J Vet Diagn Invest 17:500–502 (2005)

Isolation of equine herpesvirus–2 from the lung of an aborted fetus

Cecilia Mónica Galosi[1], Viviana Cid de la Paz, Leandro Carlos Fernández, Javier Pablo Martinez, María Isabel Craig, María Barrandeguy, María Elisa Etcheverrigaray

Abstract. This study describes the isolation of equine herpesvirus–2 (EHV-2) from the lung of an aborted equine fetus in Argentina. The isolated virus was confirmed as EHV-2 by indirect immunofluorescence using a rabbit anti–EHV-2 polyclonal antiserum and by virus-neutralization test using an equine polyclonal antibody against EHV-2. Restriction endonuclease DNA fingerprinting with *Bam*HI also confirmed the identity of the virus as EHV-2. Furthermore, viral nucleic acid was detected by polymerase chain reaction from the original lung sample and from the DNA obtained from cells infected with the virus isolate. This work constitutes the first reported isolation of EHV-2 from an aborted equine fetus. The presence of EHV-2 in the lung of the aborted fetus would indicate that this virus is capable of crossing the placental barrier. However, no cause–effect relationship was established between the EHV-2 isolate and the abortion.

Key words: Aborted fetus; equine herpesvirus–2; isolation.

The horse is the natural host for 5 recognized herpesviruses: equine herpesviruses 1, 3, and 4 (EHV-1, EHV-3, and EHV-4) belong to the Alphaherpesvirinae subfamily, whereas EHV-2 and EHV-5 are classified within the Gammaherpesvirinae subfamily.[18,20,21] The EHV-1 and EHV-4 are major causes of abortion and respiratory disease, respectively, and they are of considerable economic importance worldwide. Equine herpesvirus–3 (equine coital exanthema virus) is the causative agent of a relatively mild genital exanthema of both mares and stallions. In Argentina, EHV-1 and EHV-4 have been isolated from aborted fetuses, stillborn foals, and horses, with respiratory disease. The EHV-3 was isolated for the first time in Argentina from thoroughbred horses in training, suffering from unilateral rhinitis.[2,8,11]

The EHV-2 infection occurs soon after birth, often in the presence of maternal antibodies.[16] The prototype strain of EHV-2 (LKV), the first gammaherpesvirus recovered from the horse, was isolated in 1962 from the respiratory tract of a foal with "catarrh and coughing."[3] The virus has been isolated from leukocytes and various organs of healthy animals or horses with upper respiratory disease signs, anorexia, serous nasal discharge, conjunctivitis, fever and pneumonia, general malaise, and poor performance.[4–7] Equine herpesvirus–2 may play a role as a predisposing factor for other infections such as *Rhodococcus equi* infection.[14] The virus can establish either a persistent or a latent infection in B lymphocytes and has been isolated from peripheral mononuclear cells and mammary gland macrophages.[10,12,14,17] It has also been suggested that EHV-2 may play a role in transactivation and reactivation of latent EHV-1 and EHV-4 infections.[9] Prenatal infection with EHV-2 has not been recorded, and the virus has not been detected in colostrum or milk. There is no evidence that EHV-2 has abortigenic potential. Experimental infection of a mid-gestational equine fetus in utero resulted in normal term delivery, although the foal showed mild rhinitis and conjunctivitis, with nasal shedding of EHV-2.[13,19]

Similar to EHV-2, EHV-5 infection has been associated with upper respiratory disease, and the 2 viruses can simultaneously coinfect the same individual. Both the viruses are slow growing in primary isolation; they remain highly cell associated and cannot be differentiated by their growth properties in cell culture.[9]

A high prevalence of neutralizing antibodies against EHV-2 (up to 90%) has been reported in horses worldwide, and the virus has been isolated from circulating leukocytes in 89% of adult horses in the United States, 86% in Scotland, 90% in England, 55% in Japan, and 77% in Switzerland.[3] In Australia and the UK, 97% of examined abattoir horses harbored the virus in their lymphoid cells.[15]. In Argentina, the serological prevalence is 80%, and the virus has been isolated from animals with respiratory disease and detected by polymerase chain reaction (PCR) from nasal swab samples (Craig, unpublished data).

