ISSN 0325-7541
INFORME BREVE Revista Argentina de Microbiología (2011) 43: 273-277

Genomic study of Argentinean *Equid herpesvirus 1* strains

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ABSTRACT

Equid herpesvirus 1 (EHV-1) infection has a significant economic impact on equine production, causing abortion, respiratory disease, neonatal death and neurological disorders. The identification of specific EHV-1 genes related to virulence and pathogenicity has been the aim of several research groups. The purpose of the present study was to analyze different genomic regions of Argentinean EHV-1 strains and to determine their possible relationship with virulence or clinical signs. Twenty-five EHV-1 Argentinean isolates recovered from different clinical cases between 1979 and 2007 and two reference strains were amplified and sequenced. The sequence alignments were carried out using Clustal X version 1.92 and the putative amino acid sequences were deduced using Bio-Edit version 7.05. Minor changes were observed. No changes that could be involved in the different virulence in the mouse model of three EHV-1 Argentinean strains were found. No genetic variants were observed. The genomic regions analyzed are unsuitable for differentiation between abortigenic strains and those isolated from neonatal deaths.

Key words: *Equid herpesvirus 1*, genomic analysis, virulence

RESUMEN

Estudio genómico de cepas argentinas de Herpesvirus equino 1. La infección por Herpesvirus equino 1 (EHV-1) tiene un significativo impacto económico en la producción equina mundial al causar abortos, enfermedad respiratoria, muertes perinatales y desórdenes neurológicos. La identificación de genes específicos relacionados con la virulencia y patogenicidad de este virus ha sido el propósito de varios grupos de investigación. En este trabajo se analizaron diferentes regiones genómicas de cepas argentinas de EHV-1 para determinar la posible relación entre la estructura genómica y la virulencia o los signos clínicos producidos. Veinticinco cepas aisladas de diferentes casos clínicos observados entre los años 1979 y 2007 y dos cepas de referencia fueron amplificadas y secuenciadas. El alineamiento de las secuencias se realizó con el programa Clustal X versión 1.92; el programa Bio-Edit versión 7.05 permitió deducir la secuencia de aminoácidos. Solo se observaron cambios menores, no se encontraron variaciones que pudieran estar relacionadas con la diferencia de virulencia observada previamente en el modelo ratón. No se hallaron variantes genómicas. Las regiones genómicas analizadas no permitieron diferenciar cepas abortigénicas de aquellas aisladas de muertes neonatales.

Palabras clave: Herpesvirus equino 1, análisis genómico, virulencia

Equid herpesvirus 1 (EHV-1) is endemic in horse populations throughout the world. EHV-1 infection has a significant economic impact on equine production, causing abortion, respiratory disease, neonatal death and neurological disorders (1, 3). In Argentina, the first EHV-1 isolate was reported in 1979 from aborted fetuses (4). Neurological signs in adult horses were described in 1984 and the virus was first isolated from a horse with respiratory symptoms in 1985. Since then, other viral isolates have been obtained from abortion storms, individual cases of neonatal disease and abortion associated or not with neurological signs. Preliminary molecular studies of Argentinean isolates showed genetic homogeneity between strains and the presence of the EHV-1B genome (10). The first analysis that established phylogenetic relationships among Argentinean EHV-1 strains was conducted in 2007 (8). A retrospective study carried out in 2009 determined that 7% of Argentinean strains recovered from aborted fetuses were neuropathogenic mutants (13). The identification of specific EHV-1 genes related to virulence and pathogenicity has been the aim of several research groups. Studies of EHV-1 pathogenesis demonstrated differences among strains, correlated with the ability of strains to disseminate and establish infection at vascular endothelial sites, in particular within the endometrium and central nervous system (4, 5). Nugent *et al*. (11) showed that variation of a single amino acid of the DNA polymerase is strongly associated with neurological *versus* non-neurological disease. In addition, Ibrahim *et al*. (7) suggested that differences found in a conserved domain of the intergenic region between ORF 62 and ORF 63 might be one of the factors affecting virulence. Ghanem *et al*. (5)

