Virus Genes
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ORIGINAL ARTICLE

Genetic typing of equine arteritis virus isolates from Argentina

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Abstract We report the nucleotide sequence and genetic diversity of four Equine Arteritis Virus (EAV) ORF 5 and 6 from Argentina isolates, obtained from asymptomatic virus-shedding stallions. Nucleic acid recovered from the isolates were amplified by RT-PCR and sequenced. Nucleotide and deduced amino acid sequences from the Argentine isolates were compared with 17 sequences available from the GenBank. Phylogenetic analysis revealed that the Argentine isolates grouped together in a definite cluster near European strains. Despite the greater genetic variability among ORF 5 from different isolates and strains of EAV, phylogenetic trees based on ORF 5 and 6 are similar. Both trees showed that virus sequences from America and Europe segregate into distinct clades based on sequence analysis of either ORF 5 or 6. This study constitutes the first characterization of Argentine EAV isolates.

Keywords Equine arteritis virus · Argentina · Phylogenetic analysis

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Introduction

Equine viral arteritis (EVA) has been recognized in horses since the 19th century. The causative agent of this disease is the equine arteritis virus (EAV). It was first isolated in Ohio, USA, in 1953 [1] and has been classified as a member of the *Arteriviridae* family, along with porcine reproductive and respiratory syndrome virus (PRRSV), simian haemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV) [2].

Equine arteritis virus can be transmitted via the respiratory or venereal route. Aerosol exposure results in infection of pulmonary macrophages, with subsequent rapid spread via the circulatory system [3]. The virus can also be transmitted very effectively by artificial insemination of mares with infective chilled or fresh-frozen semen. Up to 60% of stallions acutely infected with EAV become chronically infected. Such "carrier" stallions seroconvert, but shed virus constantly in the semen and are regarded as playing a major epidemiological role in the dissemination and perpetuation of EAV between outbreaks [4]. The asymp-

tomatic ''carrier'' stallion is the principal reservoir of EAV as it can harbour the virus between breeding seasons and venereally infect susceptible mares. The majority of field strains of EAV cause subclinical or unapparent infection in horses, whereas other strains cause the clinical manifestations of equine viral arteritis [5–8]. In the course of persistent infection EAV evolves at a rate of approximately 1% nucleotide substitutions per 2822 nucleotides per year, leading to emergence of novel genotypic and phenotypic variants [9,10].

The EAV genome consists of a single positive-stranded RNA of approximately 12.7 kb, which contains nine open reading frames (ORFs) [2]. ORFs 1a and 1b encode the viral replicase while ORFs 2a, 2b, 3–6 encode the viral membrane proteins. The unglycosylated envelope protein E is encoded by ORF 2a and the three minor glycoproteins of EAV envelope designated as GP2b, GP3 and GP4 are encoded by ORFs 2b, 3 and 4, respectively. The major envelope glycoprotein GP5 is encoded by ORF 5, whereas ORF 6 encodes the unglycosylated envelope protein M. ORF 7 codes for the nucleocapsid protein N. The former G_L (GP5) envelope glycoprotein, expresses neutralization determinants of EAV [2, 11–13]. Proteins M and GP5 form disulphide-linked heterodimers –Cys-8 of M and Cys-34 of GP5- [14] and the interaction of both proteins is necessary for induction of neutralizing antibodies [15].

The prevalence of EAV infected horses in Argentina is low. During 2001–2002 was carried out a serological survey in registered stallions in Argentina; over 1774 stallions, 14 were positive (11 imported vaccinated at the country of origin) [16]. However, the first isolation of EAV (LP01 isolate) in Argentina, from the semen of a seropositive imported stallion located in one farm with high seroprevalence of EAV, was made in 2001 [17]. In the present work we sequenced part of ORF 5 and 6 of our Argentine EAV isolates and compared them with American and European strains selected from GenBank database.

Previous phylogenetic investigations in EAV around the world have focussed on ORF 5 [18, 19]. A global phylogenetic tree was built, based on the hypervariable region of the GP5 gene including both European and North American strains [20], but there is no information about South American EAV strains.

