

Characterization of *Suid herpesvirus 1* field isolates from Argentina

M. S. SERENA^{1,2*}, G. E. METZ^{1,2}, G. P. MARTÍN OCAMPOS^{1,2}, S. E. GAMBARO¹,
E. C. MÓRTOLA³, M. G. ECHEVERRÍA^{1,2}

¹Cátedra de Virología, ³Cátedra de Inmunología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118, CC 296 (1900) La Plata, Argentina, ²CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas).

*Correspondence. E-mail: solserena2000@fcv.unlp.edu.ar

ABSTRACT

The genomic characterization of *Suid herpesvirus 1* (SHV-1) isolates from Argentina was accomplished by restriction pattern analysis using the *Bam*HI, *Bst*EII and *Xho*I enzymes. Type II genome has been described only once in Argentina. This study revealed considerable homogeneity of *Bam*HI endonuclease sites in all the strains analyzed, according to the number and size of the fragments. No deletion of *Bam*HI fragment #7 among the Argentinean isolates suggests that these strains are wild-type. In addition, the main antigenic domain of glycoprotein E of all the Argentinean strains, as well as the reference strains and sequences available in the GenBank, were characterized. The similarity percent oscillated between 99 and 100%.

Key words: *Suid herpesvirus 1*, RFLP, glycoprotein E

RESUMEN

Caracterización de cepas de campo de *Herpesvirus suino 1* aisladas en Argentina. Se caracterizaron cepas argentinas de herpesvirus suino tipo 1 (HVS-1) mediante corte con las enzimas de restricción *Bam*HI, *Bst*EII y *Xho*I. La presencia del tipo genómico II había sido reportada en la Argentina una sola vez y, hasta el momento, no se han informado nuevos casos. Este estudio reveló una homogeneidad de los sitios *Bam*HI, acorde con el número y el tamaño de los fragmentos. La presencia del fragmento *Bam*HI #7 en los aislamientos argentinos analizados sugiere que éstos pertenecen al tipo salvaje (*wild-type*). Además, se caracterizó el principal dominio inmunogénico de la glicoproteína E de todas las cepas argentinas y se lo comparó con los de las cepas de referencia y con las secuencias disponibles en la base de datos GenBank. Los porcentajes de similitud obtenidos oscilaron entre 99 y 100%.

Palabras claves: *Herpesvirus suino 1*, patrones de restricción, glicoproteína E

Aujeszky's disease (AD) or Pseudorabies (Pr) is one of the most significant infectious diseases in the pig industry. The causative agent, *Suid herpesvirus 1* (SHV-1), is indigenous in swine and is probably the cause of mass mortality among piglets. AD is manifested by various degrees of respiratory distress, nervous and genital disorders, and even mortality, according to the host age and the virulence of the viral strain involved. Also, after recovery, a latent infection is established. SHV-1, a member of the *Alphaherpesvirinae* subfamily, is a double-stranded DNA virus of approximately 90 megadaltons, composed of long and short unique sequences. The short sequence is bracketed by the internal and terminal repeat sequences (IR and TR respectively) and contains a group of four glycoprotein genes: gD, gE, gI and gG. This short sequence is located in the *Bam*HI fragment #7 and is composed of 6.7 Kbp (10). Glycoprotein E, an envelope glycoprotein consisting of 577 amino acids (1.7 Kbp), is one of the SHV-1 virus virulence determinants, but is non-essential for *in vitro* replication. The main antigenic

domain of gE has been identified between amino acids 52 and 84 (7). Field isolates of SHV-1 have been subdivided into genome types I, II and III, based on restriction pattern analysis using the *Bam*HI enzyme (6). Thereafter, a subtyping has been carried out using other restriction enzymes, such as *Bst*EII and *Xho*I: genome type I (Ia, Iia, Ib, In, Ip, Is and Iip), genome type II (IIa, IIp) and genome type III (IIIa and IIIp). RFLP of the SHV-1 genome has been used to analyze strains isolated in Argentina since 1994. At that moment, type I and II genome types of SHV-1 strains were known in Argentina, although the outbreak of genome type II has occurred only once so far (2). After that, we isolated five Argentinean strains from pigs with symptoms compatible with AD, coming from different regions of the country. In 1998, the Servicio Nacional de Sanidad Animal (SENASA) established a voluntary vaccination with an inactivated-gE-deleted vaccine. However, there is no information about the vaccine strains used in our country, because its importation was discontinued in 2001. The commercially available gE-

