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Genomic and phylogenetic analysis of Argentinian *Equid Herpesvirus 1* strains

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Abstract *Equid Herpesvirus 1* (EHV-1) has long been causally implicated in the occurrence of abortion, neonatal death, respiratory disease, and neurological disorders in horses. This study analyzed for the first time the characteristics of the genomic section of Argentinian EHV-1 strains and reconstructed the phylogeny in order to establish their origin. The phylogenetic dataset included 22 Argentinian strains and four additional reference strains isolated in other countries. The intergenic region between ORF 62 and ORF 63 was amplified by PCR and sequenced. The phylogenetic analysis carried out by parsimony algorithms showed that six of the Argentinian strains had the same origin as British and Japanese strains. The mapping of symptoms caused by EHV-1 suggested that neonatal disease developed through convergent evolution, which would constitute an adaptation mechanism of the virus. This study constitutes the first analysis carried out in South-American strains that establishes the phylogenetic relationship between Argentinian strains and rebuilds the evolutionary history of symptoms. This study focuses on a very important aspect of evolution of *Herpesviridae* infecting perissodactyls and attempts to shed light on the evolution of symptoms, an issue of high clinical interest.

Keywords Equid Herpesvirus 1 · Argentinian strains · Phylogeny · Evolution

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Equid Herpesvirus 1 (EHV-1) is a member of the genus *Varicellovirus*, which is classified in the *Alphaherpesvirinae* subfamily, within the *Herpesviridae* family. This virus has long been causally implicated in the occurrence of abortion, neonatal death, respiratory disease, and neurological disorders in horses. It is distributed worldwide and has significant economic impact on the equine production [1, 13]. In addition, it has been isolated from other non-equine species such as antelopes, alpacas, camels, fallow deer, and cattle [6].

The identification of specific genes that are related with EHV-1 virulence and that determine abortigenic and neurological potential has been the aim of several researches [6, 12, 17, 18]. The first isolate from an equine fetus aborted due to EHV-1 was reported in Argentina in 1979 and then neurological signs in adult horses were described in 1984. An EHV-1 strain was isolated in 1985 from leukocyte-rich plasma from a horse with respiratory symptoms. Since then, several viral isolates have been obtained from horses that presented abortion storms, individual cases of abortion and neonatal disease. Studies with restriction endonucleases have allowed the classification of all strains and have determined that genome 1B has been present in Argentina since 1996 [3, 9].

This study analyzed for the first time the characteristics of the genomic section of Argentinian EHV-1 strains. In addition, this work used parsimony methods to reconstruct the phylogeny in order to establish that the Argentinian EHV-1 strains share ancestor with European or North-American strains. The phylogenetic dataset included 22 strains from different geographical regions of Argentina (Table 1) and two reference strains (KyB and HH1, accession numbers EU366314 and EU366313) called US and JA, respectively. In addition, sequence data for two EHV-1 strains (Ab 4 and V592) were obtained from GenBank: (accession numbers AY665713 and AY 464052) called UK 1 and UK 2, respectively. The only available *Equid Herpesvirus 4* (EHV-4) NS 80567 strain (accession number NC001844) was used as the outgroup. Each isolate was propagated for only three passages in rabbit kidney cells (RK 13) with Minimal Essential Medium (MEM) supplemented with 2% of fetal calf serum. Infected RK 13 cells were treated with proteinase K, and DNA was extracted with phenol and phenol-chloroform-isoamyl alcohol, and precipitated with cold absolute alcohol, according to the methodology previously described [3]. Four sets of primers were used for the genomic amplification by polymerase chain reaction (PCR): SF1: 5′ CCG GTC GTT CGG TTG AGC AAG TTT TTG ATG 3′ and SR1: 5′ CCT CCA GTC CAC AGA TAT GAC ATC CAA AGG 3′ (size of fragment: 655 bp).SF2: 5′ ACC GGA AGC TTG TCA TAT TTG TGA GCC TGG 3′ and SR2: 5′ TGT GAA CAT CAC CAC CAA TAC CAA GCA CGG 3′ (size of fragment: 692 bp) SF3 : 5′ CC AAT TAG CCC CCA ATT GGC ACA TGG TAA 3′ and SR3: 5′ TTA CAA AAA CCT ATG CAG GGG TGT GGG TGG 3′ (size of fragment: 605 bp) SF4: 5′ TTC CCC CGG GCC TTA TAT CTT GCA GCT TTA 3′ and SR4: 5′ TTG TTT TAG TCG ACC GAA GCT CTG AGG GAG 3′ (size of fragment: 577 bp). These primers amplified the region located between positions 108486 and 110681. However, the analysis was carried out in the portion of the intergenic region (IR) between ORF 62 and ORF 63, which is located between positions 108803 and 110385 of the genomic DNA of the Ab4 strain. This region was selected because according to other authors it is related to the viral growth and the virulence of EHV-1 and to establish differences among the strains. [6]. The DNAs were amplified with an initial denaturation step of 94°C for 4 min, followed by 30 cycles consisting of 94°C denaturation for 30 s, 60°C annealing for 20 s, and 72°C extension for 1 min [6]. The PCR products were run on 1.5 % agarose gel, purified using a gel extraction kit (Wizard SV Gel & PCR Clean Up, Promega) and sequenced (Biotechnology Resource Center,

