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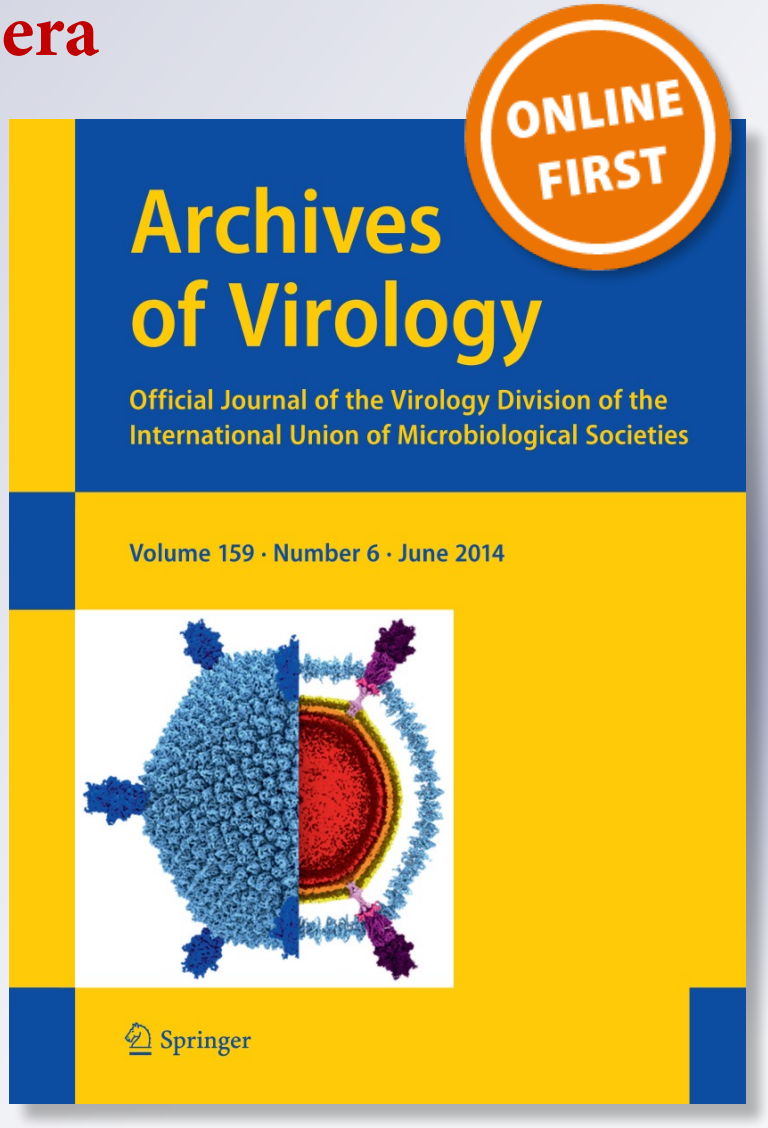
Archives of Virology
Official Journal of the Virology Division of the International Union of Microbiological Societies

ISSN 0304-8608

Arch Virol
DOI 10.1007/s00705-014-2146-8



Springer

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ORIGINAL ARTICLE

First report of isolation and molecular characterization of bubaline herpesvirus 1 (BuHV1) from Argentinean water buffaloes

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Received: 16 December 2013 / Accepted: 5 June 2014