deleted vaccine strains used worldwide include Bartha, NIA-3, NIA-4, Bucharest and Begonia strains (http://www.oie.int/esp/normes/mmanual/A_00041.htm). The latter is used in the Pr eradication program of Santa Catarina State in Brazil (13). The purpose of the present study was to investigate the possible genetic variation among SHV-1 strains from Argentina by RFLP analysis in order to know if the strains are vaccine derivatives in comparison with non-deleted reference strains. In addition, we analyzed a partial sequence of the gE gene against the gE sequences available in the GenBank (Table 1). This is the first Argentinean study comparing sequences of SHV-1 strains.

By using RFLP, we analyzed 13 SHV-1 strains, nine of which were isolated in Argentina from 1979 to 1998, and four have been previously characterized (RC/79, Mer, CL/7 and CL/15) (2). The non-deleted reference strains used were Ind-S (9), Alf (6) and S-66 (12), classified as type I, and YS-81, classified as type II (3). The CL/7, CL/15, TL/92, A/94, CL/96, CL/98 and CLP/98-10 strains were isolated in our laboratory, while the RC/79 and Mer strains were isolated in other Argentinean laboratories. All strains were propagated twice in Continuous Porcine Kidney (CPK) cell line cultures. The cells were grown in Eagle's medium (Gibco Invitrogen Corporation, USA) supplemented with 10% heat-inactivated fetal calf serum and antibiotics (penicillin-streptomycin). DNA was isolated from the cells infected with each viral strain at a multiplicity of infection between 1 and 5. CPK cells grown in 75 cm³ plastic flasks were inoculated with 1ml of supernatant of positive cell cultures, and after an extensive cytopathic effect was observed, pellets were washed and suspended in TEN buffer consisting of 100 mM Tris-HCl (pH7.5), 12.5 mM EDTA (pH 8.0), 150 mM NaCl and 1% SDS. Proteinase K at a final concentration of 0.2 mg/ml was then added and incubated at 50 °C for 4 h. The lysate was extracted once with TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0), saturated phenol, and once with a mix-

ture of phenol-chloroform-isoamylalcohol (25:24:1). Finally, total DNA was precipitated with two volumes of 99% ethanol, rinsed twice with 70% ethanol, dried and dissolved in 40 µl of sterilized distilled water. The quantity and quality of DNA was determined by measuring absorbance at an OD₂₆₀/OD₂₈₀ ratio in a spectrophotometer SmartSpec 3000 (Bio-Rad, USA). Approximately 1.5 µg of DNA solution from each viral strain was digested overnight with 2UI of *Bam*HI at 37 °C, and then separated by electrophoresis in 0.7% (w/v) agarose gel (140 x 150 x 5 mm) in TAE buffer (40 mM Tris-acetate pH 7.8, 5 mM sodium acetate, and 1 mM EDTA) at 20 V for 16 h at room temperature, stained with ethidium bromide, visualized and photographed under a UV illuminator. After classifying all the strains as genome type I or II according to the *Bam*HI pattern, the *Bst*EII and *Xho*I enzymes were used to establish the subtypes respectively. The digestion reactions were carried out according to the conditions used for *Bam*HI. Lambda DNA cleaved with *Hind*III and *Eco*RI + *Hind*III were used as size markers.

