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Detection and genotyping of equid herpesvirus 1 in Uruguay

E.R. Castro [1,2]* & J. Arbiza [3]

(1) Departamento Virología, División de Laboratorios Veterinarios 'M.C.Rubino' (DILAVE–MGAP), Ruta 8 Km. 17.500, Montevideo, Uruguay
(2) Posgrado Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay
(3) Sección Virología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
*Corresponding author: ercastro72@gmail.com; racastro@mgap.gub.uy

Summary

Infection with equid alphaherpesvirus 1 (EHV-1) 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 which results in an amino acid variation (N752/D752) of the EHV-1 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 EHV-1 has been observed in numerous countries. The purpose of this study was to detect the presence of the viral genome of EHV-1 and equid herpesvirus 4 (EHV-4) in the bronchopulmonary lymph nodes of 47 horses from various locations in Uruguay, obtained from a slaughterhouse, and to determine whether the EHV-1 genomes possessed the mutation associated with neuropathogenesis (G2254/D752). The genes encoding glycoprotein H (gH) of EHV-1 and glycoprotein B (gB) of EHV-4 were amplified by a semi-nested polymerase chain reaction. Of the samples analysed, 28% and 6% of lymph nodes contained the genes for gH and gB, respectively. The viral DNA polymerase gene was amplified and sequenced. Twelve of the 13 genomes sequenced presented the nucleotide G2254, while the remaining 1 showed both nucleotides, A2254 and G2254. The results confirm the presence of EHV-1 in Uruguay. Furthermore, there is evidence for the first time of the detection of EHV-4, and high-frequency detection of the neuropathogenic variant (G2254/D752) of EHV-1 in Uruguay. These findings provide new insights into the epidemiological situation of EHV-1 and EHV-4 in that country.

Keywords

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

Introduction

Equid alphaherpesviruses 1 and 4 (EHV-1 and EHV-4) are considered the most important alphaherpesviruses that cause a significant disease problem among horse populations throughout the world (1). Infection with EHV-1 and EHV-4 occurs in the upper respiratory tract. Soon afterwards, a primary-cell-associated viraemia, involving peripheral blood mononuclear cells, allows EHV-1 to reach other organ systems, resulting in 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, EHV-1 causes respiratory disease, abortion and neonatal foal death and, occasionally, neurological deficits associated with myeloencephalopathy.

The main mode of transmission of EHV-1 and EHV-4 is probably via aerosolised respiratory secretions from young horses (1, 3). The control of infections involving alphaherpesviruses is made more difficult by the establishment of latent infections.

The first isolation of EHV-1 from a neurological case was described by Saxegaard (4). Since then, reports of neurological diseases associated with EHV-1 have been

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increasing in both frequency and severity, particularly in recent years in the United States of America (USA) and in Europe (5, 6). Recent studies have identified a single nucleotide polymorphism (SNP), adenine (A) to guanine (G), at nucleotide (nt) 2254 of the EHV-1 gene which encodes for 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 semi-nested 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 acids persist (8, 9, 10). Latent forms, of both EHV-1 and EHV-4, 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, EHV-1/4 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 EHV-1 by molecular and immunohistochemical tests for the first time in Uruguay, in samples from an aborted foetus.

The purpose of this study was to detect the presence of the viral genomes of EHV-1 and EHV-4 in the bronchopulmonary lymph nodes of 47 horses, originating from various locations in Uruguay, obtained from a slaughterhouse, and to determine whether the genomes of EHV-1 possessed the mutation associated with neuropathogenesis (G2254/D752).

Materials and methods

Animals

Forty-seven horses, from seven locations in Uruguay, admitted to a slaughterhouse 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.

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% foetal 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 rabbit kidney (RK)13 and equine dermal (ED) 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 replated on RK13 and ED monolayers.

DNA extraction

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

Equid alphaherpesvirus 1/4 semi-nested polymerase chain reaction

Two rounds of amplifications using semi-nested PCR primers, as described by Varrasso *et al.* (15), were performed. This technique can detect specific sequences of the glycoprotein (g)H gene of EHV-1 and the gB gene of EHV-4. 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:

– EHV-1gHFw: 5′AAGAGGAGCACGTGTTGGAT3′

– EHV-1gHRw: 5′TTGAAGGACGAATAGGACGC3′

– EHV-4gBFw: 5′CTGCTGTCATTATGCAGGGA3′

– EHV-4gBRw:5′CGTCTTCTCGAAGACGGGTA3′.

The EHV-1 gH primers were expected to yield a first-round product of 636 base pairs (bp) and the EHV-4 gB primers were expected to yield a first-round product of 509 bp.

