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Phylogenetic analysis of bluetongue virus serotype 4 field isolates from Argentina

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Bluetongue is an insect-transmitted viral disease of ruminant species, which represents a major barrier to the international trade of animals and their products. Bluetongue virus (BTV) has a genome composed of ten linear segments of dsRNA, which code for at least ten different viral proteins. In South America, serological evidence for the presence of BTV has been found in Peru, Argentina, Brazil, Ecuador and Chile. Brazil and Argentina are the only South American countries where BTV has been isolated. In Brazil, only one BTV isolate, serotype 12, has been reported, whereas in Argentina five BTV serotype 4 isolates have been obtained from cattle without clinical signs. Three of these five isolates were isolated during 1999–2001, whereas two of them were obtained as part of the present work. This study describes sequence comparisons and phylogenetic analyses of segment (Seg)-2, Seg-3, Seg-6, Seg-7 and Seg-10 of the first Argentinian field isolates of BTV. The analysis of Seg-2 and Seg-6 resulted in a single cluster of Argentinian sequences into the serotype 4 clade. In addition, the Argentinian sequences grouped within the nucleotype A clade, along with reference strains. The analysis of Seg-3, Seg-7 and Seg-10 showed that the Argentinian isolates grouped into the western topotype, indicating that the circulating virus had an African/European origin. Phylogenetic analysis revealed that the Argentinian sequences present a South American genetic identity, suggesting an independent lineage evolution.

INTRODUCTION

Bluetongue virus (BTV) is the prototype member of the genus *Orbivirus* within the family *Reoviridae* (Fauquet *et al.*, 2005). The disease affects domestic and wild ruminants, and although severe forms of the disease are most frequent in sheep and white-tailed deer, clinical signs can also occur in cattle, goats and camelids (Henrich *et al.*, 2007; Koumbati *et al.*, 1999; Parsonson, 1990). Although infection is often unapparent in these other species, they can act as reservoirs, remaining viraemic for several months (particularly cattle) (MacLachlan, 1994; Maclachlan *et al.*, 2009).

Bluetongue is listed as a 'notifiable disease' by the Office International des Epizooties (OIE) (OIE, 2008). The sanitary and economic impact of BTV infection involves loss of condition, reduced milk yield, infertility and abortion, as well as indirect costs associated with international trade restrictions and the surveillance requirements imposed to limit the spread of the virus.

BTV infection of ruminants is not contagious; the virus is transmitted among hosts almost entirely by the bites of certain species of *Culicoides* biting midges. Thus, the distribution of BTV is restricted to areas where competent vector species are present, and transmission is limited to those times of the year when climatic conditions are appropriate and adult insects are active (Mellor & Boorman, 1995; Meiswinkel *et al.*, 2004; Mullens *et al.*, 2004).

The virus genome is composed of ten dsRNA segments (Verwoerd *et al.*, 1970). Seven of the viral proteins (VP1–VP7) are structural and form the double-shelled virus particle. At least four non-structural proteins (NS1, NS2 and NS3, NS3A and NS4) have been identified (Mertens *et al.*, 1984; Ratinier *et al.*, 2011; Roy *et al.*, 1990).

The internal core is formed by two layers containing VP1, VP3, VP4, VP6 and VP7 [encoded by genome segment (Seg)-1, Seg-3, Seg-4, Seg-9 and Seg-7, respectively]. These core proteins and three of the non-structural proteins (NS1, NS2 and NS4: encoded by Seg-5, Seg-8 and Seg-9, respectively) are thought to be relatively conserved and are antigenically cross-reactive between different strains and serotypes of BTV (Ratinier *et al.*, 2011; Roy *et al.*, 1990).

The outer capsid is composed of two major viral proteins, VP2 and VP5, encoded by Seg-2 and Seg-6, respectively, which determine the antigenic variability of BTV (Huismans *et al.*, 1987; Verwoerd *et al.*, 1972). Seg-2 and Seg-6 show the highest levels of sequence variation in the BTV genome

The GenBank/EMBL/DDBJ accession numbers for the Seg-2, Seg-3, Seg-6, Seg-7 and Seg-10 sequences of Argentinian BTV isolates are: JX024940–JX024964.

Two supplementary tables are available with the online version of this paper.

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(Maan *et al.*, 2007; Mertens *et al.*, 2007); VP2 correlates perfectly with virus serotype, whereas VP5 shows partial correlation with virus serotype. However, these segments vary within each serotype and correlate with the geographical origin of the virus strain (Seg-2 and Seg-6 topotypes) (Bonneau *et al.*, 1999; Maan *et al.*, 2007; Mertens *et al.*, 2007; Singh *et al.*, 2004).