For Southern blotting (SB) analysis, electrophoretically separated DNA-fragments (1% w/v agarose) were transferred to nylon membranes (Hybond N⁺ membrane, Amersham, UK) in 20X SSC buffer (3 M sodium chloride, 300 mM sodium citrate, pH 7.0) and then prehybridized at 42 °C for 4 h in a solution containing 50% formamide, 6X SSC buffer (0.9 M sodium chloride and 0.09 M sodium citrate), 5X Denhardt's solution (0.1% bovine serum albumin -BSA-, 0.1% polyvinylpyrrolidone, 0.1% Ficoll), 0.2% BSA, 1% sodium dodecyl sulfate -SDS-, 10 mM EDTA and 100 µg/ml denatured salmon sperm DNA. Probe hybridization proceeded overnight at 42 °C with the biotinylabeled *Bam*HI fragment #7 originated from the Ind-S strain (Bio Prime DNA kit labeling system, BRL Life Technology) in hybridization buffer (40 mM Tris, 1 mM EDTA, 5X Denhardt's, 6X SSC, 0.1% BSA, 0.1% SDS). The chromogenic detection streptavidin-alkaline phosphatase method was used.

To amplify the main antigenic domain of gE (286 bp), we designed the following primers: ForgEpi (5'-CCC CGA GCC TCT CCG CCG A-3') and RevgEpi (5'-TCG CAG CCG CGG ACG GGC A-3'). PCR was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). A single PCR amplification was carried out according to the manufacturer's protocols using PCR Master Mix (Promega). Denaturation, annealing and extension consisted of 30 cycles at 94 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min, respectively. PCR products were analyzed on 2.5% (w/v) agarose gels in TAE buffer, and stained with ethidium bromide. The products were visualized under UV light. The PCR products were purified according to the manufacturer's protocols using Wizard SV Gel and PCR Clean-Up System (Promega). PCR products were cloned into a pGEM-T Easy plasmid (Promega). After transformation of *E. coli* JM109 strain, the plasmid DNA was extracted and purified by the Wizard Plus

Table 1. Viral isolates from GenBank

Strain	Country	Accession number
Fa	China	AF403049
Min-A	China	AY170318
LA	China	AY173124
PRV-SH	China	AF207700
Becker	Europe	AY368490
Rice	USA	M14336
Yangsan	South Korea	AY249861
Guangdong	China	AF403050
RongA	China	AY683134
Ea	China	AF171937
GDSH	China	EF552427
P-PrV	Malaysia	FJ176390.1

Miniprep DNA Purification System (Promega). Two colonies of each strain were analyzed. Both chains of each cloned strain were sequenced using M13 standard primers by Mega BACE™ 1000 (IGEVEV, FCV-UNLP, La Plata, Argentina). Twelve sequences from different geographical origins were downloaded from the GenBank and were included in this analysis (Table 1). The sequence alignments and edition were carried out using the ClustalX version 1.92 (15) and BioEdit software version 5 (5). The percentage of similarity between sequences was calculated using the similarity index implemented in PAUP software (14).

According to the classification based on the presence of fragment 2 using *Bam*HI (6), all Argentinean strains, except for the Mer strain, were classified into genome type I. Although the Mer strain (which is classified as type II) has only been isolated once so far, the five strains isolated after 1994 are still classified as type I. In the typical pattern II, an extra *Bam*HI cleavage site, named 2a and 2b, appeared in fragment 2. Regarding type I, there were no alterations involving the gain or loss of *Bam*HI cleavage sites in any of the strains analyzed. Although all Argentinean type I strains presented almost the same pattern, there were small differences and the main variation was in the 5+14', 10 and 12 fragments. In the Argentinean strains, as well as in the Alf strain, the 5+14' fragment was smaller than in the Ind-S and S-66 strains. Fragment 10 of strains RC/79 and CL/96 shifted its migration to the position of fragment 9; also fragment 10 presented different mobility from that of the others. In addition, fragment 12 was very variable in size in all the strains analyzed. In the subtyping analysis, *Bst*EII was used to identify two subtypes: Is and Ip. Strains RC/79, CL/7, CL/15, TL/92, A/94 and CL/96 were classified as Is, because they showed an additional cleavage site in the *Bst*EII fragment 2. In contrast, strains CL/98 and CLP/98-10 were classified as Ip. Taking into account *Bam*HI and *Bst*EII patterns, the Ind-S strain was classified into subtype lia because its restriction pattern presented an additional cleavage site in fragment 5. Since the pattern of the S-66 strain was characterized by the lack of *Bam*HI cleavage sites between fragments 14' and 5', S-66 was classified as subtype lip. In addition, the Alf strain was classified as subtype lb because its pattern showed an enlarged fragment 11 that co-migrated with fragment 10. Mer and YS-81 strains were verified as genome type II, subtype Iip. The restriction analysis using *Xho*I did not show different patterns between these two strains (Figure 1 and Table 2). When we analyzed the results obtained by SB analysis, we detected that fragment *Bam*HI #7 of all strains, even type I or II, was in the same position and thus, not truncated.