Abstract Herpesviruses have mainly co-evolved with their hosts for millions of years. However, bovine herpesvirus 1 (BoHV1) and related ruminant alphaherpesviruses have been reported to cross the species barrier. Bubaline herpesvirus 1 (BuHV1) is an alphaherpesvirus closely related to BoHV1 and BoHV5. According to the serological cross-relationships between ruminant alphaherpesviruses, several surveys have studied the occurrence of BoHV1-related virus infection in wild and domestic ruminant species. Recent studies in Argentina showed an increase in serological prevalence against BoHV1 related viruses in water buffaloes (*Bubalus bubalis*) population. The aim of this study was to investigate the presence of related ruminant alphaherpesvirus in the Argentinean water buffalo population. BuHV1 was successfully isolated from 5 out of 225 buffaloes analyzed. One isolate was obtained from nasal secretions, and the others were from vaginal swabs. The buffaloes belonged to four different farms located in northeastern Argentina. The isolates were characterized as alphaherpesvirus by direct immunofluorescence using FITC-anti-BoHV1 IgG. Restriction analysis performed with *Bam*HI and *BstE*II on the complete genome showed differences between the isolates and those from BoHV1 and BoHV5 subtypes. Phylogenetic analysis on both UL27 and US6 showed similarity in tree topology. While three of the isolates grouped together with sequences of BoHV5, two other isolates clustered separately. Genetic analysis of eight concatenated sequences from all isolates and references strains showed high nucleotide sequence

S. S. Maidana (✉) · M. I. Craig · O. Zabal · S. A. Romera
Instituto de Virología, Centro de Investigaciones en Ciencias veterinarias y Agronómicas (CICVyA), Instituto de tecnología Agropecuaria (INTA), N. Repetto y Los Reseros S/N, CC25, Castelar, B1712WAA Buenos Aires, Argentina
e-mail: maidana.silvina@inta.gob.ar

M. I. Craig
e-mail: bcraig@cnia.inta.gov.ar

O. Zabal
e-mail: ozaval@cnia.inta.gov.ar

S. A. Romera
e-mail: aromera@cnia.inta.gov.ar

S. S. Maidana · M. I. Craig · S. A. Romera
Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Rivadavia 1917, C1033AAJ Ciudad Autónoma de Buenos Aires, Argentina

S. S. Maidana · S. A. Romera
Cátedra de Inmunogenética, Facultad de Ciencias exactas, Químicas y Naturales, Universidad de Morón, Cabildo 134, Morón, B1708JPD Buenos Aires, Argentina

J. L. Konrad · G. Crudeli
Cátedra de Teriogenologia, Facultad de Veterinaria, Universidad Nacional del Nordeste, 25 de Mayo 868, 3400 Corrientes, Argentina
e-mail: konradjl@hotmail.com

G. Crudeli
e-mail: gacrudelli@hotmail.com

A. Mauroy · E. Thiry
Veterinary Virology and Animal Viral Diseases, Fundamental and Applied Research on Animal Health center and Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster, 20, B43b, 4000 Liège, Belgium
e-mail: amauroy@ulg.ac.be

E. Thiry
e-mail: etienne.thiry@ulg.ac.be

S. A. Romera
Cátedra de Inmunología, Universidad del Salvador, Champagnat 1599-Ruta Panamericana-Km 54.5, B1630AHU Pilar, Provincia de Buenos Aires, Argentina

Published online: 18 June 2014

identity between BuHV1 and BoHV5. While three of the isolates clustered together with the BoHV5 reference strain, the last two isolates were closely related to an Australian BuHV1 strain. To our knowledge, this is the first report on the isolation and molecular characterization of BuHV1 in South America. Phylogenetic analysis suggested that two different BuHV1 lineages circulate in the Argentinean water buffalo population.

Introduction

Bubaline herpesvirus 1 (BuHV1) belongs to the cluster of ruminant alphaherpesviruses related to bovine herpesvirus 1 (BoHV1) [1–3]. In nature, herpesviruses are mostly associated with a single host species. However, BoHV1 and related ruminant alphaherpesviruses have been reported to cross the species barrier [2]. BoHV1 is responsible for a wide range of clinical syndromes in cattle, such as rhinotracheitis, abortion, pustular vulvovaginitis and encephalitis [4]. In contrast, BuHV1 has only been associated with subclinical disease in water buffalo (*Bubalus bubalis*) [2, 5, 6]. Recently, Amoroso et al. [7] detected BuHV1 viral DNA in an aborted fetus by PCR. Water buffalo have already been found to be seropositive for both BuHV1 and BoHV1 in different countries [7–10], while virus isolation has only been described in Australia [5] from the prepuce of buffalo, and many years later from water buffalo in southern Italy [9].

