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BRIEF REPORT

Equine arteritis virus: a new isolate from the presumable first carrier stallion in Argentina and its genetic relationships among the four reported unique Argentinean strains

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Abstract Equine arteritis virus (EAV) was isolated from a testicle of the presumable first stallion infected with EAV in Argentina. This virus isolate (named LT-LP-ARG) was confirmed by GP5-specific PCR and indirect immunofluorescence assays. The PCR product was sequenced, and the phylogenetic analysis revealed that the LT-LP-ARG strain of EAV forms a monophyletic group, together with other strains previously isolated in our laboratory (LP02 group). However, all Argentinean EAV strains belong to a polyphyletic group. We believe that the virus isolate presented in this report could be the origin of EAV infection in our country.

Equine arteritis virus (EAV) is a positive-stranded RNA virus, first isolated from fetal lung tissue during an outbreak of respiratory disease and abortion in Standardbred horses in Bucyrus, OH, USA, in 1953 [4]. It has been classified as a member of the family *Arteriviridae*, order *Nidovirales*, and grouped together with lactate dehydrogenase-elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), and simian hemorrhagic fever virus (SHFV) [2]. Only one serotype of EAV has been described worldwide, although strains of EAV have been shown to differ in their pathogenicities [15]. The EAV genome is 12.7 kb in length and has nine open reading frames (ORFs) encoding their structural and non-structural proteins. ORF 5, which encodes the GP5 protein is the most variable part of the EAV genome and it is located between nucleotides (nt) 11,146 and 11,913, based on National Center for Biotechnology Information RefSeq NC_002532. [27]. This envelope glycoprotein expresses neutralization determinants of EAV and together with the M protein induces neutralizing antibodies [1, 20]. EAV spreads by respiratory and venereal routes [16, 24]. In most cases, EAV causes only subclinical infection, but it can cause death of foals, abortions of mares and mild clinical respiratory symptomatology in adult horses [20]. Adult stallions may develop chronic infection, and they could become a major source of infection over a long period of time by spreading the virus via semen, acting as natural reservoir of the disease [25]. This virus carrier state is testosterone dependent [14]. Following standard procedures for serological identification, detection of EAV is achieved by isolation of the virus in cell culture. The RT-PCR technology has also been applied in many cases for identification of EAV in biological samples [3], acting as a support technique in this procedure. The prevalence of EAV-infected resident stallions in Argentina is very low, although there are no official records available in the country. The only seropositive horses are 16 jumping stallions belonging to breeding farms that had experienced EAV infection. In the last 3 years, 19 thoroughbred

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stallions from a total of 3,853 (0.4%) have been found positive for EAV. These animals were imported several years ago and were vaccinated in the country of origin (Barrandeguy, personal communication). The lack of any EAV import control measures in our country before 1994, minimal knowledge of the disease and economic reasons permitted the import of stallions only for sports purposes. Since 1994, new measures for EAV control have been introduced in Argentina. However, in 2001 the first isolation of EAV in our country was reported. The isolate was obtained from a semen sample from a seropositive imported stallion located in a military riding breeding farm with high seroprevalence of EAV (LP01 strain) [5]. Three other EAV isolates were reported in the country in 2007 (LP02 group), and the first phylogenetic characterization grouped them with the European EAV strains [6]. These three strains were obtained from three Warmblood stallions (registered as Zangersheide branded) located in a breeding and training farm of jumping horses. Circumstantial evidence suggested that dissemination of the strains inside the farm was initiated at least by one of these three stallions.