The percentage of similarity between gE nucleotide sequences of type I Argentinean strains and reference sequences Rice, Becker, S-66, Ind-S, Alf, Min-A and Ea was 100%. Besides, type I Argentinean strains showed

99.95% similarity with Fa, LA, Yangsan, Guangdong, RongA, GDSH and P-PrV strains. All Argentinean strains showed the lowest percentage of similarity (99.3%) with the PRV-SH strain. Likewise, type II Mer strain showed 100% similarity with its reference strain YS-81.

It is clear that all Argentinean SHV-1 strains are *Bam*HI DNA pattern type I. The only strain isolated in Argentina previously classified as type II was isolated from animals imported from Holland (2) and was not disseminated in our country because all animals had been sacrificed. It has been reported that genomic type II is common in

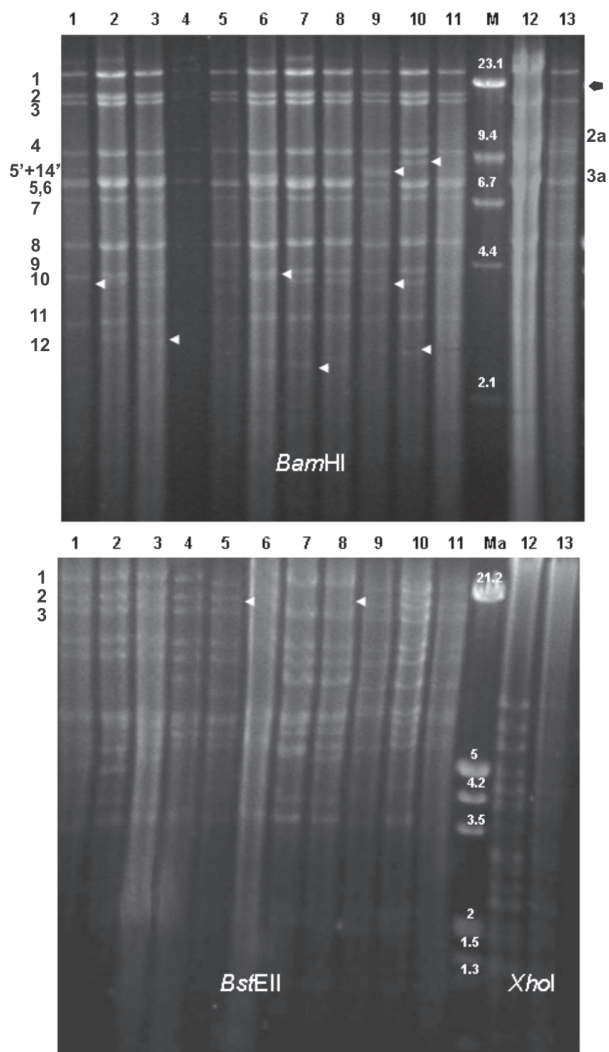


Figure 1. Restriction fragments obtained with the *Bam*HI (upper), *Bst*EII (lower left) and *Xho*I (lower right) enzymes. Lane 1: strain RC/79; Lane 2: strain CL/7; Lane 3: strain CL/15; Lane 4: strain TL/92; Lane 5: strain A/94; Lane 6: strain CL/96; Lane 7: strain CL/98; Lane 8: strain CLP/98-10; Lane 9: strain S-66; Lane 10: strain Ind-S; Lane 11: strain Alf; Lane 12: strain Mer; Lane 13: strain YS-81. Lane M, Lambda DNA cleaved with *Hind*III; Lane Ma, Lambda DNA cleaved with *Eco*RI + *Hind*III (size expressed in Kbp). Fragments are numbered following Herrmann *et al.* (13) for *Bam*HI patterns type I (left numbers) and type II (right numbers). Fragments are numbered according to Christensen (1) for *Bst*EII patterns (left numbers). Arrows indicated differences.