Water buffalo breeding represents an important economic alternative to cattle in Argentina. These animals are better adapted to wet tropical and subtropical environments than bovines. On the other hand, the main agricultural region in the center of Argentina has been devoted to soy bean production. This fact has resulted in important changes in the distribution of cattle, the intensification of cattle load in fields of lesser agricultural quality, and the appearance of mixed (buffaloes and cattle) production systems. Approximately 100,000 water buffaloes are raised under intensive conditions in wetlands of northeastern Argentina [11, 12] with a relative BuHV1 seroprevalence of 33 % [13]. Considering these data, and with the aim to gain better knowledge about BuHV1 infection, the study of herpesvirus circulation in buffalo herds becomes relevant.

Materials and methods

Samples, cells and viruses

Nasal and vaginal swabs were obtained from 225 water buffaloes from different farms in the Argentinean provinces of Buenos Aires, Chaco and Corrientes. Vaginal and nasal swabs of each buffalo were collected in minimal essential medium (MEM) containing an antibiotic-antimycotic solution (200 U/ml penicillin, 200 ig/ml streptomycin and 250 ng/ml amphotericin B; Gibco-Invitrogen). Undiluted samples in MEM were then used directly to inoculate monolayers of Madin-Darby bovine kidney (MDBK) cell cultures in 96-well plates. Cells were examined daily under an optical microscope to observe the appearance of cytopathic effects. The B6 strain of BuHV1 from Australia [4] was used as reference.

Immunofluorescence staining and electron microscopy

The isolates were characterized by direct immunofluorescence assay. Briefly, virus isolates were inoculated onto confluent MDBK cells in 8-well chambers and maintained in medium with carboxymethyl cellulose (CMC), supplemented with 1.5 % FBS at 37 °C in order to obtain viral plaques. After the cells were fixed, monolayers were incubated with FITC-anti-BoHV1 IgG (VMRD, WA, USA). Finally, the slides were buffered with glycerin and observed with an epifluorescence microscope (Olympus BX 40 + H hal.).

The morphology of the particles was observed with a JEOL 1200 EX II transmission electron microscope [14]. Briefly, 25 µl of viral suspension ($10^{7.5}$ $DICT_{50}$/ml) was placed on a collodion membrane supported on a copper grid and negatively stained with phosphotungstic acid before examination.

Viral DNA extraction and restriction enzyme analysis

MDBK cells were infected and observed until they reached complete CPE. After two successive rounds of freezing and thawing, supernatants were clarified at 3000 rpm for 20 min at 4 °C. Purification and DNA extraction steps were performed as detailed by Maidana et al. [15]. DNA was digested with *BstE*II and *BamH*I restriction enzymes (Promega, Wisconsin, USA) in a final 20-µl reaction volume. The digestion products were separated by 0.7 % agarose gel electrophoresis at 60 V for 6 hours, stained with ethidium bromide and observed under UV light.

Sequencing and phylogenetic analysis

Eight different sets of primers, spread along the genome, were used in this study. They amplified regions located in the unique long (UL) region (UL41, UL40, UL29, UL28, UL27 and UL22) [15, 16] and the short (US) region (US6 and US8) as described previously [17] (Table 1).