This study describes the isolation of EHV-2 from the lung of an aborted fetus. Pieces of lung, liver, and spleen were collected at necropsy from an aborted fetus submitted from a farm located in the Province of Buenos Aires. The samples were investigated for the presence of infectious virus by inoculation of supernatant into rabbit kidney (RK13) cells, and cytopathic effects (CPE) were monitored. On day 17 after inoculation, new cultures were established with the infected cells and by addition of fresh cells. Blood from the aborting mare was collected on the day of abortion and 20 days after abortion, and sera were prepared for use in virus-neutralization (VN) assays. The VN titers were determined by standard technique against 100 tissue culture infective doses

Department of Virology, Faculty of Veterinary Sciences, National University of La Plata, 60 y 118 La Plata, Buenos Aires Province, Argentina (Galosi, Cid de la Paz, Fernández, Martínez, Etcheverrigaray), the CICV-INTA, Castelar, Buenos Aires Province, Argentina (Craig, Barrandeguy) Member of the Scientific Research Commission (CIC) (Galosi, Cid de la Paz), Buenos Aires Province, Argentina.

[1]Corresponding Author: Cecilia Mónica Galosi, Department of Virology, Faculty of Veterinary Sciences, National University of La Plata, 60 and 118, P.O. Box 296, 1900 La Plata, Buenos Aires Province, Argentina.

50% (TCID50%) of the first EHV-2 strain isolated in Argentina (Craig, unpublished data). To confirm the isolate as EHV-2, VN test were performed using an equine polyclonal antibody against EHV-2 and polyclonal antibodies against EHV-1, EHV-4, and equine arteritis virus (Bucyrus strain)[a] as controls. In addition, standard immunofluorescence (IF) test was prepared using a rabbit anti–EHV-2 polyclonal antiserum[b] and fluorescein isothiocyanate–labeled anti-rabbit immunoglobulin G antibody.[c]

The restriction endonuclease (RE) analysis was done as described by Galosi et al.[11] The RK13-infected cells were treated with proteinase K,[d] and total DNA was extracted with phenol and phenol–chloroform–isoamylalcohol, precipitated with cold absolute alcohol and finally cleaved with *Bam*HI.[d] The resultant restriction pattern obtained by agarose gel electrophoresis was compared with patterns from 2 reference articles.[1,7] To detect EHV-2 DNA, a previously described nested PCR was used to amplify a region located upstream of the gene that encodes a product homologous to human interleukin-10.[4]

A virus confirmed to be EHV-2 was isolated from RK13 cells inoculated with the lung sample from the aborted equine fetus. The CPE consisted of foci of rounded cells and were detected about 14 days after inoculation of RK13 cell monolayers, with a 1:10 dilution of the processed lung sample. In newly established, infected cell monolayers, cytopathic changes developed slowly as has been described for EHV-2 by other authors.[7] Complete CPE was not detected even after 7–10 days of incubation. The EHV-2–positive cells were demonstrated by immunofluorescent staining. No viral CPE or virus-specific fluorescence was observed on RK13 cells inoculated with liver and spleen samples from the aborted fetus or in mock-infected cells. The EHV-2 VN titers detected on both serum samples obtained from the aborting mare were high (1:512). Identity of the isolated virus (designated BB03) was confirmed by VN assay with a polyclonal antibody against EHV-2. The virus was not neutralized by antisera against EHV-1, EHV-4, or equine arteritis virus. The RE analysis with *Bam*HI confirmed that the virus was EHV-2.[1,7] The second round of nested PCR using EHV-2–specific primers and DNA extracted from the lung sample or from infected cells amplified a visible product of the expected size (~750 bp).[4] The PCR assay was negative for DNA extracted from liver and spleen samples and for EHV-1 DNA used as a negative control.