Table 1 EHV-1 isolates, indicating origin of the isolate, date, geographical distribution, and accession number

Isolate	Origin	Date and place of isolation	GenBank accesion number
AR 1	Aborted fetus	La Plata. Buenos Aires	EU 366292
AR2	Respiratory disease	La Plata. Buenos Aires	EU 366293
AR3	Aborted fetus	25 de Mayo. Buenos Aires.	EU 366294
AR 4	Neonatal disease	Buenos Aires	EU 366295
AR 6	Aborted fetuses	Tucumán	EU366296
AR 7	Aborted fetus	Capitán Sarmiento. Buenos Aires.	EU366297
AR 8	Aborted fetus	Magdalena. Buenos Aires	EU366298
AR 9	Aborted fetuses	La Pampa	EU366299
AR 10	Aborted fetus	San Antonio de Areco. Buenos Aires	EU366300
AR 11	Abortion storm	San Antonio de Areco	EU366301
AR 12	Aborted fetuses	Trenque Lauquen. Buenos Aires	EU366302
AR 13	Aborted fetuses	General Villegas. Buenos Aires.	EU366303
AR 14	Neonatal disease	Pilar. Buenos Aires	EU366304
AR 15	Neonatal disease	San Antonio de Areco. Buenos Aires	EU366305
AR 16	Abortion storm	Entre Ríos	EU366306
AR 17	Neonatal disease	Cañuelas. Buenos Aires.	EU366307
AR 18	Aborted fetuses	General Pueyrredon. Buenos Aires	EU366308
AR 19	Abortion storm	General Pueyrredón. Buenos Aires	EU366309
AR 20	Abortion storm	San Antonio de Areco. Buenos Aires.	EU366310
AR 21	Aborted fetuses	Córdoba	EU366311
AR 22	Abortion storm	Trenque Lauquen. Buenos Aires	EU366312

University of Cornell, Ithaca, USA). The sequence alignments and the edition were carried out using Clustal X version 1.92 software and Bio-Edit version 7.05. Nucleotide diversity and identity percentage between EHV-1 strains was measured using Swaap software [14].

Phylogenetic analysis was carried out using maximum parsimony as optimality criterion. Heuristic searches with 1,000 replicates of random addition sequences (RAS) and tree bisection and reconnection (TBR) branch swapping, using TNT software [5] were carried out. The support analysis of the identified groups was carried out with Bootstrap and Parsimony Jackknifing test. The Bootstrap test included 1,000 resampled matrices, and for each resampled matrix, 1000 RAS +TBR cycles were carried out. The clinical signs produced by each strain were treated as unordered multistate character: 0 = respiratory disease, 1 = abortion, 2 = neurological disease, 3 = neonatal disease and then were mapped on the cladogram obtained using TNT and nonambiguous optimization of Winclada software [11], which displays ancestral states coincident in AccTran and Deltran optimization.

The dataset analyzed was highly conserved and consisted of 1,510 characters, 15 of which were parsimony informative. The nucleotide composition was $T = 27.4$, $C = 21.3$, $A = 26.1$, and $G = 25.2$. The identity EHV-1 strains oscillated between 99.6 and 100%. The parsimony phylogenetic tree obtained had 771 steps long. Bootstrap and jackknife trees showed similar topologies and presented very small differences in group support values. The AR10 strain was the most basal one in relation with the remaining ones, presenting high bootstrap values. Two clades and polytomy were clearly identified (Fig. 1). The first group was formed by the *AR15*, *AR18*, *AR19*, *AR20*, *AR21*, and *AR22* strains and showed low support values. A clade formed by *AR20–AR22* was identified in this group. The remaining strains formed a polytomy with this clade. The second group, which had high bootstrap values, was constituted by Argentinian, British, Japanese, and North-American strains. Two clades were identified in this group: UK1–UK2 and AR12–AR16, with bootstrap values of 60 and 42, respectively. This second clade (AR12–AR16) formed a polytomy with the *AR13*, *AR14*, and *AR17* strains.