Nucleotide sequences of the isolates were edited and analyzed with BioEdit version 7.0.5.3 [19]. Alignments

Table 1 Oligonucleotides used in the PCRs assays for BuHV1 characterization

Genome region target	Sequence 5′ 3′	Size (bp) of PCR products	Reference
UL41	(d-UL41F) GGGGCTCTTCAAGCT(A/G)CTG	236	Del Médico Zajac et al. [16]
	(d-UL41R) AAGATCGGGTAGTAGGGAGCG		
UL40	(d-UL40F) GCCAAACAGAGGAGGCAG	351	
	(d-UL40R) CATGATTCTATTCGAGGGC		
UL29	(d-UL29F) GGGCTTCGTGTACGTCTG	224	
	(d-UL29R) CAGCCACTACCACCCGAA		
UL28	(d-UL28F1) CGCAACACGTCTTCCGCCC	258	
	(d-UL28R1) CAGTCCACGCCGTCCAGC		
UL22	(d-UL22F) GACTACGACCTGGTGCCG	348	
	(d-UL22R) AGACGCTCAGGAAGCCGA		
UL27	(CR39) CACGACCTGGGCGGGCAGCAC	702	De Carlo et al. [9]
	(CR40) CTGCAACGCGAAGGTGTGGCTGTC		
US6	(CR52) CCCGMYGCCGCGATACAACTAC	615	De Carlo et al. [9]
	(CR54) CTTGTGTGCCTCCTGCGGGTA		
US8	(914F) CGARACSTGCATCTTYCACC	624	Thiry et al. [18]
	(1538R) GGSTCGTTGSTYGGM		

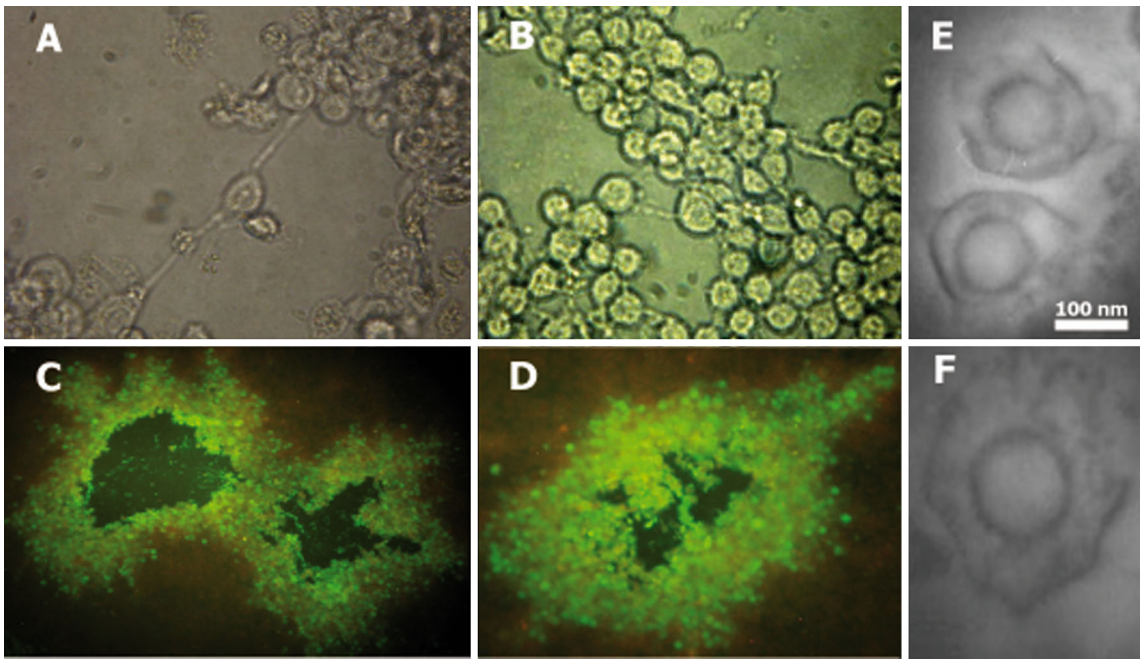

Fig. 1 Biological and morphological properties of the BuHV1 isolates. A and B: Cytopathic effects in MDBK cells by one (20287N) of the BuHV1 isolates (40X magnification). C and D: Viral plaques on MDBK cells stained for IF and monitored for epifluorescence with an Olympus BX 40 + H hal microscope (10X magnification). E and F: Electron micrographs of three purified virions of the 20287N and the 84250V isolates, which were morphologically indistinguishable from ruminant herpesviruses

were performed with Clustal W. MEGA 5.0 software [20], and phylogenetic relationships were inferred by the maximum-likelihood method, using 1000 bootstrap replicates.