Materials and methods

Semen samples and virus isolation

In winter 2002, semen samples from four horses belonging to the same farm, were received in our lab. The samples were collected from animals previously tested positive by conventional virus neutralization test [21]. The farm is a breeding and training of jumping horses. At the time of the study it had approximately 280 horses including stallions, mares, donor mares and foals. The prevalence of EAV was estimated at 45.8% between July 2001 and December 2003, and because the vaccination is forbidden, to keep the infection rate low in Argentina, the animal health control authorities closed the farm in order to stop the spreading of the virus caused by the trade of horses. Circumstantial evidence suggested that the EAV dissemination inside the farm was initiated by one or two ''carrier'' stallions imported to this farm from Europe in 2001. These animals had no evidence of clinical disease but soon after the new stallions arrived, other stallions, mares and foals on the farm became seropositive to EAV. During the time of this survey, no abortions were observed. In this study semen samples were processed immediately on arrival for the reverse transcriptase-polymerase chain reaction (RT-PCR) as described below. For isolation, the semen samples were centrifuged at 1000 *g* at 4°C for 10 min and the seminal plasma was collected. Attempted EAV isolation from seminal plasma was performed in confluent monolayers of rabbit kidney (RK13) cells propagated in 6-well plates. The cells were cultivated in Eagle's Minimum Essential Medium supplemented with 10% foetal calf serum (growth medium) or 2% foetal calf serum (maintenance medium). The cells were inoculated in duplicate wells with serial decimal dilutions (10^{-1}–10^{-3}) of seminal plasma in maintenance medium with a volume of 0.3 ml of each dilution. Plates were incubated for 60 min at 37°C in an atmosphere of 5% CO_2 [21]. After removing the inoculums, the cells were overlaid with 0.75% carboxymethylcellulose in maintenance medium. Cell cultures were observed for cytopathic effect (CPE) for 7 days. Cultures which remained negative for CPE after 6 days were subjected to two additional passages before being considered negative. When CPE was observed, cultures were passaged on to RK13 cells grown on coverslips, for subsequent examination by the immunofluorescence test (IF). The identity of EAV isolates was confirmed by one-way neutralization test using polyclonal horse antisera [21].

RT-PCR

Viral RNA was extracted directly from seminal plasma (500 µl) or infectious cell culture medium (250 µl) with 500 µl of Trizol (Invitrogen, USA), precipitated with

isopropanol and resuspended in distilled water. Five microliters of resuspended RNA was used for cDNA synthesis. A negative control was provided by substituting sample RNA with the DNA of equine herpesvirus-1. For the RT step, cDNA was obtained using reverse transcriptase and random hexamers. The sequences and locations of the pairs of primers used were based on the NCBI NC_002532 sequence. For protein M gene the specific primers used were: M1 5′-CTGAG GTATGGGAGCCATAG-3′ -11894–11913- and M10 5′-GGCCTGCGGACGTGATCG-3′ -12342–12325- flanking a 411 nt region, with a product of 449 bp [22]. For GP5 gene oligonucleotide primers GL105F 5′-GCT GACGGATCGCGGCGTTATT-3′ -11250–11271- and GL673R 5′-ATAGTGGGCCTACCTGGGACTAA-3′ -11840–11818- flanking a 546 nt region with a product of 591 bp, were used [23]. The PCR was carried out in a final volume of 50 μl containing 5 μl of the cDNA, 3 μl of $MgCl_2$ (25 mM), 5 μl 10X PCR buffer, 1.25 U *Taq* DNA polymerase (Promega, USA), 1 μl dNTP mix (0.2 mM each) and 2 μl of each primer (20 pM each). Denaturation, annealing and extension consisted of 35 cycles at 94°C 45 s, 60°C 1 min and 72°C for 90 s, respectively. Each PCR product (5 μl samples) was examined on 2% agarose gels. The gels were examined under UV light following ethidium bromide staining.

