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Partial characterization of a Brazilian strain of Aujeszky's disease virus recovered from a pig with subclinical infection

[Caracterização parcial de uma amostra brasileira do vírus da doença de Aujeszky isolada de um suíno com infecção subclínica]

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ABSTRACT

One Brazilian strain of Aujeszky's disease virus isolated from a piglet in which the disease had not been observed was studied as for its virulence in pigs. The genome of the virus was molecularly analysed as for their restriction endonuclease cleavage pattern. Fifty-day-old non-immune weanlings exposed to this strain showed no disease although the virus was present in their oropharyngeal area for at least three days. All animals developed moderate titers of neutralizing antibody. Based on number of bands and migration rate of restriction fragments the isolate was classified into Herrmann's type I group. Latent infection was detected in all pigs by PCR. Some variations were detected in the cleavage pattern of the strain ASB Piau when compared to LA031 virulent Brazilian strain, that could be related to differences in the virulence.

Keywords: Swine, Aujeszky's disease virus, virulence, latency

RESUMO

Uma amostra brasileira do vírus da doença de Aujeszky, isolada de um leitão, foi estudada quanto à virulência para suínos e seu genoma analisado no que se refere ao padrão de fragmentação pela enzima de restrição Bam HI. Leitões com 50 dias de idade, sorologicamente negativos, expostos a essa amostra, não mostraram sinais de doença e o vírus foi detectado na região orofaríngea durante pelo menos três dias após exposição. Com base no padrão de migração dos fragmentos de restrição, o isolamento foi classificado como pertencente ao grupo I da classificação proposta por Herrmann. Infecção latente foi detectada por PCR em todos os suínos. Algumas variações foram identificadas no mapa físico de Bam HI da amostra ASB Piau quando se comparou com o mapa obtido da amostra virulenta brasileira LA031.

Palavras-chave: Suíno, vírus da doença de Aujeszky, virulência, latência

INTRODUCTION

Aujeszky's disease (AD) is caused by swine herpesvirus 1 (SHV-1) which belongs to the subfamily *Alphaherpesvirinae*. Most warmblooded species are either naturally or experimentally susceptible to SHV-1. Swines are the natural hosts and the main reservoir. Infection may result in signs ranging from clinically inapparent carrier state to neurological disease or severe respiratory tract syndromes. Recovery from SHV-1 infection often results in the establishment of a carrier state in the trigeminal nerve ganglia, as well as development of humoral and cellular immunity (Carneiro & Cardim, 1947; Gustafson, 1986).

Several methods have been described for characterization of SHV-1 strains based on biological properties on monolayers of cultured cells such as rounding, fusion or lysis of cells, ability to form syncytium, trypsin and heat resistance and virulence for laboratory animals (Platt et al., 1980; Iglesias & Harkness, 1989). Restriction endonuclease (RE) assays of viral DNA are also very useful tools, because they can detect genomic diversity of SHV-1 strains and allow further distinction of virus groups (Cristensen, 1988; Jestin et al., 1990; Carvalho & Resende, 1992).

To detect acute or latent infection, the palatine tonsil and trigeminal nerve ganglia are commonly tested for the presence of viruses either by blot-hybridization methodology or polymerase chain reaction (PCR) (Rziha et al., 1986; Belàk et al., 1987; Dangler et al., 1992; Belàk & Ballagi-Pordàni, 1993; Cândido et al., 1999).

In the present study, one tissue culture cloned SHV-1 strain which was isolated from a piglet from a Brazilian Piau herd, in which clinical signs of AD had not been observed, was analyzed. The potential virulence of this strain for piglets was studied and its genome was analysed by RE cleavage pattern. Profiles obtained with *Bam HI* were compared to that found for a Brazilian virulent strain.

MATERIALS AND METHODS

The ASB Piau strain of SHV-1 was isolated from a piglet tonsil from a herd in which AD had not been clinically observed (Prof. Antônio Stocker Barbosa, Veterinary School, UFMG, personal communication). The strain was biologically cloned in a porcine kidney line (SK-6) cell culture and underwent three passages. The infectivity titer was $10^{7.0}$ median tissue

culture infectivity dosis (TCID₅₀/ml), when cytopathic effects (CPE) reached approximately 95%. The virus-containing cell supernatant was clarified by low speed centrifugation and virus titer was assayed by use of standard plaque counting methods (Dulbecco & Vogt, 1954).