The GenBank accession numbers for the reference strains are as follows: BoHV1 (UL41, UL40, UL29, UL28, UL27, UL22, US6 and US8: NC_001847.1); BuHV1 strain B6 (UL27: EF624476.1; US8: EF624469.1), BuHV1 Italian strain (UL27: AJ496607.1; US6: AJ496608.1), BoHV5 strain SV507/99 (UL41, UL40, UL29, UL28, UL27, UL22, US6 and US8:: NC_005261.2), CpHV1 strain Ba-1 (UL27: EF624477.1; US6: DQ139344.1 and US8: EF624470.1),

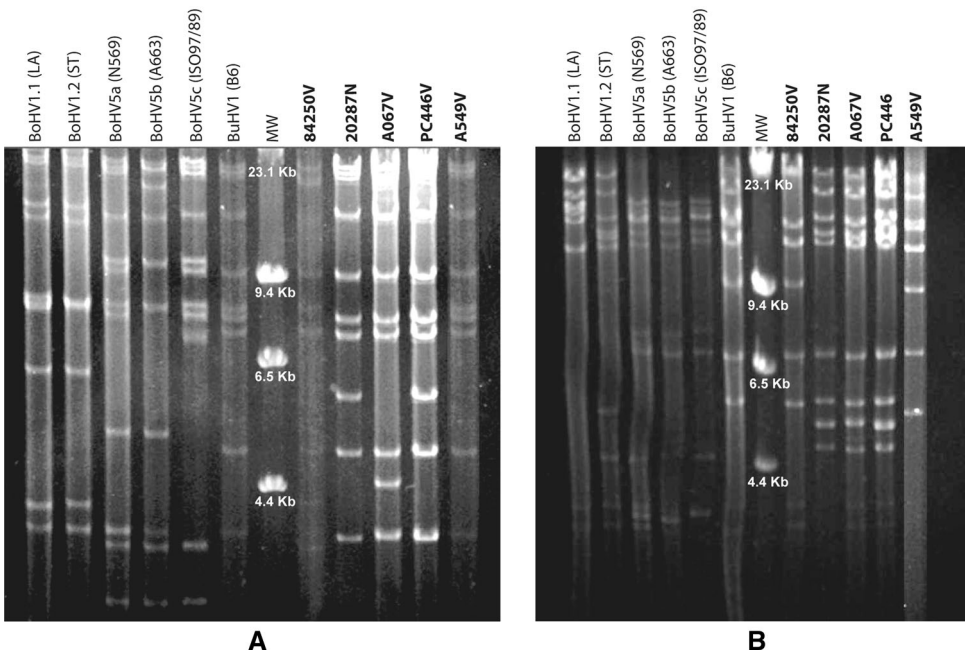

Fig. 2 Restriction profiles of the bubaline herpesvirus isolates from Argentina and the BuHV1 (B6), BoHV1 and BoHV5 subtypes, with the *BstE*II (a) and *BamH*I (b) enzymes

CvHV1 strain Banffshire 82 (UL27: AF078729.2 and US6: AF078735.1) and CvHV2 strain Salla 82 (UL27: AF078727 and US6: AF078733.1), SuHV1 (UL41, UL40, UL29, UL28, UL27, UL22, US6 and US8: NC_006151.1).