Isolation of any virus in cell culture can be achieved successfully from fresh samples or from samples stored under proper conditions. In this report, we achieved the isolation of EAV from a testicle of the presumable first stallion infected with EAV in Argentina (named "A"). This stallion was brought to Argentina from Europe in 1992 and was found to be positive for EAV in our laboratory in 1998. We do not have any report about virus isolation or test mating of this horse from any other Argentinean reference laboratories. This stallion (Warmblood registered in Zangersheide) was introduced to another farm of jumping horses before Argentinean EAV control measures took place. For sport competitions, there was movement of stallions between the three farms mentioned above. This fact suggested that horizontal transmission between stallions can occur and could be the reason for the positive serology. Horizontal transmission has been reported by Guthrie et al. [10]. Even though the Office International des Epizooties recommended either virus isolation or test mating for screening stallions, the methods for identifying EAV carrier stallions are not uniformly agreed upon among laboratories, and moreover, they vary in their ability to isolate the virus in cell culture [19]. Previous EAV phylogenetic investigations around the world have focused on the hypervariable region of the GP5 gene, including both European and North American strains [12, 13, 21]. Therefore, genetic comparison of the ORF 5 gene sequences of the Argentine isolated virus may provide insight into the genetic evolution and origin of the Argentinean EAV strains.

An archived testicle sample collected in 2000 from the "A" stallion and conserved at −20°C was analyzed for virus isolation. Doubts about the possibility of recovering EAV from this kind of sample and the abovementioned history of the stallion led us to attempt this goal, and we processed the testicle for routine virus isolation (epididimys, parenchyma and testicular liquid). The EAV isolation from the samples was performed on RK13 cells grown in 24-well plates as described previously [24]. Briefly, serial ten-fold dilutions (10^{-1}–10^{-3}) of each sample (three epididimys, two parenchyma and one testicular liquid), were inoculated in duplicate. The cells were maintained in culture medium as well as in methylcellulose medium containing 2% foetal bovine serum. The plates were incubated at 37°C in an atmosphere of 5% CO_2 and examined daily for cytopathic effects (CPE). The cultures that remained negative for CPE after 7 days were subjected to additional passages. Identification of EAV was determined by immunofluorescence test using polyclonal antibodies as described previously [5].

Viral RNA was extracted from 500 µl of the supernatant of infected RK13 cells with 500 µl of TRIzol and precipitated with isopropanol. Five microliters of RNA resuspended in distilled water was used for cDNA synthesis using reverse transcriptase and random hexamers. For PCR amplification, primers GL105F and GL673R were chosen, which flank a 546-nt region of the GP5 gene [17]. Denaturation, annealing and extension consisted of 35 cycles at 94°C for 45 s, 60°C for 1 min and 72°C for 90 s, respectively. The PCR products were examined on 2% agarose gels, observed under UV light following ethidium bromide staining and purified using a PCR purification kit. Sequences of both strands were determined in a MegaBACE™ 1000 with primers CR2 and EAV32, which flank a 519-nt region [21].

The phylogenetic dataset was built with 14 strains from different geographic regions (America, Europe and Argentina). Five strains were isolated in Argentina (including the strain reported in this paper) (Table 1). The strain GXHP-5 (accession number EU428820) of Porcine reproductive and respiratory syndrome virus (PRRSV) was used as external group (outgroup = OG), selected using Basic Local Alignment Search Tool (BLAST) http://ncbi.nlm.nih.gov/blast/Blast.cgi and bibliographical searches. The sequences alignments were performed using Clustal X version 1.92 software [26]. Sequence editing was done with Bio-Edit version 7.05 [11], and Proseq version 3.0 software [8]. The phylogenetic analysis was performed using neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) algorithms. The molecular evolution model was calculated using MrModeltest software (available in http://hiv-web.lanl.gov). The evolution model selected was HKY85. The NJ and ML trees were built using the HKY85 model with transition/transversion ratio 2.0 using PAUP software [22]. To build a

phylogenetic tree using MP as the optimality criterion, searches were performed by implicit enumeration with 1,000 replicates of random stepwise addition (RAS) and tree bisection and reconnection (TBR) branch swapping, using TNT software [9]. The support analysis of identified groups was performed with a bootstrap test [7]. The bootstrap test included 1,000 resample matrices. For each resampled matrix, 1,000 RAS + TBR cycles were done. The jackknifing test was applied to assess the support of nodes on parsimonious trees.

