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Short communication

MOLECULAR CHARACTERIZATION OF A *Herpesvirus bovino 1* CUBAN STRAIN

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ABSTRACT: **In Cuba, infections caused by *Bovine herpesvirus 1* (BoHV-1) are endemic; however clinical signs are moderate, possibly because the BoHV-1 circulating genotypes are not associated to severe infections but these are not known. In the present study a BoHV-1 strain isolated in Cuba was compared with BoHV-1 strains from other different countries. Viral DNA was analysed by adoption of a clustering system based on restriction enzyme analysis (REA) with *Hin*dIII, *Hpa*I, *Pst*I and *Sfi*I, which led to identify the Cuban isolate as a BoHV-1.1.III strain. The Cuban BoHV-1 differed from all the reference strains analysed and displayed a restriction fragment pattern more similar to strains originated from the European continent. The inclusion of these results into the clustering database is a contribution for future studies in tracing back the origin of novel infectious bovine rhinotracheitis (IBR) outbreaks.**

*(Key words: **Bovine herpesvirus 1**; infectious bovine rhinotracheitis; restriction enzyme analysis; clustering system)*

CARACTERIZACIÓN MOLECULAR DE UNA CEPA CUBANA DE *Herpesvirus bovino 1*

RESUMEN: **En Cuba, las infecciones causadas por *Herpesvirus bovino 1* (BoHV-1) son endémicas; sin embargo los signos clínicos son moderados, posiblemente porque los genotipos circulantes de BoHV-1 no están asociados con infecciones severas pero estos no se conocen. En el presente estudio se comparó un aislado de BoHV-1 proveniente de Cuba con cepas de BoHV-1 de diferentes países. El ADN viral fue analizado por adopción de un sistema de agrupamiento basado en análisis de restricción (REA) con las enzimas *Hin*dIII, *Hpa*I, *Pst*I y *Sfi*I, el cual permitió identificar el aislado cubano como una cepa de BoHV-1.1.III. El BoHV-1 cubano difirió de todas las cepas de referencias analizadas y mostró un patrón de restricción similar a cepas provenientes del continente europeo. La introducción de estos resultados en la base de datos de agrupamiento es una contribución para futuros estudios relacionados con el origen de nuevos brotes de rinotraqueitis infecciosa bovina (RIB).**

*(Palabras clave: **Herpesvirus bovino 1**; rinotraqueitis infecciosa bovina; análisis de restricción; sistema de agrupamiento)*

Bovine herpesvirus 1 (BoHV-1), classified as a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (1), is the causative agent of several infections from the respiratory and genital tracts of domestic cattle and other ruminant species. Based on Western Blot analysis of viral proteins with a panel of monoclonal antibodies, as well as on restriction endonuclease analysis of the viral nucleic acid and differential amplification by PCR (2, 3, 4), BoHV-1 strains are

classified into two subtypes, BoHV-1.1 and BoHV-1.2, BoHV-1.2 being additionally grouping into BoHV-1.2a and BoHV-1.2b.

In Cuba, studying BoHV-1 and other cattle pathogens has been limited by economic problems. Nowadays, the interest in cattle production is increasing, and gaining profound knowledge on the epidemiological status of the BoHV-1 infection is getting importance. This implies the characterization of BoHV-1 strains that are circulating in the country. The purpose of the present study was to compare a BoHV-1 isolate from Cuba with BoHV-1 strains from other different countries using a clustering system based on restriction enzyme analysis.

Madin-Darby bovine kidney cells (MDBK; ATCC CCL 22) were grown in Eagle's minimal essential medium (Eagle's MEM, BioConcept's *AMIMED*, Switzerland) supplemented with 100 IU mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and 7% foetal calf serum (FCS, *Omnilab*, Switzerland). In the maintenance medium, FCS was reduced to 2%. The cells were used for virus stocks production of the Cuban BoHV-1 strain E8, isolated in 1984 during an outbreak of keratoconjunctivitis (5) and BoHV-1 reference strains previously characterized by Wyss (6) (Table 1).

Virus purification and DNA extraction were conducted as previously described by Engels *et al* (7). DNA concentrations were determined by reading of absorbance at 260 nm (*NanoDrop Technologies, Inc., Witec* NanoDrop® ND-1000 Spectrophotometer®). The absorbance ratio 260/280 nm was always between 1.93 and 2.