Fig. 3 Phylogenetic analysis based on (a) UL27 and (b) US6 partial ▶ nucleotide sequences and (c) concatenated alignments of eight gene fragments (UL41 = 179, UL40 = 298, UL29 214, UL28 = 288, UL27 = 581, UL22 = 273, US6 = 484 and US8 = 223 nt). Phylogenetic analysis was performed using the MEGA 5 software with 1,000 bootstrap replicates. The phylogenetic analysis also included sequences of UL27 and US6 gene fragments from other Argentinean alphaherpesviruses. Argentinean bubaline isolates are indicated in bold and underlined. GenBank accession numbers are presented in Materials and methods

Results

Virus isolation and identification

Five of the 225 samples (2028N, PC446V, A549V, A067V and 84250V) showed visible cytopathic effects (CPE) at 24 h post-infection (pi) (Fig. 1A and B). These isolates were from four different farms in northeastern Argentina. One of the isolates was obtained from nasal secretions, and the others were from vaginal swabs. Some of these buffaloes were in the first stage of pregnancy or at postpartum stage; however, none of them had presented with clinical signs.

Infection of MDBK cells with all of these isolates was positive by direct immunofluorescence assay (Fig. 1C and D). The spherical morphology and size, approximately 100–300 nm, were indistinguishable from those reported for herpesviruses (Fig. 1E and F).

Comparative restriction analysis

Extracted DNA from the isolates and from different reference strains, including BoHV1, BoHV5 and BuHV1, were used in a comparative analysis of the complete genome (Fig. 2). The *BstE*II restriction profile showed differences between the isolates BoHV1 and BoHV5 (Fig. 2a). The A549V isolate exhibited an identical pattern to BuHV1 reference strain, while 20287N and PC446V showed profiles that were highly similar to each other. The A067V and 84250V isolates gave a dissimilar pattern to each other and to BuHV1, BoHV1 and BoHV5. According to the *BamH*I restriction profile, all of the isolates were different from BoHV1 and BoHV5 (Fig. 2b). However, while 84250V and A549V showed a pattern similar to that of the BuHV1 reference strain, the others showed similarity between them.

Phylogenetic analysis

Eight gene fragments were amplified by PCR. Phylogenetic analysis of both the UL27 and US6 sequences showed that three isolates (20287N, A067V and PC446V) and the Italian BuHV-1 reference strain clustered together. The other two isolates, 84250V and A459V, grouped together in a separate cluster with an Australian BuHV-1 strain (Fig. 3a and b). Simultaneously, all of these sequences