Table 2: Viral strains used in this work

Strain	Origin (City-State-Country)	Characteristic	Year of isolation	Genotype	Subtype
RC/79	Río Cuarto, Córdoba, Argentina	Isolate	1979	Type I	Is
Mer	Villa Mercedes, San Luis, Argentina	Isolate	1981	Type II	IIp
CL/7	Chañar Ladeado, Santa Fe, Argentina	Isolate	1988	Type I	Is
CL/15	Chañar Ladeado, Santa Fe, Argentina	Isolate	1988	Type I	Is
TL/92	Trenque Lauquen, Buenos Aires, Argentina	Isolate	1992	Type I	Is
A/94	Arribeños, Buenos Aires, Argentina	Isolate	1994	Type I	Is
CL/96	Chañar Ladeado, Santa Fe, Argentina	Isolate	1996	Type I	Is
CL/98	Chañar Ladeado, Santa Fe, Argentina	Isolate	1998	Type I	Ip
CLP/98-10	Chañar Ladeado, Santa Fe, Argentina	Isolate	1998	Type I	Ip
Ind-S	Indiana, United States	Reference strain	1962	Type I	lia
S-66	Sweden	Reference strain	1966	Type I	lip
YS-81	Tsukuba, Yamagata, Japan	Reference strain	1981	Type II	IIp
Alf	Alfort, France	Reference strain	Nd	Type I	Ib

Nd. Not determined

Holland (4) and that it is the predominant type in the pig population in Brazil (11, 13). The restriction patterns using *Bam*HI were the same as those obtained by others for reference strains S-66, YS-81 and Ind-S (1, 8, 9), indicating that the passage in cell cultures made in our laboratory did not modify viral DNA. In agreement with the data obtained by others (4), the most variable *Bam*HI fragments are 5 and 13, which are located within the repeat regions, and considerable variations can be seen in the sizes of *Bam*HI fragments, 10, 12 and 8', which span the repeat-unique joint regions. In this study, the most variable fragments in Argentinean strains were 5+14', 10 and 12. No great variations were obtained between the other fragments. Those variations are known from viruses isolated from the same geographic area and are characterized by a different mobility. The Argentinean type I strains had a high degree of *Bam*HI pattern identity to Alf and Ind-S strains, especially in fragment 5+14'. It is well known that the most noticeable change in the vaccine strain profile is the absence of *Bam*HI fragment #7 (10). As reported by Schaeffer *et al.* (13), the vaccine strains NIA-4, Bartha, and Begonia could be grouped as type I, although they are significantly different in their DNA pattern because they have a deletion in this fragment. Even though this fragment is transcriptionally very active, it appears to be conserved and invariable in size among the high variety of field strains, as previously corroborated by others (1, 4). No deletion of *Bam*HI fragment #7 was observed among the Argentinean strains, indicating that they are wild-type viruses. The differences in fragment position are relevant since they allow the follow-up of viral spread within the porcine population and the possible identification of vaccine strains. In reference to subtyping, six

strains (RC/79, CL/7, CL/15, TL/92, A/94, CL/96) were classified as subtype Is and two (CL/98, CLP/98-10) as subtype Ip, whereas the Mer strain was classified as IIp. This study revealed the absence of type In and type III in Argentina. When we analyzed the sequence of the fragment encoding the main antigenic domain of the gE protein, we found that the differences between the genome type I and type II strains are not significant. Due to the high similarity detected between analyzed sequences, future studies aiming to carry out a phylogenetic analysis of SHV-1 strains from Argentina, will benefit from choosing other more variable regions. In summary, the Argentinean viruses were wild type and were classified as type I, suggesting that no major variation has occurred in SHV-1 spreading in Argentina since the first outbreak was noticed. No new isolates belonging to genome type II have been reported since 1981.

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