Maan *et al.* (2007) have shown that the Seg-2 sequences of different BTV strains form distinct clades for each serotype; in addition, the sequences of certain serotypes cluster closely together, identifying distinct nucleotypes (identified as A–J). Eight nucleotypes (A–H) have also been described for Seg-6 (Maan *et al.*, 2010, 2011; Mertens *et al.*, 2005).

NS3/NS3A proteins, encoded by Seg-10, have been associated with the release of virus particles from insect cells (Hyatt *et al.*, 1991, 1993; Beaton *et al.*, 2002; Hyatt *et al.*, 1993). It has been suggested that variations in Seg-10 might be related to transmission of the virus by different insect vector populations and species (Balasuriya *et al.*, 2008; Bonneau *et al.*, 1999; Nikolakaki *et al.*, 2005).

The Seg-10 gene tree does not group BTV isolates according to serotype or geographical region. Mechanisms such as genetic drift and founder effects (Bonneau *et al.*, 2001), in combination with negative selection, are probably the most important factors governing the molecular evolution of the NS3 protein of field strains of BTV within each global ecosystem (Balasuriya *et al.*, 2008).

The internal core protein VP7 can mediate surface attachment, penetration and infection of insect cells (Mertens *et al.*, 1996). Seg-7, encoding VP7, also shows significant variations (Wilson *et al.*, 2000), despite the role of VP7 as the main serogroup-specific antigen. It has been suggested that this variation could also be related to the insect populations that act as vectors for different virus strains in different geographical areas (Bonneau *et al.*, 2000; Wilson *et al.*, 2000).

VP3 interacts with both the minor proteins of the transcriptase complexes and the dsRNA segments, helping to determine the internal structure of the virus particle (Gouet *et al.*, 1999). Both the nucleotide and amino acid sequences of VP3 and Seg-3, respectively, are highly conserved within BTV serotypes (Maan *et al.*, 2008; Tanaka & Roy, 1994). This reflects major structure/function constraints on VP3 and the protection from neutralizing antibodies, given by its internal position within the virus particle. However, significant variation can be found associated with the geographical origin of the virus. This variation divides individual isolates into eastern and western groups (topotypes) and then into a number of further geographical subgroups, based on phylogenetic analysis of their nucleotide sequences (Maan *et al.*, 2008; Nomikou *et al.*, 2009; Pritchard *et al.*, 1995).

Information about the presence of BTV in the Americas can be divided according to the amount of data available. There are substantial data about North America, Central America and the Caribbean. Serotypes 1, 2, 3, 5, 6, 10, 11, 13, 14, 17, 19, 22 and 24 have been detected in North America, whereas serotypes 1, 3, 4, 6, 8, 12 and 17 have been identified in Central America and the Caribbean (Mertens *et al.*, 2005). However, in South America, information regarding detection of BTV is limited to very few reports. Serological evidence for the presence of BTV has been reported in Peru (Rosadio *et al.*, 1984), Argentina (Puntel *et al.*, 1999), Brazil (Brown *et al.*, 1989; Castro *et al.*, 1992; Lage *et al.*, 1996), Ecuador (Lopez *et al.*, 1985) and Chile (Tamayo *et al.*, 1985). Using serological techniques, the serotypes probably present in South America are: 4, 6, 14, 17, 19 and 20 in Brazil (Groocock & Campbell, 1982); 12, 14 and 17 in Colombia (Homan *et al.*, 1985); 14 and 17 in Guyana; and 6, 14 and 17 in Suriname (Gumm *et al.*, 1984).

Brazil and Argentina are the only countries in South America where BTV has been isolated. Clavijo *et al.* (2002) reported the first isolation of BTV in Brazil, and typed it as serotype 12 by seroneutralization test (SNT).

In Argentina, two serological surveys (one between 1995 and 1996 and the other in 1998) indicated that Misiones province and two departments of Corrientes province, in the north-east of the country, were seropositive (Lager, 2004). During those surveys, BTV was isolated at the National Institute of Agricultural Technology (INTA) from the blood of sentinel cattle without clinical signs and the serotype was determined as 4 by SNT and RT-PCR (Gorsch *et al.*, 2002). Two new isolates, obtained as part of the present work, were typed by RT-PCR and sequencing.