Cloning and sequence analysis

The RT-PCR products were purified using PCR purification kit (Promega, USA). Protein M gene products were cloned into TOPO TA cloning 2.1 (Invitrogen, USA) expressed in Top10 cells, and two positive clones were selected for each isolate for nucleotide sequencing using M13 primers. Sequences of both strands of GP5 PCR products were determined using primers CR2 -11272–11294 5′-GCCAATTTGCTGCGATATG ATGA-3′ and EAV32 -11836–11814 5′-TGGGCCTA CCTGGGACTAACAAC-3′) flanking a 519 nt region [20]. The nucleotide sequences were determined using an AB1377 dye terminator kit (Cornell University, BioResource Center, DNA Sequencing Facility, Ithaca, NY). Sequence analysis of partial ORF 5 and 6 genes of Argentine isolates and those of reference EAV strains: Bucyrus (NC_002532), VBS53 (U81013), PA76 (U81018), KY77 (U81015), KY84 (AF107279), KY93 (U81017), CAN86 (U81021), CA95G (U81023), D84 (AF107266), E85 (AF107275), CW96 (AY349 167), RQ (AF118782), G1 (AF118777), BT-PA96 (AF118781), P1 (AF118775), R1 (AF118773) and A1 (AF118769) were performed using CLUSTAL W, MEGA and DNAstar programmes. The phylogenetic trees were constructed using the Neighbour Joining method and applying the ''bootstrap'' test.

Results

Virus isolation

On day 6 of the first passage on RK13 cells, a few rounded cells were observed in one well inoculated with 10^{-2} dilution (without methylcellulose), in two wells of 10^{-1} dilution and one well of 10^{-2} dilution of ''R'' seminal plasma (with methylcellulose). Sample ''C'', showed CPE on day 5 of the first passage on the following cell cultures: 1 well 10^{-1} dilution and 1 well 10^{-2} dilution without methylcellulose; 2 wells 10^{-1} dilution and 2 wells 10^{-2} with methylcellulose. The sample ''P'', showed CPE on day 2 of the second passage on: 1 well 10^{-1} dilution and 2 wells 10^{-2} dilution without methylcellulose; 2 wells 10^{-1} dilution and 2 wells 10^{-2} dilution with methylcellulose. By day 2 of the second or third passage, the CPE was extensive in most cultures, consisting of cells rounding, shrinking and detachment. Three new isolates were performed from semen samples belonging to ''R'', ''C'' and ''P'' stallions that were designated LP02/R, LP02/C and LP02/P. A working virus stock was made following the fourth passage in RK13 cells. The isolates were confirmed as EAV by one-way neutralization with an antiserum to the Bucyrus strain of EAV. The new isolates were not neutralized either by EHV-1 or EHV-4 antisera. EAV were also confirmed by IF test, where viral antigens belonging to each new isolate were localized in the cytoplasm and especially in the perinuclear area, appearing as large masses occupying most of the cytoplasm. No CPE or virus-specific fluorescence was detected in mock-infected cells.

RT-PCR

Using the two primer pairs in the RT-PCR the predicted fragments of the M and GP5 partial genes of the EAV isolates were amplified from the genomes, yielding clear and strong bands in agarose gels of the expected sizes, 449 and 591 bp, respectively.

Sequence analysis

Partial ORF 5 and 6 nucleotide sequences of the four Argentine strains were deposited into the International GenBank under accession numbers DQ435439 to

