Detection and genotyping of equid herpesvirus 1 in Uruguay

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Summary

Infection with equid alphaherpesvirus 1 (EHV1) causes respiratory disease, abortion and neurological disorders in horses. Molecular epidemiology studies have demonstrated that a single nucleotide polymorphism (A2254/G2254) in the genome region of open reading frame 30 (ORF30), which results in an amino acid variation (N752/D752) of the EHV1 DNA polymerase, is significantly associated with the neuropathogenic potential of naturally occurring strains. In recent years, an increase in the number of cases of equine neurological disease caused by neuropathogenic variants of EHV1 has been observed in numerous countries. The purpose of this study was to detect the presence of the viral genome of EHV1 and equid herpesvirus 4 (EHV4) in bronchopulmonary lymph nodes of 47 horses from various locations in Uruguay, obtained from a slaughterhouse, and to determine whether the EHV1 genomes possessed the mutation associated with neuropathogenesis (G2254 / D752). The genes encoding glycoprotein H (gH) of EHV1 and glycoprotein B (gB) of

EHV4 were amplified by a seminested polymerase chain reaction (PCR). Of the samples analysed, 28% and 6% of lymph nodes contained the genes of gH and gB, respectively. The viral DNA polymerase gene was amplified and sequenced. Twelve of the thirteen genomes sequenced presented the nucleotide G2254, while the remaining one showed both nucleotides, A2254 and G2254. The results confirm the presence of EHV1 in Uruguay. Furthermore, there is evidence for the first time of detection of EHV4, and high frequency detection of the neuropathogenic variant (G2254 / D752) of EHV1 in Uruguay. These findings provide new insights into the epidemiological situation of EHV1 and EHV4 in that country.

Keywords

Equid herpesvirus – Genotyping – Lymph node –Molecular detection – Neuropathogenic – Uruguay.

Introduction

Equid alphaherpesviruses 1 and 4 (EHV1 and EHV4) are considered the most important alphaherpesviruses that cause a major disease problem among horse populations throughout the world (1). Infection with EHV1 and EHV4 occurs in the upper respiratory tract. Soon afterwards, a primary cell-associated viraemia involving peripheral blood mononuclear cells allows EHV1 to reach other organ systems, resulting in the production of abortions in the last third of gestation, neonatal foal death and neurological syndromes. After primary infection animals remain latently infected for the rest of their lives, resulting in re-shedding and the spread of infectious viruses to susceptible animals (2, 3). Thus, EHV1 causes respiratory disease, abortion and neonatal foal death and, occasionally, neurological deficits associated with myeloencephalopathy.

The main mode of transmission of EHV1 and EHV4 is probably via aerosolised respiratory secretions from young horses (1, 3). Control of infections involving alphaherpesviruses is made more difficult by the establishment of latent infections.

The first isolation of EHV1 from a neurological case was described by Saxegaard (4). Since then, reports of neurological diseases associated with EHV1 have been increasing in both frequency and severity, particularly in recent years in the United States of America (USA) and Europe (5, 6). Recent studies have identified a single nucleotide polymorphism (SNP), adenine (A) to guanine (G), at nucleotide (nt) 2254 of the EHV1 gene encoding the viral DNA polymerase (open reading frame [ORF]30) and the consequent substitution of asparagine (N) by aspartic acid (D) at amino acid position 752. This point mutation is significantly associated with neurological disease (7).

The polymerase chain reaction (PCR) has become an important tool for detecting EHVs, and a seminested PCR assay, in particular, has demonstrated a high analytical sensitivity for detecting EHVs. It may be used in screening studies of latent infection in samples of lymphoid tissue where low levels of EHV nucleic acid persist (8, 9, 10). Latent forms, of both EHV1 and EHV4, have been most often described in lymphoid tissues and peripheral blood leucocytes (10), although latency is also established in the trigeminal ganglion (11, 12).

In Uruguay, EHV1/4 infection has been clinically diagnosed for many years and is probably endemic, as in other countries of the region (13). Easton *et al.* (14) described the detection of EHV1 by molecular and immunohistochemical tests for the first time in Uruguay, in samples from an aborted fetus.

The purpose of this study was to detect the presence of the viral genomes of EHV1 and EHV4 in bronchopulmonary lymph nodes of 47 horses obtained from a slaughterhouse, originating from various locations in Uruguay, and to determine whether the genomes of EHV1 possessed the mutation associated with neuropathogenesis (G2254 / D752).