This paper describes sequence comparisons and phylogenetic analyses of Seg-2, Seg-3, Seg-6, Seg-7 and Seg-10 from the first Argentinian field isolates of BTV. The sequences generated were further compared with those of field and prototype BTV strains from other parts of the world, in order to elucidate the molecular epidemiology of the disease and the genetic evolution of the virus in the region.

RESULTS

Virus isolation

Three BTV isolates (BTV4/ARG/2001/99, BTV4/ARG/2001/102 and BTV4/ARG/2001/829) had been previously obtained from sentinel herds during 1999–2001. Those first isolations of BTV in Argentina were identified as serotype 4 by SNT and RT-PCR, with primers that hybridized on Seg-2 of the BTV genome.

In 2007 and 2009, two blood samples from asymptomatic bovines which had seroconverted produced positive results by RT-PCR. Isolates 4/ARG/2009 and 4/ARG/2010 (Corrientes province, Argentina) were obtained in CRL1660 cells (Fig. 1). Typing of these isolates as serotype 4 was performed by RT-PCR targeting Seg-2 followed by sequencing.

Sequence analysis

Seg-2. The Seg-2 ORF encodes VP2, the immunodominant and immunodeterminant major component of the external

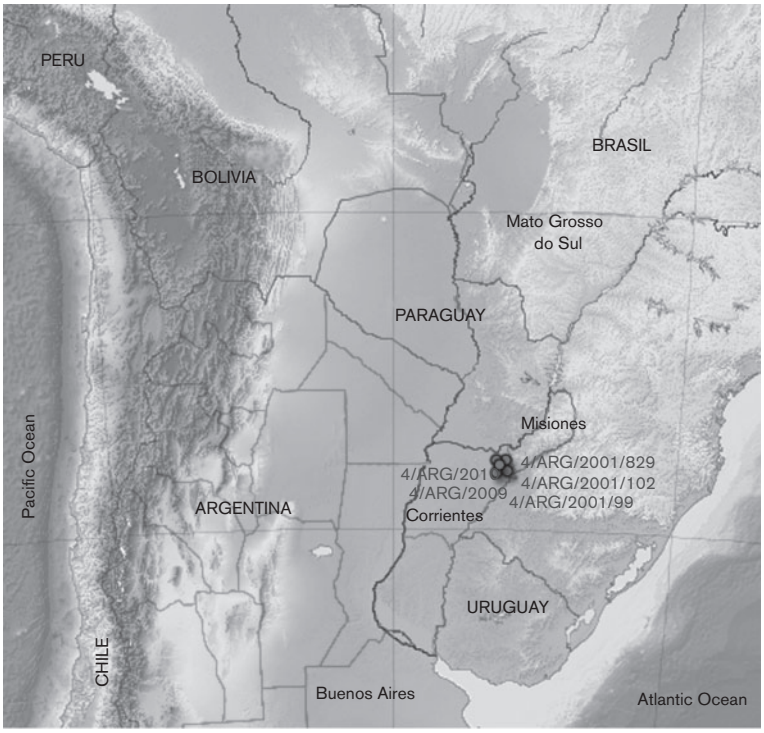

Fig. 1. Map of geographical location of Argentina's northern region. Circles indicate regions where BTV field isolates were obtained.

protein capsid (Maan *et al.*, 2007). It is also the most variable segment of the virus genome, reflecting adaptive responses to the host and vector (Mertens *et al.*, 2005).

The Argentinian Seg-2 sequences showed a 2871 nt-long ORF and a deduced amino acid sequence of 957 residues. The sequence analysis showed that the identity among the Argentinian isolates ranged from 94.7 to 99.6 % in nucleotide datasets and from 95.9 to 99.4 % in amino acid datasets. In that group, the most divergent sequence was 4/ARG/2001/829, whereas the others were closely related despite their year of isolation.

The phylogenetic analysis of the Seg-2 showed that Argentinian isolates grouped within Seg-2-based nucleotype A along with the reference strains for serotypes 10 (10/RSArrrr), 24 (24RSArrrr), 11 (11/RSArrrr), 17 (17/RSArrrr) and 20 (20/RSArrrr) (Fig. 2). The Argentinian sequences clearly segregated into this nucleotype A clade, according to serotype, grouping with the South African serotype 4 reference strain 4/RSArrrr (GenBank accession no. AJ585125.1), showing a similarity ranging from 90.4 to 91.2 % in nucleotide sequences and from 94.2 to 94.7 % in amino acid sequences. Sequences from Turkey (4/TUR/1999, 89.2–89.8 % nucleotide identity) and Greece (4/GRE/1999, 90.4–91.2 % nucleotide identity) were grouped together in the same serotype 4 cluster. A two-subclade structure was maintained by high boostrap values into the serotype 4 group, with one subclade composed of the Argentinian isolates and the other composed of the reference isolate (South Africa) and the isolates from Greece and Turkey (Fig. 2).