The EHV-2 was first isolated in Argentina from equine nasal swabs in 1997. The EHV-2 has been detected in various regions of the world; however, the exact prevalence and pathogenic role of this gammaherpesvirus are still not clear.[17] In this study, the authors isolated EHV-2 from the lung of an equine aborted fetus. A long period of time (14 days) was required for the virus to replicate and produce CPE in cell culture. The high titer of neutralizing antibodies against EHV-2 present in the mare could indicate a recent exposure to the virus. There is no evidence to indicate that EHV-2 has abortigenic potential and the virus is not known to be transmitted from mare to foal via vaginal secretions and colostrum.[14,19] Experimentally, it has been found that a pregnant mare inoculated with EHV-2 by the intrauterine route did not have any effect on the pregnancy; however, the normal newborn foal showed signs of disease at 4 days after birth.[13] Although vertical transmission of EHV-2 has not been demonstrated, it is recognized that the role of the mare in transmitting EHV-2 infections requires further investigation.[6] Viral persistence and latency are features of all herpesviruses. The EHV-2 is similar to Epstein Barr virus in that persistence and latency occur within peripheral blood mononuclear cells.[4] Some authors suggest that although EHV-2 infection is very common, the mechanism and location of persistence of the virus is not well known.[17] The authors of this study propose that the EHV-2 isolate in this study was present in the mare's leukocytes and was carried across the placenta to the fetus. Equine herpesvirus–2 is reported to belong to a heterogeneous group of viruses, and the characteristics of different strains are likely to vary and could reflect biological differences.[4,6,7] This work constitutes the first reported isolation of EHV-2 from an aborted equine fetus. The presence of EHV-2 in the lung of the aborted fetus would indicate that this virus crossed the placental barrier. However, it is not known whether EHV-2 was responsible for this abortion. Nevertheless, the finding of possible transplacental transmission of EHV-2 is a novel observation that should be taken into account in future studies on EHV-2.

Acknowledgements. This study was partially supported by grants from the Department for Science and Technology, National University of La Plata and from Japan International Copperation Agency (JICA). We are grateful to Dr. Luis Alvarez (B. Blanca Laboratory, Bs As, Argentina) by the technical assistance. The authors express appreciation to Dr. George Allen of the Department of Veterinary Science, University of Kentucky, Lexington, Kentucky, for helpful review of this manuscript.

Sources and manufacturers

a. Dr. T. Kumanomido and Dr. Y. Fukunaga, Japan Racing Association, Tochigi, Japan.
b. 341 EDV 8301, NVSL Ames, IA.
c. Kirkergaard & Perry Laboratories Inc., Maryland, MD.
d. Promega, Madison, WI.

References

1. Agius CT, Crabb BS, Drummer HE, et al.: 1994, Studies of the DNA, proteins and cellular associations of equine gammaherpesviruses 2 and 5. *In:* Equine infectious disease VII, ed. Nakajima H, Plowright W, pp. 277–285. R&W Publications (Newmarket) (Tokyo, Japan) Limited, UK.
2. Allen GP, Bryans JT: 1986, Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infection. Prog Vet Microbiol Immunol 2:78–144.
3. Allen GP, Murray JT: 2004, Equid herpesvirus 2 (EHV-2) and equid herpesvirus 5 (EHV-5) infections. *In:* Infectious diseases of livestock, ed. Coetzer J, Thomson G, Tustin R. Oxford Press, Capetown, UK. pp. 860–868.
4. Borchers K, Wolfinger V, Goltz M, et al.: 1997, Distribution and relevance of equine herpesvirus type 2 (EHV-2) infections. Arch Virol 142:911–928.
5. Borchers K, Wolfinger V, Ludwig H, et al.: 1998, Virological and molecular biological investigations into equine herpes virus type 2 (EHV-2) experimental infections. Virus Res 55:101–106.
6. Browning GF, Studdert MJ: 1987, Genomic heterogeneity of equine betaherpervirus. J Gen Virol 68:1441–1447.