The virulent SHV-1 LA031 strain was isolated from the tonsils and spleen of a 30 day-old piglet and it was obtained from Dr. C.H. Panzenhagem, LARA Pedro Leopoldo, MG (Carvalho & Resende, 1992; Flatschart & Resende, 1998) and was also produced in the SK6 cell line.

Three crossbred pigs with negative results of ELISA for SHV-1 (Carvalho et al., 1994) were exposed to 2×10^5 TCID₅₀ of the biologically cloned ASB Piau strain of SHV-1 in each nostril at 50 days of age. Clinical signs were recorded and animals were kept in isolation for 350 days postinfection (PI). Samples from the tonsils and oropharyngeal area of the pigs by means of sterile swabs for virological examination were taken after three days and every 30 days PI. Each swab was then eluted in 2.0ml of cold Dulbecco's Modified Eagle's Medium (DMEM). The eluate was clarified by low speed centrifugation at 4°C and 0.5ml of the supernatant were seeded into four 25cm² SK6 cells monolayer culture flasks. The culture flasks were incubated for seven days and examined for CPE daily. If no CPE was detected the media from the culture flasks were used for a second passage in SK6 cells monolayer. For serology, blood samples were collected at the time of exposure and at intervals of 30 days thereafter. Concentration of neutralizing antibodies were estimated using conventional techniques and were expressed as the value of the reciprocal of the highest serum dilution neutralizing 100 TCID₅₀ of SHV-1. Pigs were euthanized by intravenous injection with an overdose of sodium thiopental and the trigeminal nerve ganglia were collected and placed on ice in TEN (10mM TRIS-HCl, pH 8.0, 1mM sodium EDTA, 10mM NaCl).

Virus DNA was prepared from virus produced in cell culture. The virus-containing clarified cell supernatant was pelleted by centrifugation. The virus pellets were resuspended in TEN and layered onto 10 to 50% sucrose gradients and centrifuged at 100.000' g for two hours. The banded virions were poured over homogenization buffer (0.1M NaCl, 0.2M sucrose, 0.02M EDTA and 0.3M Tris-Cl, pH 8.0) with 62 µl/ml 10% SDS. The mixture was incubated for 30 min at 65°C. Then, 175 µl/ml 8.0M potassium acetate was added, incubated on ice for 60 min and centrifuged at 5000xg. The white precipitate was discarded and the supernatant was extracted twice with chloroform. The aqueous phase was gently poured into a collodion bag and dialyzed four times against two liters of TE (10mM Tris-Cl, pH 8.0, 1mM EDTA). DNA concentration and purity of the dialyzed solution were estimated by electrophoresis in 0.4% agarose gels.

Bam *HI* restriction enzyme was obtained from Life Technologies and used according to the manufacturer's recommendations. Two micrograms DNA were digested and electrophoresed overnight in a 0.5% agarose gel in TAE (0.04M Tris-acetate, 0.001M EDTA) at 3 V/cm and DNA bands were stained with ethidium bromide.

To obtain the RE migration pattern, 40µg of LA031 strain DNA were digested with 400 units of *Bam* *HI* (Life Technologies, Inc) for two hours and electrophoresed in agarose gels. The selected bands (*Bam* *HI* fragments numbers 7, 11, 12 and 14) were cut out of the gel and purification was performed using the kit Prep-A-Gene (BioRad) essentially in accordance with the procedure recommended by the manufacturer. Purified fragments were sonicated to reduce its size to approximately 400-800 base pairs and nick translation-labeled. Hybridization followed the procedure recommended by Cândido et al. (1999).

Southern blot hybridization studies were achieved with fragments produced after digestion of ASB Piau strain DNA with *Bam* *HI*. The fragments were transferred to positively charged nylon membrane (Hybond- N⁺ Amersham) and immobilized as recommended by the manufacturer. Hybridization was carried out as described elsewhere (Cândido et al., 1999).

Trigeminal nerve ganglia was prepared by grinding the tissue with a polytron with 2ml of homogenization buffer without SDS until no pieces of tissue were visible. The cell lysates were digested with 0.5ml of a proteinase K solution (1.0mg/ml) overnight at 37°C in the presence of 0.2% of 10% SDS. After digestion 1.0ml of 8.0M potassium acetate was added and DNA was prepared as described before. In order to assess what the SHV-1 state of a given sample was, PCR was used essentially as described elsewhere (Flatschart & Resende, 1998). To control the specificity of SHV-1 amplification in the trigeminal nerve preparation the PCR products were tested by dot-blot hybridization using probes prepared from specific amplified PCR products of LA031 strain labeled with 32 P-dCTP by nick translation (Cândido et al., 1999).