Materials and methods

Animals

Forty-seven horses admitted to a slaughter plant from seven locations in Uruguay were selected over a period of one month; their individual information is detailed in Table I. All were certified as healthy by ante-mortem inspection. Any animal with gross post-mortem lesions was excluded from the study.

Insert Table I

Tissues

Bronchopulmonary lymph nodes were sampled as aseptically as possible. These were cooled and immediately sent to the laboratory where they were conditioned at −70°C until processed.

Virus isolation

Approximately 0.2 g of tissue was ground and homogenised in 2 ml of virus isolation medium (Dulbecco's Modified Eagle's medium [DMEM] with 2% fetal calf serum, antibiotics and fungizone) and centrifuged at 800 × *g* for 15 min. Tissue supernatants were aliquoted, stored at −70°C and later tested in parallel for virus isolation on RK13 (rabbit kidney) and ED (equine dermal) cells, as described elsewhere (12), and for DNA extraction. Cultures were sub-cultured once when no visible cytopathic effect (cpe) developed after one week. Monolayers were scraped off, frozen and thawed twice, and centrifuged at 800 × *g* for 10 min. Supernatant aliquots were re-plated on RK13 and ED monolayers.

DNA extraction

Samples of DNA from bronchopulmonary lymph nodes were extracted using the QIAamp® DNA Mini Kit Kit (QIAGEN Australia Pty Ltd.) according to the manufacturer's instructions.

Equid alphaherpesvirus 1/4 seminested polymerase chain reaction

Two rounds of amplifications using seminested PCR primers, as described by Varraso *et al.* (15), were performed. This technique can detect specific sequences of the glycoprotein (g)H gene of EHV1 and the gB gene of EHV4. For the first round of amplification, 5 µl of DNA extract samples was taken and PCR was performed using a Taq DNA Polymerase Kit (Tiangen Beijing Biotech ET101) with the following set of primers:

EHV1gHFw: 5′AAGAGGAGCACGTGTTGGAT3′

EHV1gHRw: 5′TTGAAGGACGAATAGGACGC3′

EHV4gBFw: 5′CTGCTGTCATTATGCAGGGA3′

EHV4gBRw:5′CGTCTTCTCGAAGACGGGTA3′

The EHV1 gH primers were expected to yield a first round product of 636 bp and the EHV4 gB primers were expected to yield a first round product of 509 bp.

Samples of DNA extracted from Cornell University reference strains EHV1 040907 (fourth passage) and EHV4 171104 (fourth passage), kindly provided by Dr Barrandeguy from the Instituto de Virología, CICVyA, INTA, were used as positive controls. Molecular biology grade water (AmrescoInc® Solon, Ohio) was used as the negative control. Amplification was performed in a thermocycler (Applied Biosystem Gene Amp 9700 PCR system) with the following parameters: initial 95°C/5 min denaturation, followed by 35 cycles of 95°C/30 sec, 60°C/30 sec and 72°C/1 min, with a final extension at 72°C/5 min.

For the second round of amplification, 2 µl aliquots of the amplicon of the first reaction were taken (these were used as a template) and the PCR was performed under the same conditions as the first reaction, but the Rw primers for EHV1 and EHV4 were replaced by RN: 5′AGTAGGTCAGGCCGATGCTT3′ and RN:

5′CGCTAGTGTCATCATCGTCG3′, respectively. The EHV1 gH primers were expected to yield a second round product of 287 bp, and the EHV4 gB primers were expected to yield a second round product of 323 bp.

Finally, 10 µl aliquots of the seminested PCR products were analysed by 2% agarose gel electrophoresis, coloured with Good View™ (Beijing SBS Genetech Co., Ltd. Haidian, Beijing) and visualised by transillumination with ultraviolet (UV) light.

Equid alphaherpesvirus 1 open reading frame 30 polymerase chain reaction and sequence analysis

All samples that were positive for the detection of EHV1 by the seminested PCR were amplified according to the conditions reported by Nugent *et al.* (7). An aliquot of 5 µl from each sample was run by PCR using a Taq DNA Polymerase Kit (Tiangen Beijing Biotech ET101) and the following set of primers:

ORF30 Fw: GCGCTACTTCTGAAAACG

ORF30 Rw: CCACAAACTTGATAAACACG

The EHV1 ORF30 primers were expected to yield a PCR product of 650 bp.