Aliquots containing 2 mg DNA were digested for 2 hr with *Hin*dIII, *Hpa*I, *Pst*I (*Roche*) and *Sfi*I (*BioLabs*), respectively, under conditions recommended by the supplier in a final volume of 20 mL. The digestion products were separated by electrophoresis in 0.8% agarose gels containing 1 mg mL^{-1} ethidium bromide solution (*BioRad*). Gels were electrophoresed in TAE electrophoresis buffer (4 mM Tris-acetate, 1 mM EDTA; pH 8.0) between 18 and 24 hr at 60 V. For determinating the DNA fragment sizes, 1 Kb DNA Ladder (*Gibco*) with a size range of 500 bp to 12 kb and high molecular weight DNA markers (*Invitrogen*) with a size range of 9 kb to 48 kb were included. The bands were visualized and photographed using Quantity One® (*Bio-Rad Laboratories, BioRad*) and the molecular characterization was based on the clustering system by Wyss (6).

Comparative analysis of the resulting restriction enzyme patterns revealed that E8 *Hin*dIII pattern was more similar to one Canadian strain (BoHV-1 V214) and two European strains (BoHV-1 599/97 and BoHV-1 Cu7) (see Fig. 1A). As all those strains belonged to the BoHV-1.1 genotype, it was concluded that E8 belonged to the same genotype. Furthermore, isolate E8 was identified as BoHV-1.1.III strain, based on *Hin*dIII fragments E and F which appeared merging,

TABLE 1. BoHV-1 reference strains./ *Cepas de referencia de BoHV-1*

Designation	Year	Country of origin	Subtype	Numeric code			
				***Hin*dIII**	***Hpa*I**	***Pst*I**	***Sfi*I**
Cu7	1977	Belgium	1.1.III	931	421	125123	431231
V214	1993	Canada	1.1.I	931	421	125124	431221
VI 273/97	1997	Germany	1.1.IIb	931	421	122224	421221
7624/97	1997	Germany	1.1.IIa	921	421	122224	421221
9389/97	1997	Germany	1.2.a.III	931	421	122124	421221
VI 599/97	1997	Germany	1.1.II	931	421	023224	531221
Roma	1963	Italy	1.1.IIIc	921	4	112xxx	xxxxxx
Jura	1978	Switzerland	1.1.I	931	421	122124	431221
Miggi	1982	Switzerland	1.2a.IV	931	4	113125	331221
94-12508 (USA 3)	-	U.S.A	1.1.I	931	322	124124	421221
85-24083 (USA 11)	1985	U.S.A	1.1.Ib	931	421	023224	630231
Bovilis®	-	Vaccine	1.2a.I	931	421	121124	431231
Riemser®	-	Vaccine	1.1.Ia	931	4	132114	321242