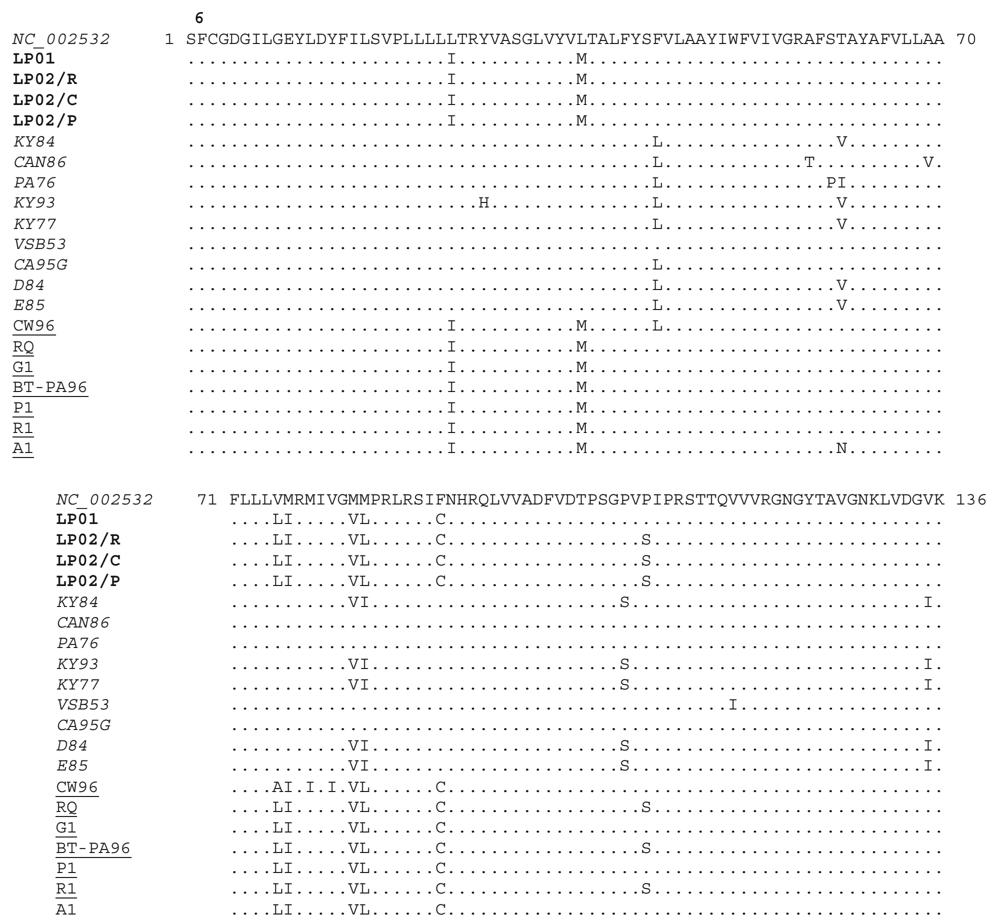

Fig. 1 Alignment of the deduced amino acid sequences (one letter code) of ORF6 of Argentine isolates (in bold) in comparison to strains of America (in italic) and Europe (underlined). The numbers indicate the amino acid position in the mature protein

DQ435442 and DQ333586 to DQ333589, respectively. No deletions or insertions were detected, only several point mutations were seen. The alignments of the deduced amino acid sequences predicted from the 411 and 519 nucleotide fragments, corresponding to M and GP5 genes (136 and 172 amino acids, respectively), are illustrated in Fig. 1 and 2.

Protein M gene: the sequences alignment revealed identity with the European strains at nucleotide as well as amino acid level. The highest level of nucleotide identity was found between the LP01 and P1 strains (97.6%). Nucleotide identity values were 97.8% between LP02/R and RQ, G1, BT-PA96 and R1 strains; LP02/C showed 97.8% identity to G1; and between the LP02/P and G1 strains they were of 98.1%. Complete identity was found at amino acid level for LP01, G1 and P1, and for LP02 strains with RQ, BT-PA96 and R1. Nucleotide substitutions of Argentine EAV strains were similar and only differ in one proline in LP01 and a serine in LP02 strains at position 114. The Argentine isolates exhibited close homology to the European isolates at ORF 6. When the Argentine EAV isolates were compared to each other, the lowest level of nucleic and amino acid identity were found between LP01 and LP02 strains (96.1 and 99.26%, respectively). The predicted M ectodomain contains a single Cys residue at position 8 in all strains analyzed, and another Cys residue is present in European and Argentinean EAV strains at position 90.

Analysis of the variable and conservative regions of GP5 gene allowed us to compare the genetic relatedness of the local EAV isolates. The deduced amino acid sequences of the most variable region (V_1) of the amplified GP5 fragments are illustrated in Fig. 2. Argentinean strains have almost the same substitutions at amino acid level with the exception of LP01 strain involving the first variable region V_1 (amino acids 61–121) specially in the neutralization sites B, C and D. LP02/R, LP02/C and LP02/P differ each other at 3 amino acids. All the strains analyzed, with the exception of NC_002532, posses the glycosylation site at amino acid 81. It is remarkable the presence of a threonine at position 90 in six out of seven European and all Argentinean strains, and the substitution of alanine, present in all American strains (exception for