A sample of DNA extracted from an EHV1 reference strain, kindly provided by Dr Barrandeguy from the Instituto de Virología, CICVyA, INTA, was used as a positive control. Molecular biology grade water (AmrescoInc® Solon, Ohio) was used as the negative control. The PCR was performed in a thermocycler (Applied Biosystem Gene Amp 9700 PCR system) under the following conditions: an initial denaturation at 94°C/4 min, followed by 35 cycles of amplification at 94°C/30 sec, 48°C/1 min and 72°C/2 min, followed by a final extension at 72°C/10 min. After amplification, 5 µl of each PCR product was analysed on a 2% agarose gel, coloured with Good View™ and visualised by transillumination with UV light. Finally, the PCR products were submitted for sequence analysis to Macrogen Inc., Korea (www.macrogen.com). The nucleotide

sequences obtained were assembled and aligned using the BioEdit Sequence Alignment Editor V7.0.5 system (16).

Results

A total of 47 bronchopulmonary lymph nodes samples were processed by the EHV1/4 seminested PCR test. When the first round products were amplified in a second round (seminested) PCR, the products of predicted sizes of 287 and 323 bp for EHV1 and EHV4, respectively, were obtained (Fig. 1). The EHV1 gH gene was detected in 28% (13/47), and the EHV4 gB gene was detected in 6% (3/47) different samples (as shown in Table I). All samples were negative on the virus isolation test.

Insert Figure 1

The neuropathogenic genotype (G2254) was detected in 92% (12/13), and both non-neuropathogenic and neuropathogenic genotypes (A2254 and G2254) were detected in one EHV1-positive sample (Table I).

Discussion

The results of this study showed that 28% of bronchopulmonary lymph node samples were EHV1 positive, and that 6% were EHV4 positive. Other authors have studied the prevalence of EHVs in other countries and found different results (9, 10, 17, 18, 19, 20).

Those findings could be explained by factors such as the equine population, geographical location and sensitivity of the test used (19).

In this study, the virus was not isolated from any of the lymph node samples, suggesting a low level of acute infections of lytic virus and that most PCR positives may be in a latent form. Whether all positive samples analysed here represented true latent forms or a low-level persistent infection cannot be fully determined, since in this work there was no focus on the presence of EHV1 latency-associated transcripts (LATs) in total RNA (11). Further studies are necessary to screen LATs.

Our study demonstrated that the EHV1 neuropathogenic genotype (G2254) is circulating in the horse population in Uruguay. Of the 13 sequenced genomes 12 amplicons had the mutation. The remaining one had both nucleotides A and G at position 2254, probably due to a mixed infection (19, 21).

Different studies have been conducted to detect variants having SNP in the 2254 ORF position 30 of EHV1 in various geographical regions and in different types of tissue (5, 17, 19, 20, 21, 22).

Apparently the frequency and distribution of biovars may be influenced by the breed of horse and the type of tissue studied (19).

The finding that 92% of the EHV1 studied in Uruguay corresponded to the neuropathogenic genotype is not consistent with estimates reported in other regions (5, 21, 22). In Uruguay, the occurrence of equine herpesvirus myeloencephalopathy (EHM) is sporadic; similarly, therefore, as expressed by other authors (23), it is probable that determinants of viral virulence other than G2254 genotype exist, but remain to be identified.

It is also possible that this population of horses did not fairly represent equine populations as a whole, but that does not detract from the fact that these results are the first to indicate unequivocally that EHV1 and 4 are widespread in the horse population and that there is a high frequency of the neuropathogenic variant (G2254 / D752) of EHV1 in Uruguay. This finding provides new epidemiological knowledge related to the situation of EHV1 and EHV4 in the country.

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Table I

Equine sample data sheet for equid alphaherpesvirus 1/4 seminested polymerase chain reaction, and equid alphaherpesvirus 1 genotyping results