The trigeminal nerve ganglia preparation were also tested for the presence of SHV-1 by dot-blot hybridization using as a probe the *Bam HI* 7 fragment which was prepared and labeled as described before. The positive control for each test (PCR or dot-blot hybridization) was the DNA purified from the SHV-1 LA031 strain and the negative control was DNA from the bovine BHV-1 BH83 strain.

RESULTS AND DISCUSSION

No strong signs of AD were observed in the pigs exposed to ASB Piau strain except a temperature response, observed 24 to 48 hours after infection. The pigs exhibited a rectal temperature slightly below 40°C. A mild transient anorexia between two and three days after exposure was also observed. More virulent strains are able to cause severe nervous disorders in young pigs or respiratory problems in older pigs (Andries et al., 1978).

The results of virus isolation from the oropharyngeal and tonsil area are shown in [Table 1](#). The virus was detected in all of the three pigs 72 hours after exposure. No virus was detected by swabbing at later intervals of examination.

Table 1. Survey of virus excretion and antibody titers in serum of three pigs after nasal exposure with ASB Piau strain of swine herpesvirus-1.

Days PI	Swine number					
	1		2		3	
	VI	SN	VI	SN	VI	SN
0	-	<2	-	<2	-	<2
3	+	<2	+	<2	+	<2
30	-	64	-	128	-	64
60	-	128	-	256	-	256
90	-	128	-	128	-	128
120	-	32	-	64	-	32
150	-	16	-	16	-	16
180	-	16	-	16	-	8
210	-	8	-	8	-	8
240	-	<2	-	8	-	8
270	-	<2	-	8	-	<2
310	-	<2	-	8	-	<2
350	-	<2	-	8	-	<2

PI: Post inoculation.

VI: Virus isolation from oropharyngeal and tonsil swabs; positive (+) and negative (-). SN: Titers of neutralization antibody in serum (dilution reciprocals).

DNA extracted from SK-6 cells infected with ASB Piau and LA031 SHV-1 isolates were digested with *Bam HI* and the cleavage patterns were analyzed. The endonuclease restriction patterns with *Bam HI* are shown in [Figure 1](#). The numbering of the fragments was done according to maps published for the SHV-1 Ka strain (Ben- Porat et al., 1984). According to

the four types of RE cleavage patterns of SHV-1 DNA described by Herrmann et al. (1984), the ASB Piau isolate can be considered as belonging to *Bam* *HI* cleavage type I with a slight difference in the migration rate of fragments 12, 14 and 15. The two strains showed RE cleavage patterns similar to each other, except for the fragment 7 of ASB Piau strain which was shorter than that of the LA031 strain. The fragment sizes detected in the *Bam* *HI* RE assay and the physical map of ASB Piau strain DNA with *Bam* *HI* are shown in [Figure 2](#). The physical map of strain ASB Piau was basically the same as to Herrmann et al. (1984) type I, although there was variation in the length of fragment 7. This fragment corresponds to a gene block where gE, gI, gD and most of the gG genes are located. Genes for the glycoproteins gE, gI and gG can be deleted from the viral genome without preventing autonomous viral replication (Mettenleiter, 1991). Deletion mutants for gD can also replicate autonomously once they penetrate the cell, as for example by phenotypic complementation for primary infection and retain infectiousness by direct cell-to-cell transmission, presumably by fusion of contiguous cell membranes (Heffner et al., 1993; Mulder et al., 1995). In the present study, the RE analysis and hybridization were established in order to detect markers to distinguish ASB Piau from LA031 strain. It is possible that the difference in size in the fragment 7 is responsible for the virulence reduction of ASB Piau strain. It is known that genes gE-gI located in this fragment affect virulence by changing the spreading of virus in the host and in vitro (Whealy et al., 1993). The gG gene, also in this fragment, codes for the only non-structural glycoprotein and its disruption of the gG gene may result in marginal attenuation (Balan et al., 1994) although the function of the protein still is not known. Symptoms of AD had not been reported in pigs naturally infected with ASB Piau strain and most animals reacted negatively in the ELISA (Prof. A.S. Barbosa, personal communication). So, it appears that the variation in the length of the *Bam* *HI* 7 fragment may account for the decreased virulence of ASB Piau. Several studies indicated that the degree of virulence can be influenced by genomic changes (Gielkens & Peters, 1994).