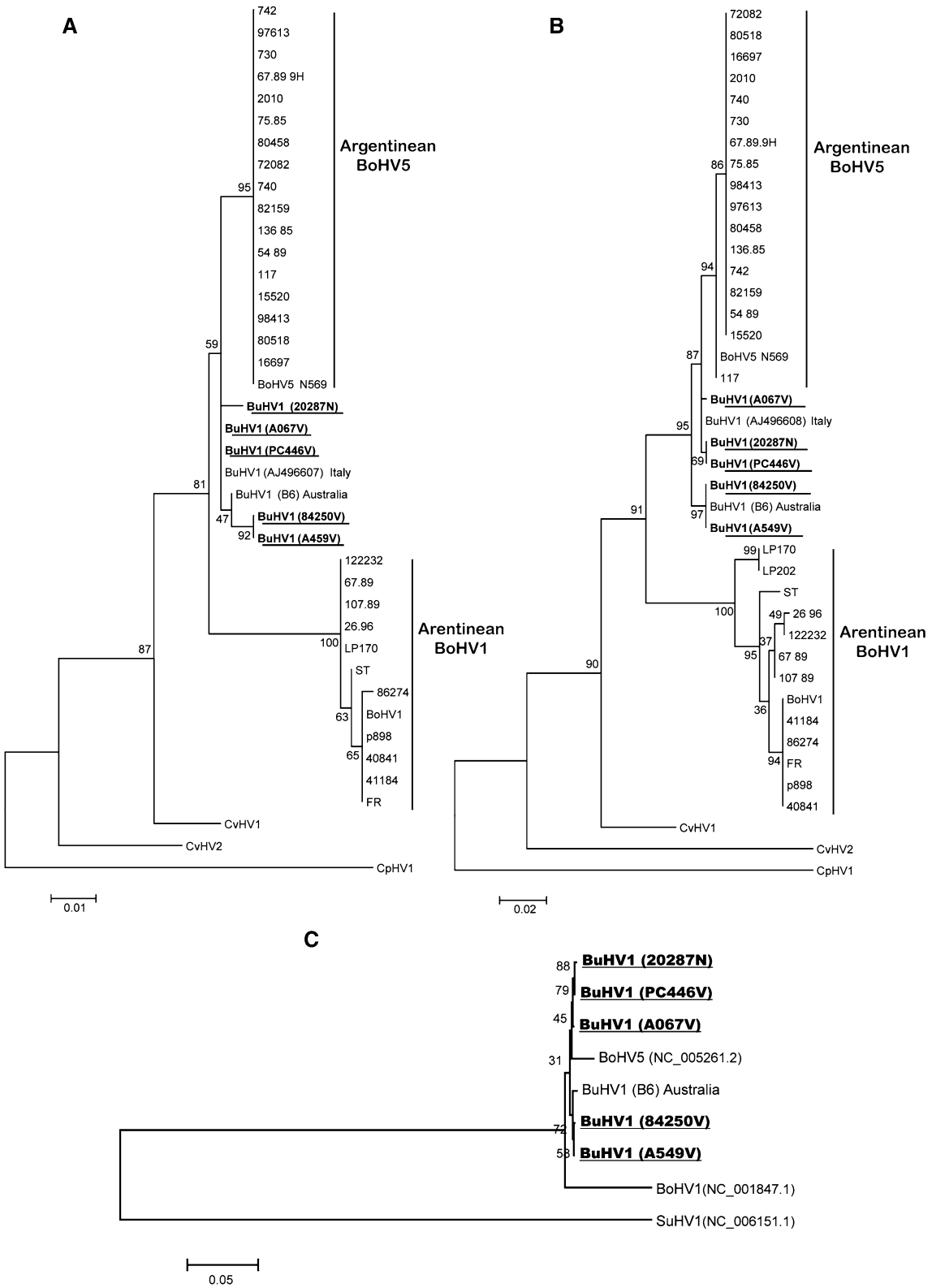

Table 2 Comparison of identity percentages in concatenated nucleotide sequences of eight gene fragments (UL41 = 179, UL40 = 298, UL29 = 214, UL28 = 288, UL27 = 581, UL22 = 273, US6 = 484 and US8 = 223 nt) of BuHV1 isolates and related alphaherpesviruses

Isolates	Ref Strains			
	BoHV1	BoHV5	BuHV1	SuHV1
20287N	93	97.3	99.1	53.4
84250V	93.1	97.1	99.6	53.4
A067V	93.1	97.5	99.2	53.4
A549V	93.1	97.2	99.2	53.4
PC446V	93.1	97.4	99.1	53.3

appeared to be more closely related to BoHV5 than to BoHV1.

The phylogenetic analysis of the eight concatenated sequences (UL41,UL40, UL29, UL28, UL27, UL22, US6 and US8) of five isolates together with the reference strains showed the same relationships that were obtained by the analysis of UL27 and US6 separately (Fig. 3c). Also, the analysis of nucleotide sequence identity confirmed that the sequences were more closely related to BuHV1 and BoHV5 than to BoHV1 (Table 2).

Discussion

In the present study, the isolation and molecular characterization of BuHV1 from water buffaloes were reported. These viruses were isolated from nasal and vaginal swabs from five of 225 animals sampled from different farms in northeastern Argentina. None of these buffaloes had manifested clinical signs. This observation supports previous studies in which other authors detected the presence of this virus in asymptomatic buffaloes [9]. However, Amoroso et al. [7] found an association between BuHV1 and abortions.