Fig. 2 Alignment of the deduced amino acid sequences (one letter code) of ORF5 of Argentine isolates (LP01, LP02/R, LP02/C and LP02/P, in bold) in comparison to strains of America (in italic) and Europe (underlined). The numbers indicate the amino acid position in the mature protein. The variable region (V_1) at the deduced amino acid sequences of GP5 fragments is indicated in grey. B, C and D are the neutralization sites. The glycosylation site at amino acid 81 (N) is bolded and underlined. Note the threonine (T) at position 90 in European and Argentinean strains. See text for details

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                     B            C                 D
           51   56                81
NC_002532  HTALYNCSASKTCWYCTFLDEQIITFGTDCDDTYAVPVAEVLEQAHGPYSALFDDMPPFI
LP01       ..........E.....V.....V.....G.NN..S...ST..........V.........
LP02/R     ......W.........E...D.......G.N...S...ST..........V.........
LP02/C     ................V...D.......G.N..HS...ST..........V.........
LP02/P     ................E...D.......G.NN.HS...ST..........V.........
KY84       ..........Q.................N.N........V..........V.........
CAN86      ..........E...........V.....G.N..H....SV..........V.........
PA76       ................A...D.........NN.H....SV..........V.........
KY93       ..........E...........V.....N.N...S...SV..........V..N......
KY77       ......................V.......N........V..........V.........
VBS53      ..............................N...................V.........
CA95G      ..........E.................G.NN.H....SV..........V.........
D84        ..........Q.................N.N........V..........V.........
E85        ..........Q...........V.....N.N..H.....V..........V.........
CW96       ..........VD..........V.....G.N..FS...SV..........T.........
RQ         ..........N.....V...........G.N...S...ST..........V.........
G1         ................K.....V.....G.NN..S...ST..........V.........
BT-PA96    ..........N.....V...........G.N...S...ST..........V.........
P1         ................E...........G.N...S...ST..........V.........
R1         ..........N.....V...........G.N...S...ST..........V.........
A1         ................V...........G.N...S...ST..........V.........

NC_002532  YYGREFGIVVLDVFMFYPVLVLFFLSVLPYATLILEMCVSILFIIYGIYSGAYLAMGIFA
LP01       ........F.M...................V............VV..L............
LP02/R     ........F.M................................VV..L............
LP02/C     ........F.M................................VV..L............
LP02/P     ........F.M................................VV..L............
KY84       ........L........................F...L......V...............
CAN86      ........F......................................V............
PA76       ............................................................
KY93       ........L........................F...L......V...............
KY77       ........L........................F..........V...............
VSB53      ............................................................
CA95G      ........F...................................................
D84        ........L........................F...L......V...............
E85        ........L........................F...L......V...............
CW96       ........F.M.............................V...................
RQ         ........F.M................................VV..L............
G1         ........F.M...................T............VV..L............
BT-PA96    ........F.M................................VV..L............
P1         ........F.M...................V.........V..V...L............
R1         ........F.M................................VV..L............
A1         ........F.M................................VV..L............

NC_002532  ATLAIHSIVVLRQLLWLCLAWRYRCTLHASFISAEGKVYPVDPGLPVAAVGN
LP01       T..VV..V.............................I........I..A..
LP02/R     T..VV..V.............................I........I..A..
LP02/C     T..VV..V.............................I........I..A..
LP02/P     T..VV..V.............................I........I..A..
KY84       ...V...V......................................I.....
CAN86      ....V.............................................M..
PA76       ..............................................I.....
KY93       ...I...V............................................
KY77       ...V...V..................................S...I.....
VBS53      .................................................A..
CA95G      ....V.............................................M..
D84        ...V...V......................................I.....
E85        ...V...V......................................I.....
CW96       ...I......F...................................I.VA..
RQ         T..VV..V.............................I........I..A..
G1         T..VV..V.............................I........I..A..
BT-PA96    T..VV..V.............................I........I..A..
P1         T..VV..V.............................I........I..A..
R1         T..VV..V.............................I........I..A..
A1         T..VV..V.............................I........I..A..
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KY93), for serine at 85 position, in all European and Argentinean strains. All analyzed strains except NC_002532 and CW96 had a valine at position 101. An extra putative N-linked glycosylation site was found only in KY93 strain at amino acid 104. With the exception of American isolates, all other analyzed strains had methionine at position 121. In the second variable region V_2 (amino acids 141–178) the major features were the presence of valine at position 154 and leucine at position 158 in all Argentinean and