- Not reported
x No results

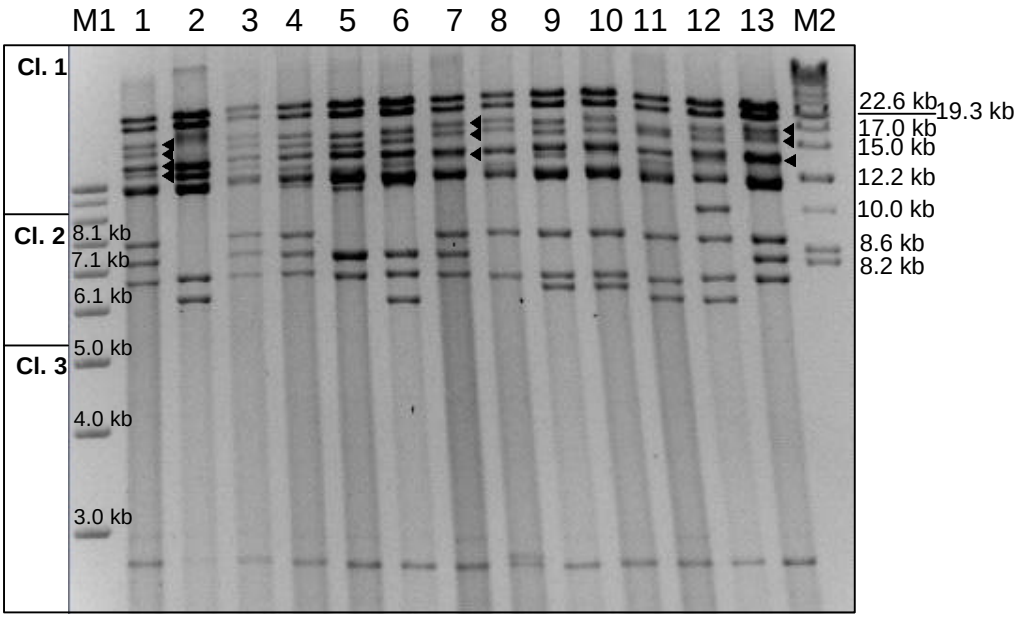

FIGURE 1A. Restriction enzyme analysis of DNA from BoHV-1 strains with *Hin*dIII. **1**: V214 strain; **2**: Vaccine strain Riemser; **3**: USA 11 strain; **4**: VI 599/97 strain; **5**: 7624/97 strain; **6**: VI 273/97 strain; **7**: Cu7 strain; **8**: Roma strain; **9**: Vaccine strain Bovilis; **10**: 9389/97 strain; **11**: Miggi strain; **12**: Sm2 strain; **13**: E8 strain; **M1**: 1 kb molecular weight marker; **M2**: High Molecular Weight DNA Markers. **Cl.** Cluster. Arrows indicate the differences between BoHV-1 strains./ *Análisis con enzimas de restricción del ADN de cepas de BoHV-1 con HindIII. **1**: Cepa V214; **2**: Cepa vacunal Riemser; **3**: Cepa USA 11; **4**: Cepa VI 599/97; **5**: Cepa 7624/97; **6**: Cepa VI 273/97; **7**: Cepa Cu7; **8**: Cepa Roma; **9**: Cepa vacunal Bovilis; **10**: Cepa 9389/97; **11**: Cepa Miggi; **12**: Cepa Sm2; **13**: Cepa E8; **M1**: Marcador de peso molecular de 1 kb; **M2**: Marcador de ADN de alto peso molecular. **Cl.** Grupo. Las flechas indican lãs diferencias entre cepas de BoHV-1.*

and fragments C and D which showed higher molecular weight than the corresponding fragments of the other BoHV-1 strains.

Seven bands were obtained by E8 digestion with *Hpa*I (see Fig. 1B). These results corresponded to the numeric code 421 based on the number of bands observed in clusters 1, 2 and 3, respectively.

E8 *Pst*I pattern corresponded to the numeric code 125124. This code was also obtained from BoHV-1 V214, but differences in the molecular weight of the band 4 of cluster 6 were found (see Fig. 1C). Five fragments from E8 viral DNA were observed in cluster 3 and these were not observed from the American strain USA3 with 2 and 4 bands, respectively. Furthermore, bands 3, 4 and 5 of cluster 3 were observed at similar intensity for E8, and differences were found in BoHV-1 strains V214 and Cu7.

As it can be seen in Fig. 1D, the numeric code 421221 can be assigned to E8 *Sfi*I pattern. It was more similar to the BoHV-1 strains V214 but only two bands in cluster 2 were obtained.

Restriction fragment pattern analysis has mostly been used for classification purposes knowing that the highest differences between BoHV-1.1 and BoHV-1.2 are restricted to distinct genomic regions characterized by loss or gain of restriction sites (8). It has also been used to differentiate between vaccine and field strains of BoHV-1 according to Hamelin *et al* (9). Moreover, according to systematic sequencing of individual isolates will be difficult to perform due to the high G + C content of BoHV-1 genomes (1), it is recommended the adoption of a clustering system based on restriction endonuclease analysis with enzymes *Hin*dIII, *Hpa*I, *Pst*I and *Sfi*I in order to identifying the possible origins of BoHV-1 strains (6). This methodology was previously used to the identification of BoHV-1 strain origin, which had been isolated from imported semen and which had led to a new outbreak in 1983 in Switzerland (10).