Seg-3. Seg-3 encodes a highly conserved protein, located in the subcore internal capsid. Argentinian isolates have an ORF of 2706 nt and 902 deduced amino acid residues. All the isolates grouped in a single cluster when they were compared with other Seg-3 sequences available. The Argentinian isolates' sequences for Seg-3 showed 92.2–99.5 % nucleotide identity and 98–99.5 % amino acid identity.

Phylogenetic analysis based on Seg-3 showed significant divergence, which reflected the geographical origin of the virus. Based on that observation, isolates could be divided into eastern and western groups/topotypes, and a number of subgroups (Fig. 3) (Nomikou *et al.*, 2009). In this work, we observed that Argentinian sequences grouped within the western topotype along with isolates from the Mediterranean Basin, Africa and North America. The nucleotide identity of the Argentinian isolates to the above-mentioned western clade ranged from 86.9 to 90.5 %. On the other hand, the nucleotide identity of the isolates to the main eastern topotype used in this analysis ranged from 74.1 to 81 %. The Argentinian isolates were genetically most closely related to isolates 9/RSArrrr (89.2–89.9 % nucleotide identity), 1/RSArrrr (89.1–89.8 % nucleotide identity), 6/RSArrrr (89.8–90.5 % nucleotide identity) and 2/RSArrrr (89.2–90 % nucleotide identity), all of which are reference strains isolated from South Africa. Argentinian field isolates shared a close genetic relationship with isolates from the USA (17/USA/1997, 90.4–92.1 % nucleotide identity; 6/USA/2006, 90.6–92 % nucleotide identity) and The Netherlands (6/NET/2008, 89.5–90.4 % nucleotide identity), which were closely related to a live-vaccine strain reported by Maan *et al.* (2010). Also, the five Argentinian