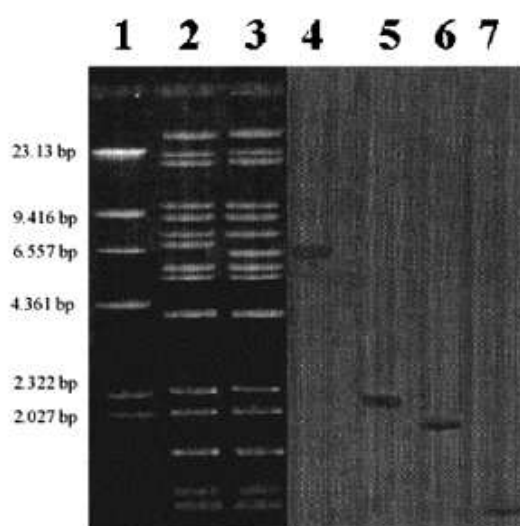


Figure 1. Comparison of *Bam* *HI* restriction enzyme cleavage pattern and Southern Blot hybridization of Brazilian isolates of swine herpesvirus 1. Track 1, lambda phage DNA restricted with *Hind* *III*; track 2, LA 031 strain; track 3, ASB Piau strain; tracks 4, 5, 6 and 7 Southern Blot hybridizations of strain ASB Piau with P-labeled 031 virus DNA probe; track 4 fragment 7; track 5 fragment 11; track 6 fragment 12 and track 7 fragment 14.

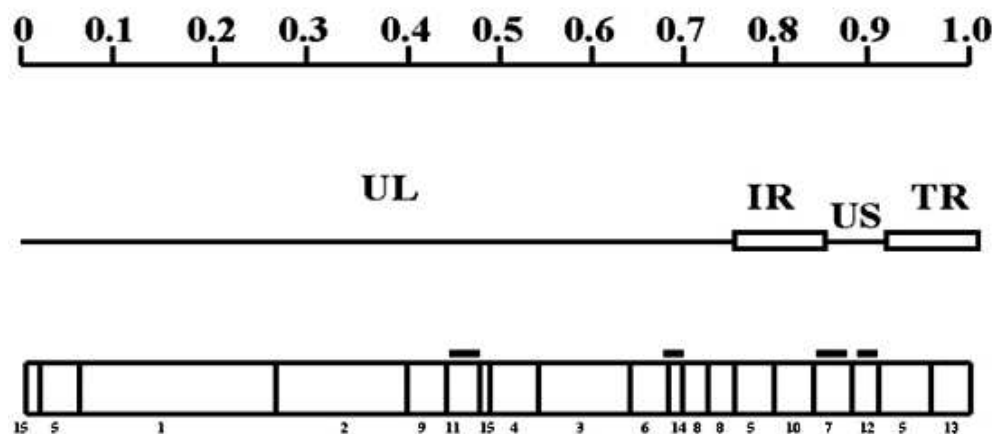


Figure 2. *Bam HI* restriction map of the ASB Piau strain of swine herpesvirus 1. The number and size in kilobase pairs (Kb) of each fragment were: 1 (31.2); 2 (19.2); 3 (18.9); 4 (9.5); 5 (8.2); 6 (7.8); 7 (6.4); 8 (5.0); 9 (4.2); 10 (4.0); 11 (3.0); 12 (2.0); 13 (1.6); 14 (1.3); 15 (0.8). The position of fragments hybridized to the fragments of swine herpesvirus 1 strains LA 031 are illustrated as thickened lines above the individual fragments.

In order to compare some RE fragments of the ASB Piau strain with virulent LA031 strain Southern Blot hybridizations were carried out using fragments 7, 11, 12 and 14 of SHV-1 LA031 strain as probes. As shown in hybridization in [Figure 2](#) fragments 7, 11, 12 and 14 of Piau strain corresponded reasonably well in size to the fragments of LA031 strain. A signal was observed only in the band homologous to the fragment used as probe. The signal produced with *Bam HI* 7 was different in size between the two isolates. The most likely explanation for this difference is the occurrence of deletions within this fragment in the ASB Piau strain. The *Bam HI* fragment 7 of Bartha strain was also smaller than that of LA031 strain while fragment 5 was larger (Carvalho & Resende, 1992).