proposed that point nucleotide differences in ORF 46 and the gene encoded for glycoprotein D in RacL11 and Kentucky D strains could be responsible for their pathogenicity in a rodent model. Gupta *et al*. (6) postulated that ORFs 1, 2 and 3 could be markers for differentiating abortigenic strains from those causing neonatal disease. Osterrieder *et al*. (12) proposed that the IR6 protein, encoded by ORF 67, is a major determinant of EHV-1 virulence and might play a role in virus maturation and egress of the cell. Carvalho *et al.* (2) analyzed the ORF37 of the EHV-1 that encodes a nucleus-associated protein homolog to the UL24 gene product of human herpesviruses and related with virulence in animal models. This protein also plays a role in viral replication in ganglionic neurons, which may be related to the reactivation of latency. The purpose of the present study was to analyze different genomic regions of Argentinean EHV-1 strains and to determine their possible relationship with virulence or clinical signs.

To this end, 25 Argentinean EHV-1 isolates recovered from different clinical cases between 1979 and 2007 and two reference strains (HH1 Japanese strain and KyB American strain) were used in this study (Table 1). In previous studies, the AR1 strain showed low virulence in a respiratory and abortion mouse model (4). In addition, AR2 and AR8 showed different virulence in the abortion mouse model (9). All strains were isolated in primary tissue culture of equine fetal kidney or in rabbit kidney epithelial (RK13) cells. Each strain was then propagated for only three passages in RK13 cells in Minimal Essential Medium (MEM) supplemented with 2 % of fetal calf serum before DNA extraction. Infected RK13 cells were treated with proteinase K and total DNA was extracted with phenol and phenol-chloroform-isoamylalcohol and precipitated by adding 1/10 volume of sodium acetate (3 mol/l) and 2.5 volumes of cold absolute alcohol. The DNA pellets were washed twice with 70 % ethanol and resuspended in distilled water (10). A polymerase chain reaction (PCR) was carried out in an Eppendorf thermal cycler in a final volume of 25 µl, using an amplification reagent kit (PCR Master Mix, Promega Lab., Madison, WI, USA). Six sets of primers amplifying different regions of EHV-1 were used. The DNAs were amplified with an initial denaturation step

Table 1. *Equid herpesvirus 1* strains used in this study

Strain	Clinical Signs	Origin		
		Year	Place	Source
AR1	Abortion	1979	La Plata. Bs As	CM Galosi
AR2	Rhinopneumonitis	1985	La Plata. Bs As	CM Galosi
AR5	Abortion	1991	Corrientes	CM Galosi
AR6	Abortion	1990	Tucumán	CM Galosi
AR7	Abortion	1990	Capitán Sarmiento. Bs As	CM Galosi
AR8	Abortion	1996	Magdalena. Bs As	CM Galosi
AR10	Abortion	2002	San Antonio de Areco. Bs As	CM Galosi
AR11	Multiple abortions	2004	San Antonio de Areco. Bs As	M Barrandeguy
AR12	Abortion	2001	Trenque Lauquen. Bs As	CM Galosi
AR13	Abortion	1997	General Villegas. Bs As	M Barrandeguy
AR14	Neonatal disease	1998	Pilar. Bs As	M Barrandeguy
AR15	Neonatal disease	1999	San Antonio de Areco. Bs As	M Barrandeguy
AR16	Multiple abortions	1997	Entre Ríos	M Barrandeguy
AR17	Neonatal Disease	1999	Cañuelas. Bs As	CM Galosi
AR18	Abortion	1999	General Pueyrredón. Bs As	M Barrandeguy
AR19	Multiple abortions	2000	General Pueyrredón. Bs As	M Barrandeguy
AR20	Multiple abortions	1999	La Plata. Bs As	CM Galosi
AR21	Abortion	2001	Córdoba	M Barrandeguy
AR22	Abortion	1998	Trenque Lauquen. Bs As	M Barrandeguy
AR50	Neonatal Disease	2004	Bavio. Bs As	CM Galosi
AR51	Abortion	2004	Chascomús. Bs As	CM Galosi
AR52	Neonatal Disease	2005	Chascomús. Bs As	CM Galosi
AR53	Abortion	2007	Brandsen. Bs As	CM Galosi
AR103	Abortion associated with neurological signs	1990	25 de mayo. Bs As	CM Galosi
AR104	Neonatal Disease	1990	Gral Belgrano. Bs As	CM Galosi
HH1	Abortion	1970	Japan	Y Kawakami
KyB	Abortion	1954	USA	ER Doll