European strains, with the exception of CW96 and CAN86. It was found the presence of alanine at position 171 in all American and CW96 strains and threonine in all European and Argentinean strains. In the V_3 region (amino acids 202–222) valine was replaced by an isoleucine at amino acid 208 in all Argentinean and European strains with the exception of CW96 strain. When the Argentine EAV isolates were compared to each other, the LP01 and LP02/R isolates showed the lowest level of nucleotide and amino acid identity, 94.6 and 95.93%, respectively. The LP02/C and LP02/P Argentine isolates were found to be more closely related to each other with nucleotide and amino acid identities of 99.42 and 98.83%, respectively. When the Argentine EAV isolates were compared to each other the highest level of nucleic acid identities were shown between A1 strain and LP01 and LP02/C (97.3%), with LP02/R at 96.9 and with LP02/P at 97.1%, whereas at amino acid level, between A1 strain with LP02/R, LP02/C and LP02/P strains at 98.3, 98.8 and 97.7%, respectively, and between G1 strain with LP01 strain at 98.3%. According with the partial analyzed sequence, the putative GP5 ectodomain contains 4 Cys residues at positions 57, 63, 66 and 80. Among the partial conserved Region C_1 the Cys in position 57 is replaced by tryptophan only in LP02/R.

Phylogenetic analysis

In order to illustrate phylogenetic relationships among the Argentine strains as well as the 17 sequences obtained from GenBank, phenograms were constructed based on ORF 6 and ORF 5 nucleotide or amino acid sequences (Fig. 3 and 4). The phylogenetic analysis showed that Argentine strains are grouped with European strains and that LP02 strains were clustered together (Boostrap = 95%). The trees showed that EAV isolates and strains fall into two major groups. Group I contained viruses from North America and group II was composed of viruses from Europe.

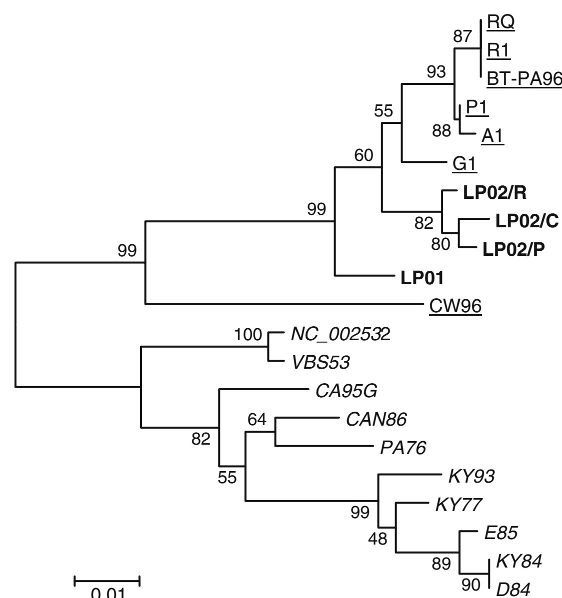

Fig. 3 Phylogenetic tree showing the relationships among ORF6 nucleic acid sequences from EAV isolates from Europe (underlined), America (italic) and Argentina (bold). The tree was constructed by the Neighbour Joining method using the Kimura-2 parameters distance estimates (MEGA version 2.0), applying the bootstrap test with 1000 iterations