7. Collison PN, O'Rielly JL, Ficorilli N, Studdert MJ: 1994, Isolation of equine herpesvirus type 2 (equine gammaherpesvirus 2) from foals with keratoconjunctivitis. JAVMA 205:329–331.
8. Crabb BS, Studdert MJ: 1995, Equine herpesviruses 4 (Equine rhinopneumonitis virus) and 1 (Equine abortion virus). Adv Virus Res 45:153–190.
9. Drummer HE, Reubel GH, Studdert MJ: 1996, Equine gammaherpesvirus 2 (EHV-2) in latent in B lymphocytes. Arch Virol 141:495–504.
10. Dutta SK, Campbell DL: 1978, Pathogenicity of Equine herpesvirus: in vivo persistence in equine tissue macrophages of herpesvirus type 2 detected in monolayer macrophage cell culture. Am J Vet Res 39:1422–1427.
11. Galosi CM, Norimine J, Echeverría MG, et al.: 1998, Diversity of genomic elecropherotypes of naturally occurring equine herpesvirus 1 isolates in Argentina. Braz J Med Biol Res 31:771–774.
12. Gleeson LJ, Coggins L: 1985, Equine herpesvirus type 2: cell-virus relationship during persistent cell-associated viremia. Am J Vet Res 46:19–23.
13. Gleeson LJ, Studdert MJ: 1977, Equine herpesviruses. Experimental infection of a foetus with type 2. Aust Vet J 53:360–362.
14. Murray MJ, Eichorn ES, Dubaui EJ, et al.: 1996, Equine herpesvirus type 2: prevalence and seroepidemiology in foals. Equine Vet J 28:432–436.
15. Nordengrahn A: 2000, Differential diagnosis and aspects on epidemiology and pathogenesis of Equine Herpesvirus. Doctoral Thesis, Swedish University of Agricultural Sciences (Uppsala, Sweden).
16. Nordengrahn A, Klingeborn B, Lindholm A, Merza M: 2001, The use of a neutralizing monoclonal antibody to detect infections of equine herpesvirus type 2 (EHV-2). J Vet Diagn Invest 13:389–393.
17. Nordengrahn A, Merza M, Ros C, et al.: 2002, Prevalence of equine herpesvirus type 2 and 5 in horse populations by using type-specific PCR assays. Vet Res 33:251–259.
18. Roder HJ, Bugert JJ, Handerman M, et al.: 1994, Molecular characterization and determination of the coding capacity of the genome of Equine herpesvirus type 2 between the genome coordinates 0.235 and 0.258 (the Eco RI DNA fragment N; 4.2 kbp). Virus Genes 9:61–75.
19. Smith KC: 1997, Herpesviral abortion in domestic animals. Vet J 153:253–268.
20. Studdert MJ: 1974, Comparative aspects of equine herpesviruses. Cornell Vet 64:94–122.
21. Telford EAR, Watson MS, Aird JP, Davison AJ: 1995, The DNA sequence of Equine Herpesvirus 2. J Mol Biol 249:520–528.

J Vet Diagn Invest 17:502 (2005)

Erratum

***J Vet Diagn Invest* 17:58–61 (2005)**

The PCR primers listed in the article entitled, "Diagnostics of Infectious Canine Hepatitis Virus (CAV-1) Infection in Puppies with Encephalopathy" (*J Vet Diagn Invest* 17:58–61 (2005)) were incorrect. The correct primers as listed in the source reference for the article should be:

5′-GCCGCA (G/A) TGGT C(T/C) TACATGCAC ATC−3′
5′-CAGC(A/G) (C/T) (G/A) CCGCGGATGTCAAA (G/A) T−3′

The authors and Editor regret any confusion caused by publication of incorrect primer sequences.

J Vet Diagn Invest 17:502 (2005)

Submitting manuscripts to the *Journal of Veterinary Diagnostic Investigation*

All manuscripts to the *Journal of Veterinary Diagnostic Investigation* (JVDI) should be submitted through our online submission site at **http://mc.manuscriptcentral.com/jvdi.**

Instructions to authors are downloadable at the journal's website **(http://jvdi.org)** and the online manuscript submission site (http://mc.manuscriptcentral.com/jvdi) under the ***Instructions and Forms*** tab.