of 94 °C for 5 min, followed by 30 cycles of amplification, using denaturation at 94 °C for 1 min, annealing at a temperature determined for each primer pair (Table 2), and followed by a final extension at 72 °C for 1 min. The PCR products were run on a 1.5 % agarose gel in 1X TBE Buffer (45 mM Tris-borate, 1 mM ethylenediamine tetra-acetic acid –EDTA–, pH 8). The bands were visualized by ultraviolet transillumination after staining with ethidium bromide at a final concentration of 0.5 µg/ml. Each band was purified using a commercial kit (Wizard SV Gel & PCR Clean Up, Promega), quantified by a spectrophotometer (Smart spec™ 3000, Bio-Rad, Hercules, CA, USA) and sequenced (Biotechnology Resource Center, University of Cornell, Ithaca, USA). The sequence alignments were carried out using Clustal X version 1.92 and the putative amino acid sequences were deduced using Bio-Edit version 7.05.

When primer pairs I, II, III, V and VI were used, single PCR products of 794, 850, 911, 864 and 1188 bp were respectively obtained in all strains analyzed (Table 2). Single PCR products of different sizes between 500 and 800 bp were obtained by ORF 71 amplification when using primer pair IV in all strains. Combination of primer IF and IIIR rendered a single band of ~ 2529 bp in all strains. The nucleotide and amino acid sequences of the Argentinean strains and the AB4 (accession number AY665713) and V592 (accession number AY464052) strains obtained from GenBank were compared.

The sequence analysis of ORF 1 showed a 13 nt deletion (nt 1630-1643) in the AR14 strain, isolated from neonatal disease (Figure 1). This deletion generates a frame shift. Three changes were found when ORF 2 was compared: 1) AR5 and AR8 code for glycine at residue 136 rather than for arginine, as found in other Argentinean strains and AB4 and V592; 2) all Argentinean strains and V592 encode aspartic acid in residue 59, while AB4 encodes glycine, 3) V592 differs from all Argentinean strains and AB4 in one nucleotide at position 2162. ORF 3 was identical among all isolates, including AB4 and V592. Two changes were found when ORF 67 was compared: 1) AR51 strain differs in one nucleotide with respect to all Argentinean strains and AB4 (position 124664) and V592 (position 124141). However, this is a synonymous substitution; 2) V592 differs from all Argentinean strains and AB4 in one nucleotide at position 123890 encoding serine instead of phenylalanine. ORF 46 was identical among all isolates including AB4.

Previous research conducted by Gupta *et al*. (6) reported the finding of an additional band when ORF 1-2-3 was amplified using a single primer pair. In our study, this band was not observed in the Argentinean and reference strains analyzed. These authors hypothesized that ORFs 1-2-3 could be used as potential markers for differentiating the EHV-1 isolates obtained from abortion cases from those obtained from perinatal foal mortality. However, they examined only one strain derived from neonatal disease and suggested that more strains should be analyzed to test this hypothesis. When six Argentinean strains isolated from neonatal diseases were studied, only a 13 nt deletion in the ORF 1 of AR14 was found, allowing to infer that this region might not be used as a marker for differentiation (Figure 1). According to the results obtained by the same authors, the amplification of ORF 71 (a membrane glycoprotein) is variable among all strains because this region is known to contain two 15 bp repeat sequences. Furthermore, from the analysis of ORF 71, these authors