Sample number	Breed	Sex	Age (years)	Location	History of vaccination	Results: seminested PCR EHV1	Results: genotype	Results: seminested PCR EHV4
1	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Negative
2	Mixed	Male	>15	Artigas	Unknown	Positive	G2254	Negative
3	Mixed	Female	>15	Artigas	Unknown	Positive	G2254	Negative
4	Mixed	Female	>15	Lavalleja	Unknown	Positive	G2254	Negative
5	Mixed	Male	5–10	Lavalleja	Unknown	Positive	G2254	Negative
6	Mixed	Female	>15	Lavalleja	Unknown	Negative	–	Positive
7	Mixed	Female	5–10	Artigas	Unknown	Positive	G2254	Negative
8	Mixed	Male	>15	Artigas	Unknown	Positive	G2254	Negative
9	Mixed	Female	>15	Artigas	Unknown	Positive	Mixed A2254 / G2254	Negative
10	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Negative
11	Mixed	Male	5–10	Paysandú	Unknown	Negative	–	Negative
12	Mixed	Male	5–10	Paysandú	Unknown	Negative	–	Negative
13	Mixed	Female	>15	Paysandú	Unknown	Negative	–	Negative
14	Mixed	Female	5–10	Paysandú	Unknown	Negative	–	Negative
15	Mixed	Female	5–10	Paysandú	Unknown	Negative	–	Negative
16	Mixed	Female	5–10	Maldonado	Unknown	Positive	G2254	Negative
17	Mixed	Female	>15	Paysandú	Unknown	Negative	–	Negative
18	Mixed	Female	5–10	Paysandú	Unknown	Negative	–	Negative
19	Mixed	Male	5–10	Maldonado	Unknown	Negative	–	Negative
20	Mixed	Male	5–10	Paysandú	Unknown	Negative	–	Negative
21	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Negative
22	Mixed	Male	>15	Florida	Unknown	Negative	–	Negative
23	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Negative
24	Mixed	Male	>15	Tacuarembó	Unknown	Negative	–	Negative

25	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Negative
26	Mixed	Male	>15	Maldonado	Unknown	Negative	–	Negative
27	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Negative
28	Mixed	Male	5-10	Canelones	Unknown	Positive	G2254	Negative
29	Mixed	Female	>15	Tacuarembó	Unknown	Positive	G2254	Negative
30	Mixed	Male	>15	Tacuarembó	Unknown	Negative	–	Negative
31	Mixed	Male	>15	Tacuarembó	Unknown	Negative	–	Negative
32	Mixed	Female	5–10	Canelones	Unknown	Negative	–	Positive
33	Mixed	Female	5–10	Maldonado	Unknown	Negative	–	Negative
34	Mixed	Female	5–10	Tacuarembó	Unknown	Negative	–	Negative
35	Mixed	Female	>15	Tacuarembó	Unknown	Positive	G2254	Negative
36	Mixed	Female	5–10	Paysandú	Unknown	Negative	–	Negative
37	Mixed	Female	>15	Paysandú	Unknown	Positive	G2254	Negative
38	Mixed	Male	5–10	Paysandú	Unknown	Negative	–	Negative
39	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Positive
40	Mixed	Male	>15	Paysandú	Unknown	Negative	–	Negative
41	Mixed	Female	>15	Paysandú	Unknown	Negative	–	Negative
42	Mixed	Female	>15	Paysandú	Unknown	Negative	–	Negative
43	Mixed	Female	>15	Paysandú	Unknown	Negative	–	Negative
44	Mixed	Female	>15	Paysandú	Unknown	Negative	–	Negative
45	Mixed	Female	>15	Paysandú	Unknown	Negative	–	Negative
46	Mixed	Female	5-10	Paysandú	Unknown	Negative	–	Negative
47	Mixed	Female	>15	Paysandú	Unknown	Positive	G2254	Negative

EHV: Equid alphaherpesvirus
PCR: Polymerase chain reaction

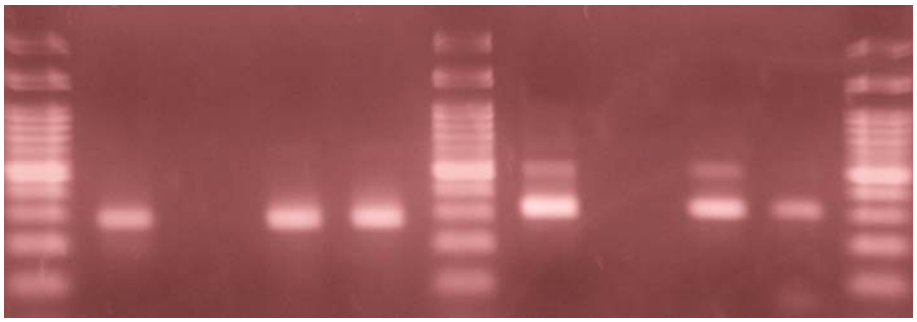

Fig. 1
Polymerase chain reaction products of gH (equid alphaherpesvirus 1) and gB (equid alphaherpesvirus 4) in bronchopulmonary lymph node samples using seminested polymerase chain reaction assays

287 bp EHV1 Positive control (lane 2)

323 bp EHV4 Positive control (lane 7)

EHV1 Positive samples (lanes 4, 5)

EHV4 Positive samples (lanes 9, 10)

Negative control (lanes 3, 8)

100 bp DNA ladder (lanes 1, 6, 11)