Seven passages in a confluent monolayer of RK13 cells were performed with the inoculums, and CPE was observed in the 4th passage only in cells inoculated with the testicular liquid sample when compared to the cell control. Although three more passages were done in order to increase the virus titer, but the magnitude of the CPE remained constant. The isolated virus strain was named LT-LP-ARG. After retro-transcription, cDNA was obtained from the epididimys (E-cDNA), parenchyma (P-cDNA) and testicular liquid (TL-cDNA). An immunofluorescence test confirmed the presence of EAV particles in the testicular liquid. No virus-specific fluorescence was observed in mock-infected cells. Using specific primers for the GP5 gene, only the TL-cDNA gave a visible 591-bp band in a ethidium-bromide-stained agarose gel. No bands were observed in the E-cDNA or P-cDNA samples or in the negative control used in the PCR. The 519-bp sequence was analyzed and aligned with the four Argentinean EAV isolates reported in literature, and also with American and European reference strains. The data set was variable, consisting of 519 characters, of which 91 were informative characters. The distance tree obtained by the NJ algorithm showed that the LT-LP-ARG and LP02 strains are clustered together with high bootstrap values. According to the NJ analysis, LT-LP-ARG and LP02 are closely related to the American group, with LP01 being the most basal strain of EAV. By NJ, the relationship among European strains could not be established. The tree obtained by the ML algorithm revealed that two groups can be identified: the first group is formed by the LT-LP-ARG and LP02 strains. The second group is formed by the American and European strains and one Argentinean strain (LP01). In this second group we distinguished an exclusive subgroup formed by American strains with high bootstrap values. NJ and ML trees described in this study are provided as supplementary material (supplementary material on line). Parsimony analysis resulted in 26 equally parsimonious trees 405 steps long. A strict consensus tree of this tree showed two large clades. The first clade is formed by American strains and the second clade is formed by Argentinean and European strains. In this last clade, the LP01 strain was the most basal of the groups in this study and remained with the other strains. The rest of the Argentinean strains constitute a monophyletic group. Bootstrap and jackknife analysis showed the same topology and presented only minimal differences in support values (Fig. 1).

We report here the isolation of EAV from a testicle, conserved for 7 years at −20°C, from a stallion testing positive by serology. Based on the fact that virus isolation from semen stored at −20°C is possible, we decided to attempt the virus isolation from a testicle sample conserved under the same conditions. We argued that the difference

Table 1 EAV strains, accession numbers, year of isolation and geographic distribution

Strain	Year of isolation	Region	Accession no.
PRRSV-GXHP-5 (OG)	Unknown	China	EU428820
KY93	1993	USA	U81017
KY84	1984	USA	AF107279
D84	1984	USA	AF107266
E85	1985	USA	AF107275
R1	1996	Europe	AF118773
P1	1996	Europe	AF118775
G1	1995	Europe	AF118777
A1	1995	Europe	AF118769
LP01	2001	Argentina	DQ435439
LP02/R	2002	Argentina	DQ435440
LP02/C	2002	Argentina	DQ435441
LP02/P	2002	Argentina	DQ435442
LT-LP-ARG	2007	Argentina	EU622859

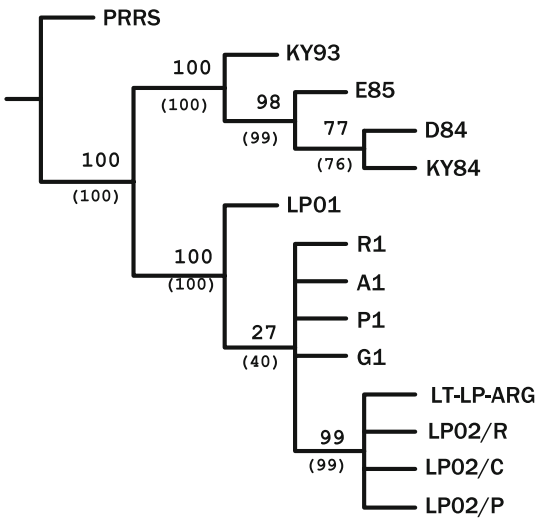

Fig. 1 Strict consensus of the 26 most parsimonious trees found through implicit enumeration searches of 1,000 RAS + TBR cycles. Tree length = 405. Group support assessed with 1,000 bootstraping and jackknifing replicates. *Numbers above branches* correspond to bootstrap support. Jackknife support is given in *parentheses*