Table 2. Oligonucleotide primer pairs used for PCR amplification of 25 Argentinean EHV-1 strains and reference strains (HH1 Japanese strain and KyB American strain). Annealing temperature and expected product size for each primer pair are indicated

Primer pair	Sequence (5' to 3')	Annealing temperature	Position in EHV-1 genome (bp)	Product size
I (ORF1)	F: TTTCCATCTCCTCTCCAACT R: CGGCTCTACAGTAAAACCTT	48 °C	1271 2064	794
II (ORF2)	F: CCGCAAAGGTTAATCGCATT R: CAATCACGGGGACAGCTATT	48 °C	1920 2769	850
III (ORF3)	F: CACGTACTACGGTTGTTCTA R: ACCGTATATGGTGTTTTGCT	48 °C	2730 3640	911
IV (ORF71)	F: CTACAACCACAGCTGTTACT R: TAGTAGCCGCAGCTGATGTTG	50 °C	129488 129991	504
V (ORF67)	F: ATTTACACCGTTCCAACTTC R: CCTTTGAAGATAACAGACGT	46 °C	124352 125215	864
VI (ORF46)	F: GATCATCTCTACGTGCCGCA R: GCAAGGCTGTCAGTATCGAG	52 °C	86601 87789	1188

F: forward R: reverse

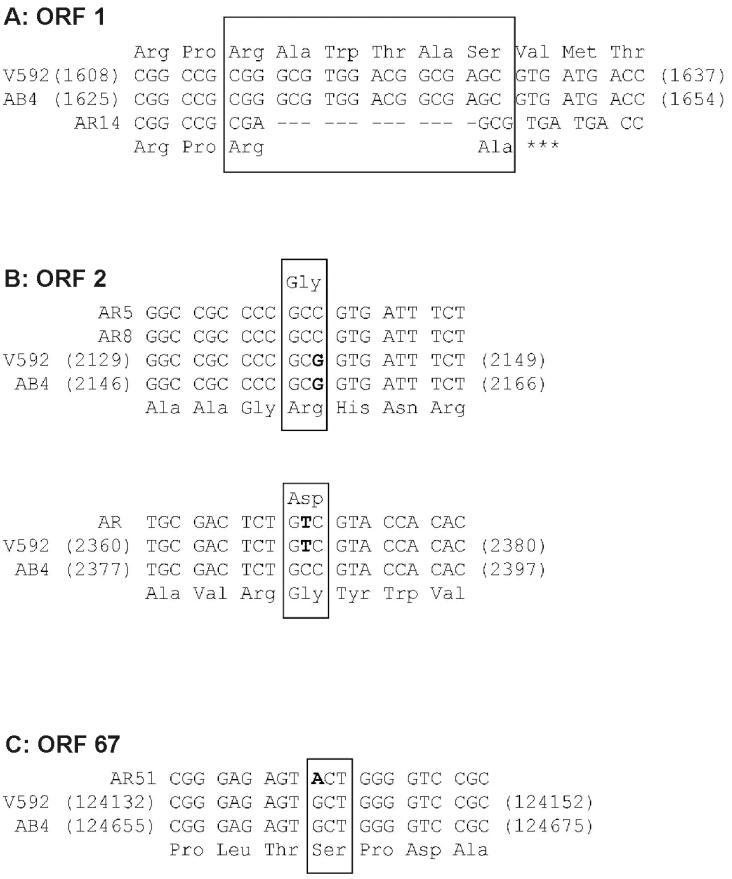

Figure 1. Sequences of regions with changes in the Argentinean strains. Dashes (-) indicate nucleotide deletion.