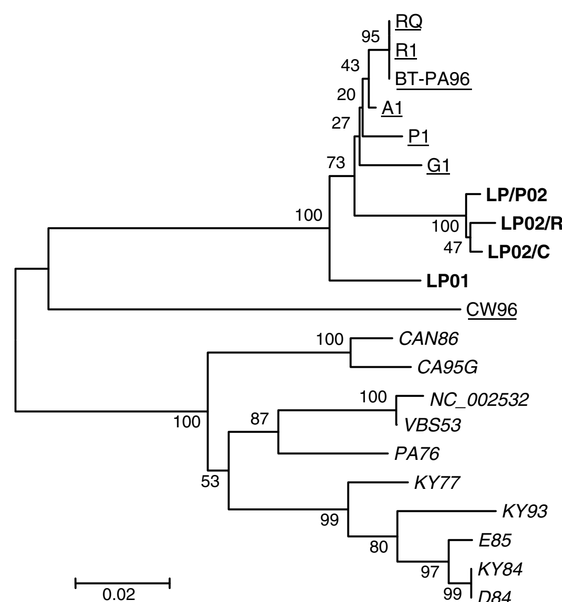

Fig. 4 Phylogenetic tree showing the relationships among ORF5 nucleic acid sequences from EAV isolates from Europe (underlined), America (italic) and Argentina (bold). The tree was constructed by the Neighbour Joining method using the Kimura-2 parameters distance estimates (MEGA version 2.0), applying the bootstrap test with 1000 iterations

Discussion

Three isolates out of four semen samples, "R", "C" and "P" were obtained during first or second passage in tissue culture, and were designated as LP02/R, LP02/C and LP02/P. EAV "carrier" stallions were determined by isolation of the virus from semen samples. These animals never suffered clinical respiratory disease, nor has the virus ever been recovered from abortion material or nasal swab samples. As in other countries, cases with clinical signs suggestive of EAV are not frequently reported [24, 25]. Positive results were obtained with reference polyclonal antisera, unequivocally establishing that the isolates were EAV by

one-way neutralization and IF tests. As well as demonstrated by others [26], the addition of carboxymethylcellulose to the medium enhanced viral recovery.

The goal of this study was to compare ORF 5 and ORF 6 sequences from Argentine isolates and 17 sequences from American and European strains. Based on the results of previous phylogenetic studies, there is agreement on the differences between American and European EAV strains, even comparing different ORFs [27–29] where two groups were identified: group I contained viruses from North America and New Zealand, while group II represented viruses from Europe.

Analyzing M ectodomain, the Cys residue at position 8 was found in all isolates analyzed, which is also conserved in other arterivirus [30]. In contrast, the putative GP5 ectodomain contained in the analyzed fragment, 4 Cys residues at positions 57 (except LP02/R), 63, 66 and 80. Among the three variable regions of the GP5 gene, the first coding for amino acids 61-121 is still hypervariable in Argentine isolates [9]. The second GP5 putative glycosylation site was conserved in all analyzed strains, as well as most non-laboratory strains. However, this mentioned site has been lost in all cell culture-adapted strains [7].

Despite the greater genetic variability of ORF 5 of isolates and strains of EAV, phylogenetic trees based on ORF 5 and 6 are similar with only few differences. Both trees showed that viruses from America and Europe segregate into distinct clades based on sequence analysis of either protein M or GP5 genes. It is important the fact that both proteins interact by forming disulphide-linked heterodimer that are necessary for induction of neutralizing antibodies. In summary, phylogenetic analysis of both ORFs of the same isolates gave similar but not identical results.

The high seroprevalence of EAV infection in the two horse breeds where the strains were isolated, contrasted with the sporadic occurrence of the disease, and indicate that asymptomatic and subclinical EAV infection in horses is very common. This concept is consistent with previous clinical reports that many ‘‘carrier’’ stallions venereally transmit viruses of low virulence [5, 29].

Previous studies carried out in our laboratory using virus neutralization assay with a panel of polyclonal equine sera, confirmed that nucleotide variations in ORF 5 of the Argentine strains correlated with phenotypic differences between viruses and the Bucyrus laboratory strain. Our data reported here indicate that there are some antigenic variations among virus isolates derived from the same year or even the same farm.

Argentinean EAV isolates clustered in two branches on the European clade. LP01 isolate was obtained from a seropositive stallion imported from Europe, but located in a military riding breeding farm [17]. Even though the isolations were made from two different farms, the phylogenetic affiliation of Argentinean strains strongly indicated that the stallions were infected by European source. These are at least the two ‘‘populations’’ of EAV circulating in Argentina. It is interesting to consider, as well reported by others, that viruses introduced by an imported horse may become endemic in a new country [26].

It is very important to remark that in Argentina exists an intensive international movement of horses, and international transportation of semen for insemination. This is the first report of an investigation of the origin of Argentine EAV strains. The phylogenetic analysis of Argentine EAV strains confirmed that the stallions were infected with a European strain of EAV, which is consistent with the fact that the stallions were imported from Europe.

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