>Abortion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>06-I-73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(thymus)</td>
<td>Abortion</td>
</tr>
<tr>
<td>2009</td>
<td>1</td>
<td>TPD240</td>
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<td>EHM</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sample materials are represented in parentheses. <sup>b</sup> 06-I-68 and 06-I-73 contained ORF30 A2254 strains.
19.4% during the years 2000–2006 [26]. Therefore, it will be necessary to continue surveys of the prevalence of ORF30 G2254 strains in the Hidaka district to evaluate the risk of neurological EHV-1 outbreaks in this area.

Notably, samples 05-I-310 and TPD111, which came from horses with EHM, contained the ORF30 A2254 genotype. Additionally, in 1989 a relatively large EHM-associated outbreak caused by the ORF30 A2254 strain occurred in the Japanese racehorse population [19, 30]. Therefore, regardless of the G/A 2254 genotype, strict disease control measures should be implemented in the case of an EHV-1 outbreak.

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REFERENCES