classified Indian isolates into two genetic variants (6), but in our study of Argentinean strains this difference was not observed. Osterrieder *et al.* (12) postulated that a mutation or deletion of the IR6 gene (ORF 67) is correlated with loss of virulence in different isolates, and demonstrated this postulate by converting the non-pathogenic RacH virus into a highly virulent form by insertion of a wild type IR6 gene. However, all Argentinean strains, including AR1, which is less virulent in the mouse model (4), showed no differences in this gene. Other researchers have proposed that some tegument proteins might be involved in the pathogenicity of herpesviruses. Ghanem *et al.* (5) analyzed several of these genes and found that the serine residue at position 140 in ORF 46 was conserved among RacL11, Kentucky D, and V592. In addition, they found that RacL11 and Kentucky D showed different pathogenicity with respect to AB4 and Japanese strains in a hamster infection model, but were unable to find any reports on the pathogenicity of V592 strains for the same model. Moreover, they demonstrated that AB4 and Japanese strains encode phenylalanine instead of serine in the same position. All Argentinean strains, including AR1, which proved to be less virulent than other strains, encode phenylalanine. This result suggests that changes in ORF 46 are irrelevant to differentiate among virulent strains. The analysis of ORF 37, a region closely related to viral latency, of two Brazilian EHV-1 isolates and reference strains showed that this genomic region is highly conserved (2). However, this region was not studied in Argentinean strains. The Argentinean strains showed genetic homogeneity when they were studied from the analysis of the intergenic region (ORF62-ORF63) postulated by Ibrahim *et al.* (7) as an indicator of virulence. In addition, phylogenetic analysis performed using parsimony as an optimality criterion and equal weighting strategies based on this region revealed that these Argentinean EHV-1 strains constitute a polyphyletic group (8, 9). A new study analyzed the genetic distance among the same EHV-1 strains based on the ORFs 1-2-3 and confirmed our previous results (Martín Ocampos GP *et al.*, unpublished data).

We conclude that the genomic regions analyzed are unsuitable for differentiation between abortigenic strains and those isolated from neonatal deaths. In addition, in these regions there were no changes that could be

involved in the different virulence of EHV-1 Argentinean strains AR1, AR2 and AR8 shown in the mouse model. Further studies are needed to know and to determine which genomic regions are involved in the virulence of strains and whether virulence is related to other factors not yet determined. This report describes the first genomic study performed with Argentinean strains to determine the cause of the different virulence or clinical signs produced.

Accession Numbers: The coding sequences of the strains showing variations have been deposited in the GenBank database under the accession numbers: JN562754 (ORF1 AR14), JN613350 (ORF2 AR5), JN613351 (ORF2 AR8), JN613352 (ORF67 AR51), JN613371 (ORF71 AR1), JN613363 (ORF71 AR2), JN613368 (ORF71 AR5), JN613364 (ORF71 AR6), JN613354 (ORF71 AR7), JN613369 (ORF71 AR8), JN613362 (ORF71 AR10), JN613366 (ORF71 AR11), JN613353 (ORF71 AR12), JN613357 (ORF71 AR13), JN613377 (ORF71 AR14), JN613367 (ORF71 AR15), JN613372 (ORF71 AR16), JN613355 (ORF71 AR17), JN613358 (ORF71 AR18), JN613370 (ORF71 AR19), JN613359 (ORF71 AR20), JN613360 (ORF71 AR21), JN613365 (ORF71 AR22), JN613373 (ORF71 AR50), JN613374 (ORF71 AR51), JN613375 (ORF71 AR52), JN613376 (ORF71 AR53), JN613361 (ORF71 AR103), JN613356 (ORF71 AR104).

Acknowledgements: CMG is a researcher of the Scientific Research Commission (CIC) Buenos Aires Province (PBA); NAF and MLE are fellows of CCT-CONICET-La Plata, Argentina. We thank Dr. María Barrandeguy for providing some of the strains used in this study. This study was supported by grants from CIC-PBA and the Department for Science and Technology at the Universidad Nacional de La Plata.

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Recibido: 21/05/11 – Aceptado: 11/11/11