Samples of DNA extracted from Cornell University reference strains EHV-1 040907 (fourth passage) and EHV-4 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, USA) 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

Table I
Equine sample data sheet for equid alphaherpesvirus 1/4 semi-nested polymerase chain reaction, and equid alphaherpesvirus 1 genotyping results

Sample number	Breed	Sex	Age (years)	Location	History of vaccination	Results: semi-nested PCR EHV-1	Results: genotype	Results: semi-nested PCR EHV-4
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

were used as a template) and the PCR was performed under the same conditions as the first reaction, but the Rw primers for EHV-1 and EHV-4 were replaced by RN: 5′AGTAGGTCAGGCCGATGCTT3′ and RN: 5′CGCTAGTGTCATCATCGTCG3′, respectively. The EHV-1 gH primers were expected to yield a second-round product of 287 bp, and the EHV-4 gB primers were expected to yield a second-round product of 323 bp.

Finally, 10 µl aliquots of the semi-nested PCR products were analysed by 2% agarose gel electrophoresis, coloured with Good View™ (Beijing SBS Genetech Co. Ltd, Haidian, Beijing, People's Republic of China [China]) 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 EHV-1 by the semi-nested 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 Biotech [Beijing] ET101, China) and the following set of primers:

– ORF30Fw: GCGCTACTTCTGAAAACG
– ORF30Rw: CCACAAACTTGATAAACACG.

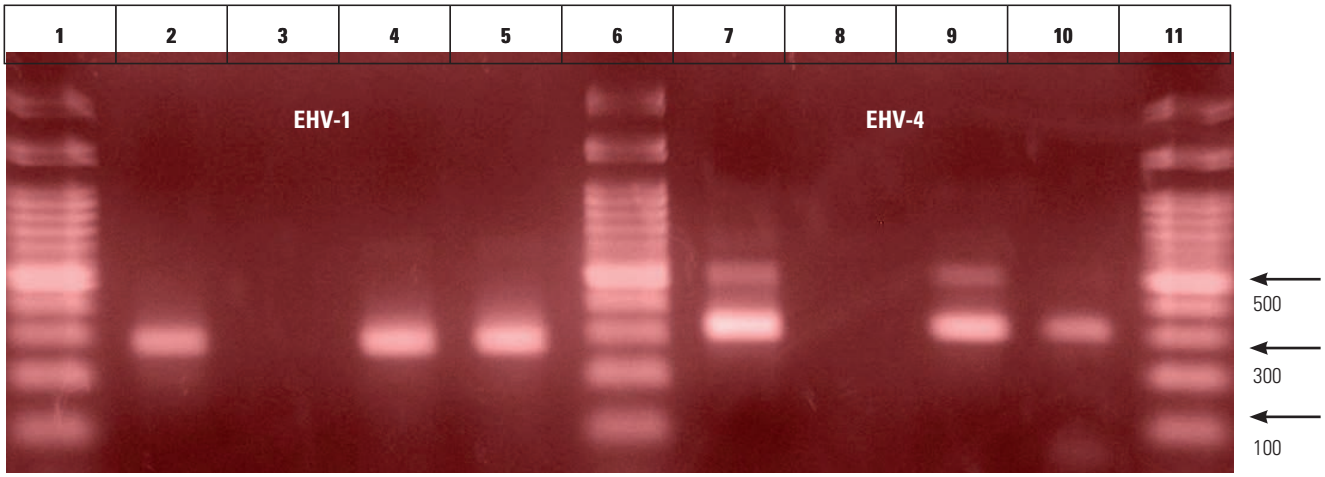
The EHV-1 ORF30 primers were expected to yield a PCR product of 650 bp.

A sample of DNA extracted from an EHV-1 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, USA) 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., Republic of 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 node samples were processed by the EHV-1/4 semi-nested PCR test. When the first-round products were amplified in a second round (semi-nested) PCR, products of the predicted sizes of 287 bp and 323 bp for EHV-1 and EHV-4, respectively, were obtained (Fig. 1). The EHV-1 gH gene was detected in 28% (13/47), and the EHV-4 gB gene was detected in 6% (3/47) of samples (as shown in Table I). All samples gave a negative result on the virus isolation test.

The neuropathogenic genotype (G2254) was detected in 92% (12/13), and both non-neuropathogenic and neuropathogenic genotypes (A2254 and G2254) were detected in one EHV-1-positive sample (Table I).



bp: base pair
EHV: equid alphaherpesvirus
Lanes 1, 6, 11: 100 bp DNA ladder
Lane 2: 287 bp EHV-1 (positive control)
Lanes 3, 8: negative control
Lanes 4, 5: EHV-1 (positive samples)
Lane 7: 323 bp EHV-4 (positive control)
Lanes 9, 10: EHV-4 (positive samples)

Fig. 1
Polymerase chain reaction products of glycoprotein H (equid alphaherpesvirus 1) and glycoprotein B (equid alphaherpesvirus 4) in bronchopulmonary lymph node samples using semi-nested polymerase chain reaction assays

Discussion

The results of this study showed that 28% of bronchopulmonary lymph node samples were EHV-1 positive, and that 6% were EHV-4 positive. However, studies elsewhere on the prevalence of EHVs have attained 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 EHV-1 latency-associated transcripts (LATs) in total RNA (11). Further studies are necessary to screen LATs.