The results of mapping the symptoms in bootstrap and jackknife trees indicated that neonatal disease caused by EHV-1 infection developed independently (Fig. 2). Phylogenetic analysis revealed that the Argentinian strains constitute a polyphyletic group. Argentinian (*AR11*, *AR12*, *AR13*, *AR14*, *AR16*, and *AR17*), British (UK1 and UK2) and Japanese (JA) strains showed the same origin; this result is supported by the high bootstrap values obtained.

The polyphyletic origin of the Argentinian strains of EHV-1 might be explained by the continuous animal transit from countries where EHV-1 is common. From the

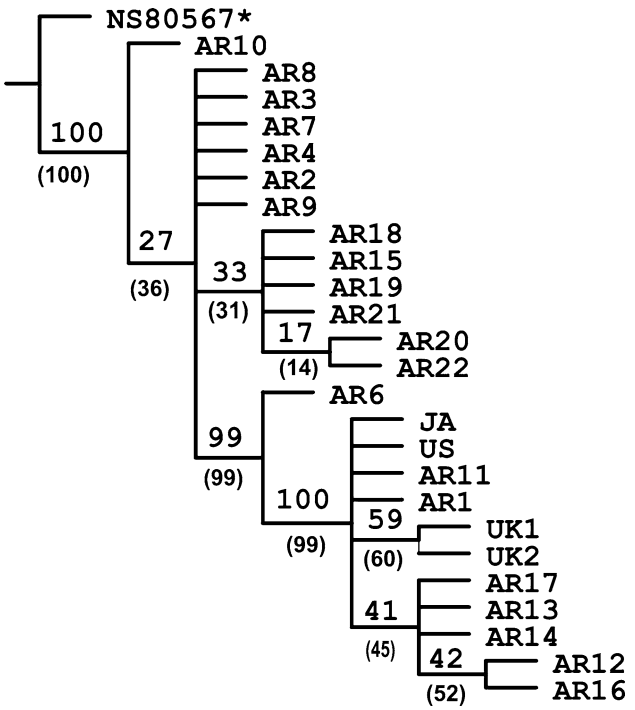

Fig. 1 Phylogenetic tree obtained by parsimony method from the analysis of a section of intergenic region amplified by polymerase chain reaction of Argentinian EHV-1 strain. Group support obtained with 1,000 bootstrapping and jackknifing replicates. Numbers above branches indicate support values. Jackknife supports in parentheses. Terminal with asterisk is the EHV-4 strain as external group (OG)

evolutionary point of view, the polyphyletic origin may be due, among other things, to the fact that, since groups appeared almost at the same time, there is no existing evidence of diversification events or of the fact that the genomic region analyzed lacked phylogenetic information for this taxonomic level. However, considering that the taxon sample of non-Argentinian strains was poor, these strains might also be polyphyletic and may be evolutionary nested within the Argentinian strains.

Previous phylogenetic analysis carried out with Herpes simplex virus 1 (HSV-1) isolates from a geographically restricted area (western Sweden) and from Caucasian individuals demonstrated that two strains from North America and one strain from Scotland were closely related [10]. These data have also allowed the determination of the relationship between HSV-1 circulating strains and constitute an important feature in order to establish the phylogenetic relationship between HSV-1 isolates worldwide.

Ibrahim et al. [7] also carried out a phylogenetic analysis of EHV-1 using the genomic sequence of glycoprotein G, and based on the neighbor-joining (NJ) algorithm. Trees built by the NJ algorithm are based on genetic distances and allow the obtainment of similarity groups. However, the groups obtained through this method do not necessarily