between the magnitude of CPE observed in this case compared with other EAV laboratory strains could be explained by shedding of not fully infectious immature virus particles in the sample, or low virulence or low titer of the sample [18]. In some reports [23, 25], it has been argued that asymptomatic carrier stallions are the principal reservoir of EAV with reduced virulence. During persistent infection, genetic changes may occur that compromise virulence. These genetic changes are one of our proposed explanations for the different CPE observed between EAV strains. Our results contrast with a previous report [17] in which the authors argued that EAV from samples stored several months at −20°C could only be recovered by transfection of extracted RNA. In this work it was unnecessary to transfect RNA, as we observed CPE with the inoculum itself. The toxic properties inherent in the kind of tissue sample could affect the viability of the virus particle present in the epididimys and parenchyma inoculums, explaining why isolation was not successful with these two samples. In a PCR reaction using GP5-specific primers, we only obtained a visible band with the TL-cDNA, which correlates with the results obtained with cell cultures and with the immnunofluorescence test.

The different phylogenetic approaches used in this study yielded the following results: when comparing the sequence obtained with the LT-LP-ARG strain to the sequences available in GenBank, this isolate grouped with the European EAV strains as well as with the Argentinean EAV strains. However, our results obtained by ML, NJ and MP analysis showed that the LT-LP-ARG and LP02 strains are grouped together. Therefore, the strains included in this group can be considered a monophyletic group, and this is supported by high bootstrap values. These results allow the conclusion that LT-LP-ARG and the LP02 group (LP02/R, LP02/P and LP02/C) have a close common ancestor. Despite the results obtained in this study, the Argentinean strains have a polyphyletic origin. The analysis also showed that the LP01 strain is separated from the remaining Argentinean EAV strains, suggesting that this strain could have a different origin. The continuous transportation of equines between Europe and America could be the cause of the polyphyletic origin of Argentinean EAV strains.

We demonstrated here the isolation of EAV from a testicle stored 7 years at −20°C. We believe that the storage conditions of the sample delayed the appearance of CPE until the fourth cell passage, and the magnitude of CPE was not typical of what is normally observed for EAV. The special emphasis on this sample is because of its history. Since the prevalence of EAV has increased in Argentina since 2001, we believe that the virus isolate presented in this report could be the origin of EAV infection in our country.