The clustering data base includes about 100 different BoHV-1 strains previously analyzed by Wyss (6); in there, BoHV-1.1.III subtype corresponds with the 16% of BoHV-1.1 strains. It is important to note

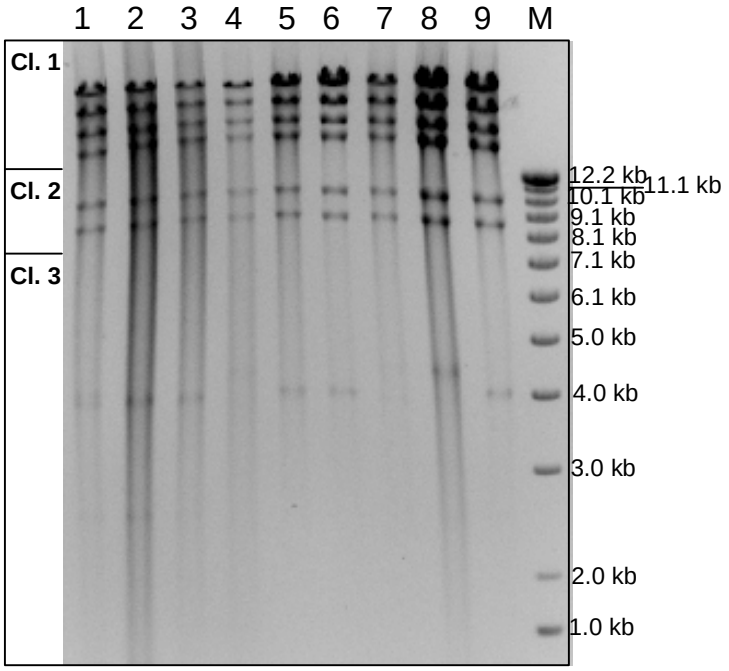

FIGURE 1B. Restriction enzyme analysis of DNA from BoHV-1 strains with *Hpa*I. **1**: V214 strain; **2**: USA 11 strain; **3**: VI 599/97 strain; **4**: 7624/97 strain; **5**: VI 273/97 strain; **6**: Cu7 strain; **7**: Vaccine strain Bovilis; **8**: 9389/97 strain; **9**: E8 strain; **M**: 1 kb molecular weight marker. **Cl.** Cluster./ *Análisis con enzimas de restricción del ADN de cepas de BoHV-1 con HpaI. **1**: Cepa V214; **2**: Cepa USA 11; **3**: Cepa VI 599/97; **4**: Cepa 7624/97; **5**: Cepa VI 273/97; **6**: Cepa Cu7; **7**: Cepa vacunal Bovilis; **8**: Cepa 9389/97; **9**: Cepa E8; **M**: Marcador de peso molecular de 1 kb. **Cl.** Grupo.*

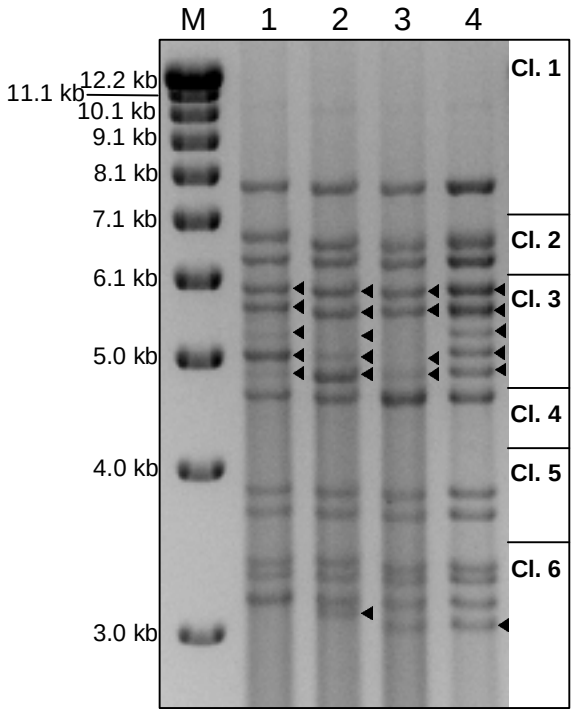

FIGURE 1C. Restriction enzyme analysis of DNA from BoHV-1 strains with *Pst*I. **1**: Cu7 strain; **2**: V214 strain; **3**: USA3 strain; **4**: E8 strain; **M**: 1 kb molecular weight marker. **Cl.** Cluster. Arrows indicate the differences between BoHV-1 strains./ *Análisis con enzimas de restricción del ADN de cepas de BoHV-1 con PstI. **1**: Cepa Cu7; **2**: Cepa V214; **3**: Cepa USA3; **4**: Cepa E8; **M**: Marcador de peso molecular de 1 kb. **Cl.** Grupo. Las flechas indican las diferencias entre cepas de BoHV-1.*