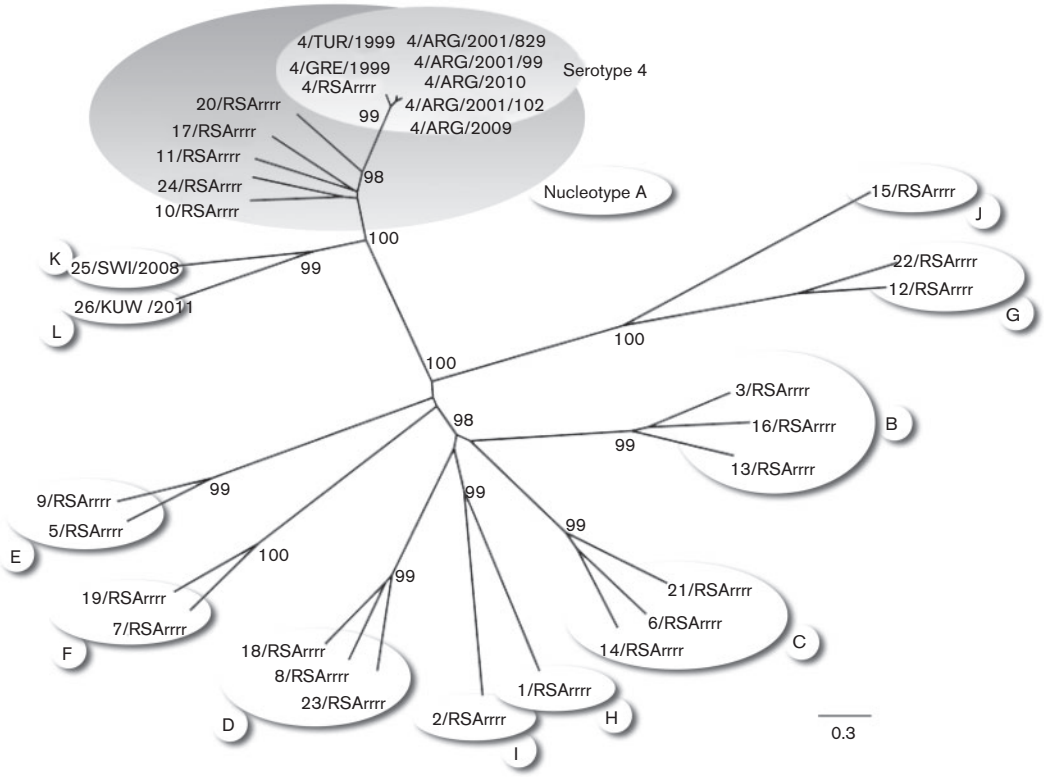

Fig. 2. Neighbour-joining tree showing phylogenetic relationships between Seg-2 from Argentinian field isolates and different strains (reference and field isolates) isolated all over the world. Evolutionary analysis was conducted in MEGA5 (Tamura *et al.*, 2011). Bootstrap test values (1000 replicates) are shown near the tree nodes. Further information about Argentinian field isolates and reference and field isolates' strains from the rest of the world is available in Table 1 and Table S1 (available in JGV Online), respectively. Bar, 0.3 nucleotide substitutions per site.

isolates shared a unique feature mutation at the amino acid level at position 81, which changed an arginine for a lysine. This residue was conserved in all isolates except for 25/SWI/2008 (glycine in position 81) and 26/KUW/2011 (glutamic acid in position 81).

Seg-6. Seg-6 encodes the second main component of the capsid outer layer (VP5), and is also the second most variable segment in the BTV genome. Previous phylogenetic studies have demonstrated that Seg-6 sequences do not correlate perfectly with virus serotype, but segregate partially according with different serotypes, since VP5 influences the conformational features of VP2 and is indirectly involved in serotype determination (Mertens *et al.*, 2005).

In concordance with the results obtained in the analysis of Seg-2, Argentinian Seg-6 sequences were highly conserved. The level of nucleotide/amino acid identity ranged from 98.6 to 99.6 % and from 99.4 to 99.8 %, respectively. Argentinian Seg-6 sequences showed an ORF of 1581 nt which encoded 527 deduced amino acids. Comparisons of sequence data showed that Argentinian sequences clustered within the nucleotype A group along with (in decreasing order of identity) an isolate from Turkey (4/TUR, 92.6–93.1 % nucleotide identity), an isolate from South Africa (4/SA/2002, 92.5–93.1 % nucleotide identity), BTV-24 (24/RSArrrr, 92–92.6 % nucleotide identity), BTV-11 (11/RSArrrr, 91.8–92.7 % nucleotide identity), BTV-4 (4/RSArrrr, 91.8–92.6 % nucleotide identity), an isolate from Greece (4/GRE/2000, 91.8–92.6 % nucleotide identity), an isolate from Turkey (4/TUR/1978, 91.8–92.5 % nt identity) and BTV-17 (17/RSArrrr, 90.2–91 % nucleotide identity) (Fig. 4).

It is notable that the only Argentinian sequence available at GenBank (4/ARG/2002, GenBank accession no. AJ586682.1) (Lager *et al.*, 2004) is almost identical to the five Argentinian isolates presented and analysed in this work. In fact, the nucleotide identity of the sequences ranged from 98.9 to 99.6 %, whereas the identity of the deduced amino acid sequences ranged from 99.4 to 100 % (totally identical to 4/ARG/2001/829).

Seg-7. Seg-7 encodes VP7, one of the components of the two inner layers of the BTV capsid (Mertens *et al.*, 2005); in particular, VP7 is part of the outer surface of the core shell. Argentinian isolates had an ORF of 1128 bp and 376 deduced amino acids. These isolates clustered together in a single group with an internal nucleotide identity that