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References

1. Balasuriya UBR, Heidner WH, Hedges JF, Williams JC, Davis NL, Johnston RE, MacLachlan NJ (2000) Expression of two major envelope proteins of equine arteritis virus as a heterodimer is necessary for induction of neutralizing antibodies in mice immunized with recombinant Venezuelan equine encephalitis virus replicon particles. J Virol 74:10623–10630
2. Cavanagh D (1997) Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 142:629–633
3. Chirnside ED, Wearing CM, Binns MM, Mumford JA (1994) Comparison of M and N gene sequences distinguishes variation amongst equine arteritis virus isolates. J Gen Virol 75:1491–1497
4. Doll ER, Bryans JT, McCollum WH, Crowe MEW (1957) Isolation of a filterable agent causing arteritis of horses and abortion in mares. Its differentiation from the equine abortion (influenza) virus. Cornell Vet 47:3–41
5. Echeverría MG, Pecoraro MR, Galosi CM, Etcheverriagaray ME, Nosetto EO (2003) The first isolation of equine arteritis virus in Argentina. Rev Sci Tech 22:1029–1033
6. Echeverría MG, Díaz S, Metz GE, Serena MS, Panei CJ, Nosetto EO (2007) Genetic typing of Equine Arteritis Virus isolates from Argentina. Virus Genes 35:313–20
7. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
8. Filatov DA (2002) Proseq: a software for preparation and evolutionary analysis of DNA sequence data set. Mol Ecol Notes 2:621–624
9. Goloboff P, Nixon K, Farris J (2003) (TNT) Tree analysis using new technology (Published by the authors). Tucumán, Argentina
10. Guthrie AL, Howell PG, Hedges JF, Bosman AM, Balasuriya UB, McCollum WH, Timoney PJ, MacLachlan NJ (2003) Lateral transmission of equine arteritis virus among Lipizzaner stallions in South Africa. Equine Vet J 35:596–600
11. Hall TA (1999) BioEdit: a user friendly biological sequences alignment editor and analysis program for Window 95/98/NT. Nucleic Acids Sym Ser 41:95–98
12. Hornyak A, Bakonyi T, Tekes G, Szeredi L, Rusvai M (2005) A novel subgroup among genotypes of Equine Arteritis virus: genetic comparison of 40 strains. J Vet Med Sci B 52:1–7
13. Larsen LE, Storgaard T, Holm E (2001) Phylogenetic characterization of the GL sequences of Equine Arteritis Virus isolated from semen of asymptomatic stallions and fatal cases of Equine Viral Arteritis in Denmark. Vet Microbiol 80:339–346
14. Little TV, Holyoak GR, McCollum WH, Timoney PJ (1992) Output of equine arteritis virus from persistently infected stallions is testosterone dependent. In: Plowright W, Rossdale PD, Wade JF (eds) Proceedings of the 6th international conference on equine infectious diseases, Cambridge, 1991. R & W Publications, Newmarket, pp 225–229
15. McCollum WH (1969) Development of a modified virus strain and vaccine for equine viral arteritis. JAVMA 155:318–322
16. McCollum WH, Prickett ME, Bryans JT (1971) Temporal distribution of equine arteritis virus in respiratory mucosa, tissues and body fluids of horses infected by inhalation. Res Vet Sci 2:459–464
17. Mittelholzer C, Johansson I, Olsson AK, Roneus M, Klingeborn B, Belak S (2006) Recovery of Swedish equine arteritis viruses from semen by cell culture isolation and RNA transfection. J Virol Meth 133:48–52

18. Mittelholzer C, Stadejek T, Johansson I, Baule C, Ciabatti I, Hannant D, Paton D, Autorino GL, Nowotny N, Belak S (2006) Extended phylogeny of equine arteritis virus: division into new subgroups. J Vet Med B 53:55–58
19. O. I. E. (2004) Equine viral arteritis. In: Manual of standards for diagnostic test and vaccines for terrestrial animals, 5th edn. Office International des Epizooties, OIE Standards Commission, Paris
20. Snijder EJ (2001) Arteriviruses. In: Fields virology. Knipe DM, Howley PM (eds) Lippincott Williams y Wilkins, Philadelphia, pp 1205–1220
21. Stadejek T, Bjorklund H, Ros Bascunana C, Ciabatti IM, Scicluna MT, Amaddeo D, McCollum WH, Autorino GL, Timoney PJ, Paton DJ, Klingeborn B, Belak S (1999) Genetic diversity of equine arteritis virus. J Gen Virol 80:691–699
22. Swofford DL (1998) PAUP*. Phylogenetic analysis using parsimony (*and others methods). Version 4. Editorial Sinauer Associates, Sunderland
23. Timoney PJ, McCollum WH, Roberts AW, Murphy TW (1986) Demonstration of the carrier state in naturally acquired equine arteritis virus infection in the stallion. Res Vet Sci 41:279–280
24. Timoney PJ, McCollum WH, Roberts AW (1987) Detection of the carrier state in stallions persistently infected with equine arteritis virus. Proc Am Assoc Equine Pract 32:57–65
25. Timoney PJ, McCollum WH (1993) Equine viral arteritis. Vet Clin N Am Equine Pract 9:295–309
26. Thompson J, Gibson T, Pleuniak F, Jeanmougin F, Higgins D (1997) CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
27. Ziebuhr J, Snijder EJ, Gorbalenya AE (2000) Virus-encoded proteinases and proteolitic processing in the Nidovirales. J Gen Virol 81:853–879