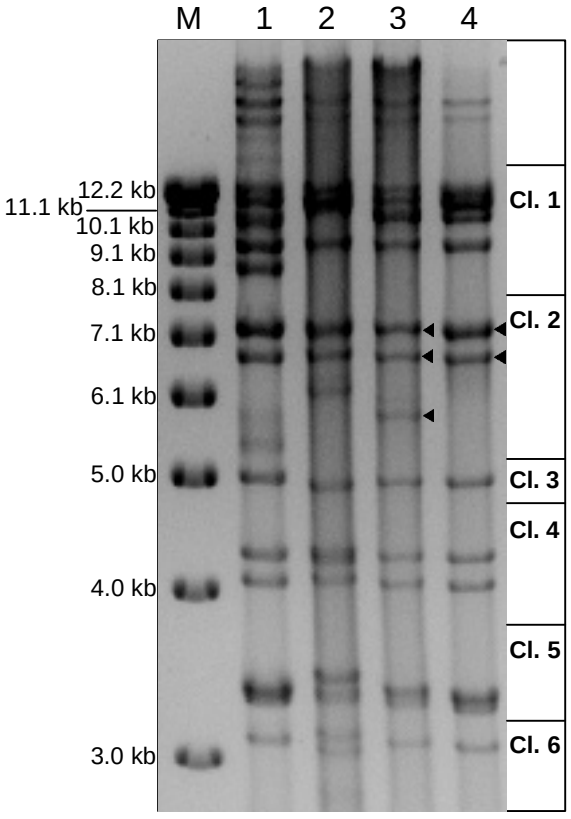

FIGURE 1D. Restriction enzyme analysis of DNA from BoHV-1 strains with *Sfi*I. **1**: VI 273/97 strain; **2**: Miggi strain; **3**: V214 strain; **4**: E8 strain; **M**: 1 kb molecular weight marker. **Cl.** Cluster. Arrows indicate the differences between BoHV-1 strains./ *Análisis con enzimas de restricción del ADN de cepas de BoHV-1 con SfiI. **1**: Cepa VI 273/97; **2**: Cepa Miggi; **3**: Cepa V214; **4**: Cepa E8; **M**: Marcador de peso molecular de 1 kb. **Cl.** Grupo. Las flechas indican las diferencias entre cepas de BoHV-1.*

that all strains from Canada and U.S.A were 1.1.I subtype while 1.1.III subtype was only found in Europe. The highest number of strains with the same restriction fragment pattern (71%) was observed by digestion with *Hpa*I. This can be explained by the small number of cleavage sites produced by this enzyme, which has been used for identification of the four major genotypes of BoHV-1 (11).

The main differences between E8 and the other bovine herpesviruses analyzed were found in the restriction fragment patterns obtained by digestion with *Pst*I and *Sfi*I. This is due to the high number of restriction sites for these enzymes that can be found in the genome of BoHV-1. The number of bands obtained in cluster 3 of E8 strain by digestion with *Pst*I is not common and it was only found in strains from Canada, U.S.A, Holland and Belgium according to the table of classification performed by Wyss (2001). Taking into account the results obtained by Whetstone *et al* (12), it is possible to predict that the differences in E8 *Pst*I pattern are associated with changes in the inverted repeat regions. These changes were observed in restriction endonuclease *Pst*I digestion patterns of BHV-1.1 and BHV-1.2 isolates after one passage in host animal. Changes were also observed when virus was reactivated from latency or after superinfection with another strain of BHV-1 (13).

It was not possible to find a reference strain with the same four restriction fragment patterns as the BoHV-1 E8. The largest number of similar strains was originated from the European continent, but it has to be considered that comparison was made with strains mainly from this region. Considering that the animals and semen have been imported to the country only from Canada or USA, it is recommended to increase the number of entries from de American continent into the clustering data base. E8 DNA fragment pattern introduction in this database is a contribution for future studies in tracing back the origin of novel IBR outbreaks and is also a contribution to the updating of the Cuban surveillance epidemiological program but it is recommended to continue this study with other Cuban strains.

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