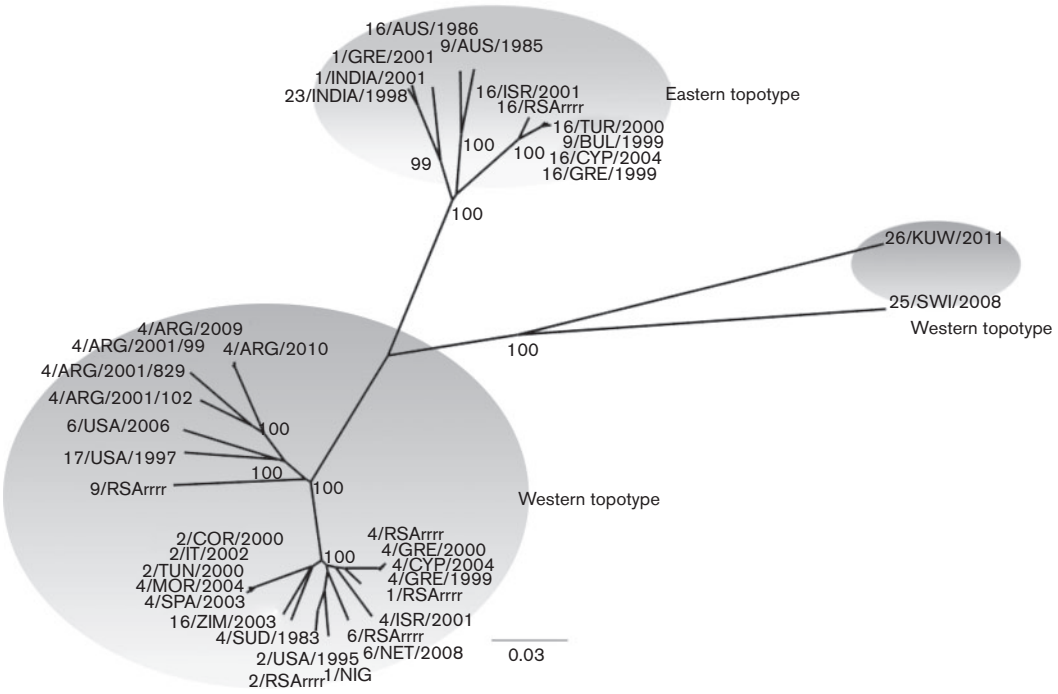

Fig. 3. Neighbour-joining tree showing phylogenetic relationships between Seg-3 from Argentinian field isolates (Table 1) and different strains (reference and field isolates) isolated all over the world (Table S1). Evolutionary analysis was conducted in MEGA5 (Tamura *et al.*, 2011). Bootstrap test values (1000 replicates) are shown near the tree nodes. Bar, 0.03 nucleotide substitutions per site.

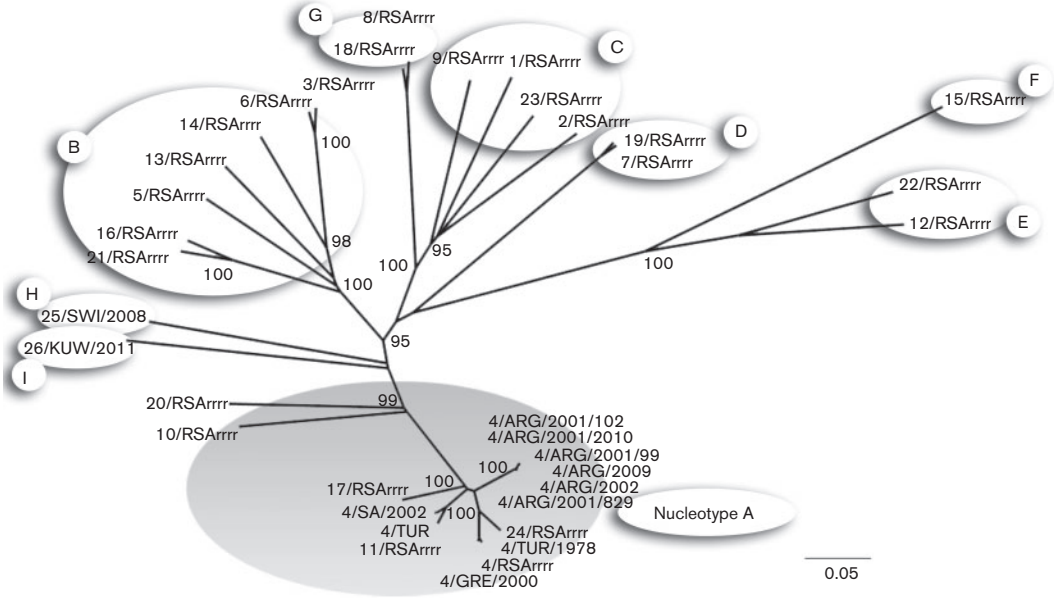

Fig. 4. Neighbour-joining tree showing phylogenetic relationships between Seg-6 from Argentinian field isolates (Table 1) and different strains (reference and field isolates) isolated all over the world (Table S1). Evolutionary analysis was conducted in MEGA5 (Tamura *et al.*, 2011). Bootstrap test values (1000 replicates) are shown near the tree nodes. Bar, 0.05 nucleotide substitutions per site.