The authors' study demonstrated that the EHV-1 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 EHV-1 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 EHV-1 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 is sporadic; similarly, therefore, as expressed by other authors (23), it is probable that determinants of viral virulence other than the 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 EHV-1 and 4 are widespread in the horse population and that there is a high frequency of the neuropathogenic variant (G2254/D752) of EHV-1 in Uruguay. This finding provides new epidemiological knowledge related to the situation of EHV-1 and EHV-4 in the country.

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Détection et génotypage d'un herpèsvirus équin de type 1 en Uruguay

E.R. Castro & J. Arbiza

Résumé

L'infection par l'herpèsvirus équin de type 1 (EHV-1, sous-famille des alpha-Herpesvirinae) provoque chez les chevaux des maladies respiratoires, des avortements et des troubles neurologiques. Des études d'épidémiologie moléculaire ont montré une corrélation significative entre la présence d'un polymorphisme nucléotidique simple (A2254 / G2254) dans la région génomique

du cadre de lecture ouvert 30 (ORF30), se traduisant par une variation des acides aminés (N752 / D752) de l'ADN polymérase du virus EHV-1, et la neuropathogénicité potentielle des souches présentes sur le terrain. On constate depuis quelques années dans de nombreux pays une augmentation du nombre de chevaux atteints de troubles neurologiques dus aux variants neuropathogènes de l'EHV-1. Les auteurs présentent les résultats d'une étude visant à détecter la présence du génome viral de l'EHV-1 et de l'herpèsvirus équin de type 4 (EHV-4) dans des échantillons de ganglions lymphatiques broncho-pulmonaires de 47 chevaux provenant de diverses régions d'Uruguay, collectés à l'abattoir, et à déterminer si les génomes de l'EHV-1 présentaient la mutation associée avec cette neuropathogénicité (G2254 / D752). Dans un premier temps, une amplification en chaîne par polymérase semi-nichée a permis d'amplifier les gènes codant pour la glycoprotéine H (gH) de l'EHV-1 et la glycoprotéine B (gB) de l'EHV-4. Les gènes gH et gB étaient présents respectivement dans 28 % et 6 % des échantillons de ganglions lymphatiques analysés. Le gène de l'ADN polymérase virale a été amplifié puis séquencé. Au total, 12 des 13 génomes séquencés contenaient le nucléotide G2254 tandis que le treizième génome présentait à la fois les nucléotides A2254 et G2254. Ces résultats confirment la présence de l'EHV-1 en Uruguay. En outre, il s'agit du premier rapport faisant état de la présence de l'EHV-4 et de la fréquence de détection du variant neuropathogénique (G2254 / D752) de l'EHV-1 en Uruguay. Ces résultats apportent un nouvel éclairage sur la situation épidémiologique de l'EHV-1 et l'EHV-4 dans ce pays.

Mots-clés
Détection moléculaire – Ganglion lymphatique – Génotypage – Herpèsvirus équin – Neuropathogénicité – Uruguay.

Detección y tipificación genética del herpesvirus equino 1 en el Uruguay

E.R. Castro & J. Arbiza

Resumen
La infección por alfa-herpesvirus equino 1 (HVE1) causa en el caballo enfermedades respiratorias, abortos y trastornos neurológicos. Los estudios de epidemiología molecular han demostrado la existencia de una correlación significativa entre el potencial neuropatogénico de cepas presentes en la naturaleza y la presencia de un polimorfismo de nucleótido único (A2254/G2254) en la región genómica del marco abierto de lectura 30 (ORF30). Este polimorfismo se traduce en la variación de un aminoácido (N752/D752) en la ADN-polimerasa del HVE1. En los últimos años se ha observado en muchos países un aumento del número de casos de enfermedad neurológica equina causados por variantes neuropatógenas del HVE1. Los autores describen un estudio encaminado a detectar la presencia de genoma vírico del HVE1 y del herpesvirus equino 4 (HVE4) en ganglios linfáticos broncopulmonares de 47 caballos de varias localidades del Uruguay a partir de muestras obtenidas en mataderos, y a dilucidar después si el genoma de esos HVE1 poseía la mutación ligada a la neuropatogénesis (G2254/D752). En primer lugar se empleó una técnica de reacción en cadena de la polimerasa (PCR) semianidada para amplificar los genes que codifican la glucoproteína H (gH) del HVE1 y la glucoproteína B (gB) del HVE4. De las muestras de ganglios linfáticos analizadas, los genes de la gH y de la gB estaban presentes,

respectivamente, en un 28% y un 6%. Se amplificó y secuenció el gen de la ADN-polimerasa vírica. Doce de los trece genomas secuenciados presentaban el nucleótido G2254, mientras que el restante contenía ambos nucleótidos, A2254 y G2254. Los resultados confirman la presencia del HVE1 en el Uruguay. Además, por primera vez, quedó demostrada la presencia del HVE4, así como la elevada frecuencia de la variante neuropatógena (G2254/D752) del HVE1, en el Uruguay. Estos resultados arrojan nueva luz sobre la epidemiología de los virus HVE1 y HVE4 en el país.

Palabras clave
Detección molecular – Ganglio linfático – Herpesvirus equino – Neuropatogénico – Tipificación genética – Uruguay.

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