Ganglion DNA from all three pigs yielded a PCR product of approximately 260 base pairs (bp) using primers and conditions previously described (Flatschart & Resende, 1998). This product was clearly visualized as a sharp band in agarose gels. Amplification products were transferred to nylon membranes and hybridized with probes prepared from specific PCR products from LA031 strain. No sequences homologous to SHV-1 were detected using the *Bam HI* 7 fragment in dot-blot hybridization ([Figure 3](#)).

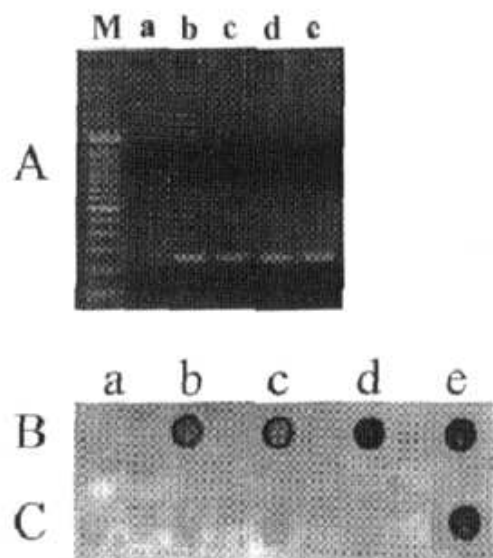


Figure 3. Panel A: PCR amplification of SHV 1 (swine herpesvirus 1) genome in trigeminal nerve ganglia of infected swine. Lna M is 100bp DNA ladder. Lane a: bovine herpesvirus 1 DNA strains BH83; lanes b, c and d trigeminal nerve ganglia of infected swine; lane e SHV 1 DNA. Panel B: Dot Blot hybridization using SHV1 *Bam* HI fragment 7 to determinate specificity of SHV 1 amplification product. Sample a, no signal was evident in negative control (BHV 1 DNA). Sample b, c and d represent amplification product of samples of latent infected pigs. Sample e represent the positive control of amplification of SHV 1 DNA. Panel C: dot Blot hybridization using SHV 1 *Bam* HI fragment 7 of trigeminal samples of infected pigs. Sample a, negative control (BHV 1 DNA). Samples b, c and d trigeminal samples of latent pigs. Sample e, positive control (SHV 1 DNA).

The results of this study showed that SHV-1 latency could be confirmed by PCR in all experimentally infected pigs. Under the amplifications conditions used the test has enough sensitivity to detect latent infected animals. The results obtained with dot-blot hybridization were clearly different and showed that the level of sensitivity of the test was not sufficient to detect SHV-1 sequences in latently infected tissues. Therefore dot-blot hybridizations do not seem to be a reliable laboratory method for examining the presence of latent SHV-1 infections. All pigs developed moderate amounts (1:124 to 1:256) of viral neutralizing (VN) antibodies following exposure to the ASB Piau strain. Based on the VN titer determined 150 days after exposure one can detect a pronounced decrease in serum neutralization titer (1:16). Thereafter, the VN titers went through a progressive decline with time until they were either very low or negative by day 240. None of the three infected pigs had a visible increase in VN titer during the observation period which may be attributed to reactivation of latent virus. A fourfold increase in VN titer could be interpreted as a natural reactivation of latent virus. The higher concentration of VN titer was detected between 30 and 90 days after exposure to SHV-1 ASB Piau strain. This can be clearly associated with the failure to isolate virus from these pigs from the third sampling at 30 days and so forth. The present study shows that screening swine with serological tests may not be sufficient to detect latent infected animals since VN titers were low or not detected 180 days after exposure to SHV-1 ASB Piau strain.

In conclusion, the analysis of restriction fragments revealed that ASB Piau strain had DNA pattern which allowed it to be classified as SHV-1 type I (Herrmann et al., 1984). This study

also suggests that the degree of virulence of this strain is low considering both the clinical and serological observations from the three exposed pigs.

A more detailed characterization of the strain as well as the cloning and sequencing of the *Bam* *HI* fragment 7 of this particular strain are of great interest. Hopefully it will be possible to gain some insight into the types of management practices that could favor the appearance of better adapted or less virulent strains in pig population. Also, in a frame of an eradication program of SHV-1, one will be able to use sensitive methods to detect the infection in apparently uninfected herds.

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