ranged from 96.9 to 99.6 % and an amino acid identity that ranged from 97.9 to 99.4 % (Fig. 5). A Brazilian Seg-7 sequence (12/BRA/2001), the only South American Seg-7 sequence reported so far (Clavijo *et al.*, 2002), clustered tightly to this group. The nucleotide variability between the Argentinian and Brazilian sequences was 95.4–96 % in the nucleotide dataset and 96.8–97.1 % in the amino acid dataset. This South American subgroup was included in a previously known topotype group. The Brazilian sequence has been reported as part of the western 1 (Maan *et al.*, 2008) and western 4 (Maan *et al.*, 2010) topotype subgroups. In the same clade, Argentinian sequences also grouped with isolates from the USA (2/USA/1982, 92.3–92.5 % nucleotide identity), Jamaica (3/JAM/1989, 91.5–91.5 % nucleotide identity), Guatemala (3/GUA/1991, 92.4–93 % nucleotide identity), Honduras (6/HOND/1990/02, 91.4–92.1 % nucleotide identity), the USA (17/USA, 91.1–91.6 % nucleotide identity) and Puerto Rico (17/PR/1990, 91.6–91.9 % nucleotide identity,) all within the main western topotype group (Maan *et al.*, 2008, 2010) (Fig. 5).

Seg-10. Seg-10, one of the most conserved segments in the BTV genome, encodes two non-structural proteins, NS3 and NS3A, formed by 229 and 216 aa, respectively. Analysis based on Seg-10 of the Argentinian isolates showed that this sequence had two ORFs of 687 and 648 bp coding for 229 and 216 deduced amino acids (NS3 and NS3A proteins, respectively). An identity of 95.9–99.8 % in the nucleotide dataset and 98.2–100 % in the amino acid dataset was observed among the Argentinian isolates. All the Argentinian sequences presented in this work clustered together in a single clade along with sequences previously reported within the western topotype group (Fig. 6). The sequences most closely related to the Argentinian group were isolates from Guatemala (3/GUA/1990, 92.4–93 % nucleotide identity), Costa Rica (3/COS/1988, 91.8–92.8 % nucleotide identity), Honduras (1/HOND/1989, 91.8–92.5 % nucleotide identity), Jamaica (12/JAM/1988, 91.1–92 % nucleotide identity), and The Netherlands (8/NET/2006, 90.2–91.4 % nucleotide identity).

DISCUSSION

In Argentina, BTV4 was isolated from sentinel herds during 1999–2001 (Lager *et al.*, 2004). In addition, BTV was further detected and isolated in 2007 and 2009. All those isolates represented the first non-serological evidence of virus circulation in Argentina. BTV4 has been isolated in Africa, Europe, the Middle East, Pakistan, India, South-east Asia, Indonesia, China, and North, Central and South America (Mertens *et al.*, 2002). Recent disease reports have shown BTV4 circulation in Morroco, Cyprus, Greece and Spain (OIE, 2011).

Since no BTV outbreaks have been reported in South America, the sanitary and economic impact of BTV infection is associated with the interference in the international movement of animal products and subproducts. The first report of the disease in this continent was in 1889, but the