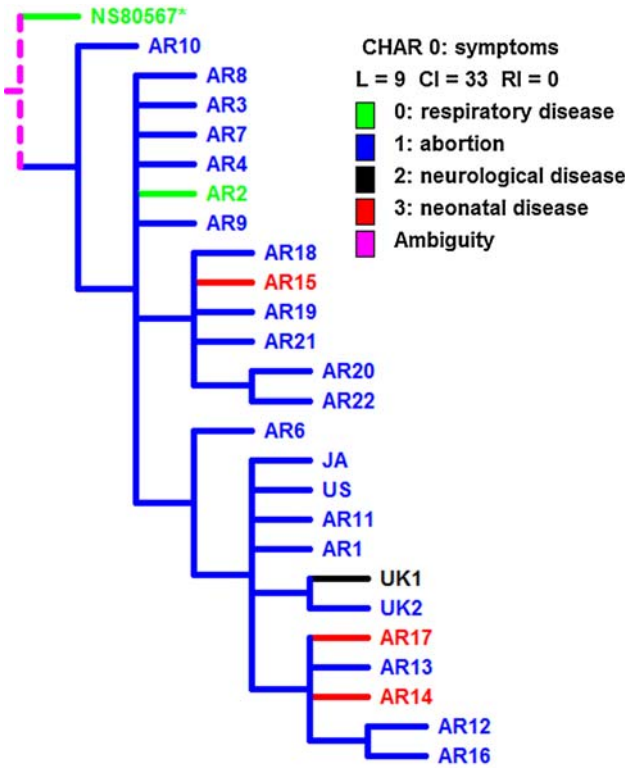

Fig. 2 Phylogenetic tree obtained by parsimony method from the analysis of a section of intergenic region amplified by polymerase chain reaction of Argentinian EHV-1 strain. Results of optimizing the symptoms on the jackknife/bootstrap tree. The history of change for the symptoms is depicted in color. Terminal with asterisk is the EHV-4 strain as external group (OG)

represent the evolutionary history of the group. Although the NJ algorithm is widely used and that the results obtained with it are widely accepted, this method has received criticism concerning the fact that a previous evolutionary model must be assumed to build phylogeny [2]. Instead, from a cladistic point of view, the assumption of such a previous model is not completely correct since it presumes the knowledge of how the evolutionary process operates before knowing the phylogeny [4]. In addition, another criticism that the NJ has received is that the conversion of discreet data such as the DNA sequences in distance matrices is not recommendable.

Character mapping is a procedure that uses parsimony as optimality criterion allowing the discovery of the evolution of states of character. This procedure provides information on the number of times a character is originated and on its ancestral condition. This method allows to establish if characters under analysis had followed a historical evolution (nearest common ancestor, and the strains are forming the same clade) or convergent evolution (independent development of characters, and the scattered strains in this tree). The study of character evolution by optimization in phylogenetic trees has been useful in many biological disciplines. This approach (i.e., character mapping) has also been used to study the evolution of pathogenesis in other viruses [8]. To our knowledge, no mapping of the symptoms produced by the *Herpesviridae* family had been carried out up to now.

In this work, symptoms character mapping indicated that the symptomatology caused by EHV-1 is not related to the historical evolution of the virus but developed independently through convergent evolution and that the abortion state constitutes an ancestral condition of EHV-1. The evolutionary patterns of a virus may be constrained by immune selection [16] or simply by genetic drift in geographically separated areas. Researches in *Vesicular Stomatitis Virus* have detected strong evidence of the importance of ecological factors in viral evolution [15]. The results shown here may suggest that the differences observed in the symptomatology caused by EHV-1 may constitute an adaptation mechanism of the virus to different environmental pressures of tissues. Different host factors such as different target cells, active antibody populations, and physical and chemical conditions might act as different selective pressures that may increase the frequency of the most efficient genetic variants in the new environmental conditions.

The adaptation of populations takes place by the appearance and subsequent fixation of beneficial mutations. In asexual systems, the lineages resulting from the appearance of different beneficial mutations compete with each other and such genetic changes with a better adaptive effect become fixed [8].

Nugent et al. [12], for example, found that non-neuropathogenic EHV-1 strains encode A in the position 2,254 (amino acid N752) of the genome, whereas neurological isolates encode G in that position (amino acid D752). This suggests that the ancestral EHV-1 virus probably encoded D752 and the variants expressing N 752 arose subsequently, possibly due to a selective advantage.

Ibrahim et al. [6] analyzed the IR between ORF 62 and ORF 63 to establish its relation between the growth and virulence of EHV-1, but the phylogeny of EHV-1 strains had not been studied before.

The reconstruction of the evolutionary history of the symptoms produced by HSV-1, EHV-1, and other Herpesvirus could help for a better understanding of the pathogenesis mechanisms.

This study constitutes the first analysis carried out in South-American EHV-1 strains that establishes the phylogenetic relationship between these strains and rebuilds the evolutionary history of symptoms based on maximum parsimony methods. In summary, this study focuses on a very important aspect of the evolution of *Herpesviridae* infecting perissodactyls and attempts to shed light on the evolution of symptoms, an issue of high clinical interest.

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