Not only were all of our isolates positive by IF assay, but specific sera against BoHV1 and BoHV5 were also able to neutralize them *in vitro* with different ranges of activity (data not shown).

The REA assay showed clear differences at the molecular level between our isolates, BoHV1 and BoHV5. In accordance with this, De Carlo et al. [9] demonstrated the presence of cross-reactivity between BoHV5 and BuHV1 in neutralization assays.

We were able to characterize these isolates at the molecular level by partial amplification and subsequent sequencing of eight different regions from their genome. Six of the gene fragments were located in the unique long region (UL), and the other two were in the unique short region (US) of the genome. Although the sequences of the fragments obtained from the isolates showed similarity between them and to other BuHV1 sequences previously reported from Australia [5, 17] and Italy [9], they were not identical. This result is in accordance with the hypothesis of different ways of introduction of water buffaloes into Argentina. Water buffaloes are not native to Latin America, and, to our knowledge, the first introduction happened around the 1900s, directly from Asia, passing through Brazil [11]. At the same time, Australia also imported water buffaloes from Asia. This event could have led to the existence of two isolates that were closely related to the Australian strain. The second introduction event occurred directly from Italy during the seventies [11], allowing the appearance of isolates genetically related to the Italian strain (AJ496607.1, AJ496608.1).

Phylogenetic analysis based on alignments of the nucleotide sequences of the UL27 and US6 genes and with eight concatenated genes demonstrated that the isolates clustered closely with the BoHV5 group. Phylogenetic analysis of the eight concatenated nucleotide sequences showed a tree similar to those obtained from the separate gene sequences. We studied various genomic regions that were different from those commonly used for phylogenetic characterization of field isolates because it allowed us to confirm their identity and monitor potential recombination events in BuHV1 isolates.

The phylogenetic position of the Argentinean buffalo isolates might be correlated with the antigenic cross-reactivity observed by cross-neutralization studies with anti-BoHV1 and anti-BoHV5 sera. The high degree of genetic homology between BuHV1 and BoHV5 has been reported previously by others [7, 9, 17]. In this way, the analysis of a larger number of BuHV1 isolates and sequences could address the hypothesis of a common ancestor for BuHV1 and BoHV5 and their recent adaptation during evolution to their respective hosts, buffaloes and cattle, respectively.

Most of our isolates were obtained from vaginal secretions. This supports the hypothesis of a preferential genital tropism for BuHV1, like many other ruminant herpesviruses (BoHV1; CpHV1, CpHV2 and CvHV1), which thereafter evolved to gain respiratory tropism through more intensive farming conditions (BoHV1, CvHV1 and CpHV1) [2, 21]. In general, genital transmission can be considered less effective for interspecies transmission of viruses. This could be the reason why BuHV1 has not yet been isolated from cattle – or BoHV1 from buffaloes – although the susceptibility of buffaloes to BoHV1 has been demonstrated experimentally [6]. In addition, a more controlled experimental study on BuHV1 transmission from buffalo to cattle should be performed to elucidate the epidemiological role of buffalo in cattle and buffalo populations. To our knowledge, this is the first report of BuHV1 isolation in South America. Our results extend the geographical distribution of BuHV1 from Australia and Italy to the South American continent. Although

phylogenetic analysis showed that BuHV1 is more closely related to BoHV5 than to BoHV1, further studies are necessary to support the hypothesis of a common ancestor.

Acknowledgments The authors wish to thank Mónica Florin (INTA) and Matías Ottaviani (INTA) for help in drafting the manuscript and figures. This work was supported by Fonds de la Recherche Scientifique (FRS-FNRS) and CONICET. SM is a fellow of the CONICET (Argentina).

Conflict of interest The authors declare that they have no conflict of interest.

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