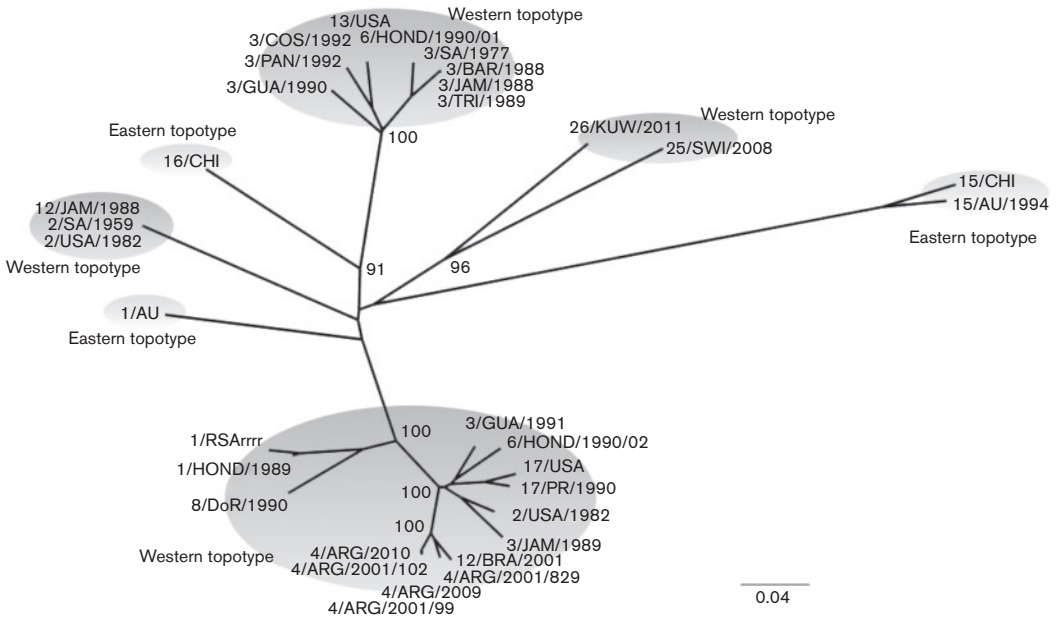

Fig. 5. Neighbour-joining tree showing phylogenetic relationships between Seg-7 from Argentinian field isolates (Table 1) and different strains (reference and field isolates) isolated all over the world (Table S1). Evolutionary analysis was conducted in MEGA5 (Tamura *et al.*, 2011). Bootstrap test values (1000 replicates) are shown near the tree nodes. Bar, 0.04 nucleotide substitutions per site.

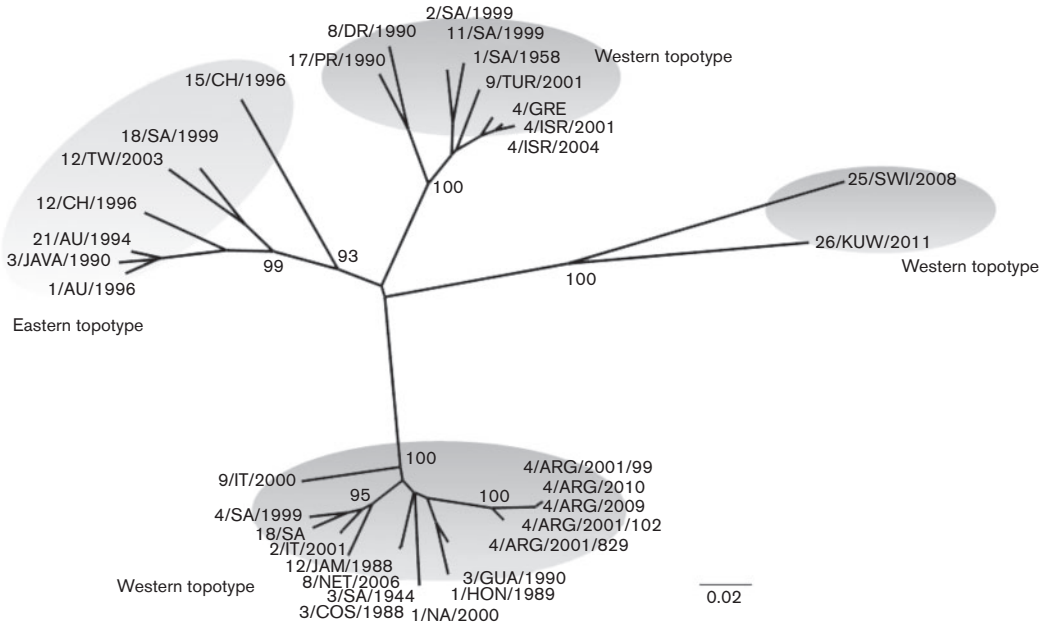

Fig. 6. Neighbour-joining tree showing phylogenetic relationships between Seg-10 from Argentinian field isolates (Table 1) and different strains (reference and field isolates) isolated all over the world (Table S1). Evolutionary analysis was conducted in MEGA5 (Tamura *et al.*, 2011). Bootstrap test values (1000 replicates) are shown near the tree nodes. Bar, 0.02 nucleotide substitutions per site.

first isolation was not until 1958 (Clavijo *et al.*, 2002). The only South American countries where BTV has been isolated are Brazil (BTV12) and Argentina (BTV4). However, serotypes 4, 6, 12, 14, 17, 19 and 20 have been detected all over South America by serological tests.

In this work, we describe for the first time the genetic features of Argentinian BTV field isolates. We present genetic data from BTV Seg-2, Seg-3, Seg-6, Seg-7 and Seg-10 from virus isolated in 1999–2001, 2009 and 2010. Until this work, the only genetic data available were a BTV12 Seg-7 sequence from a Brazilian isolate (Clavijo *et al.*, 2002) (GenBank accession no. AY263377.1) and a BTV4 Seg-6 from an Argentinian isolate (obtained during surveillance in 1999).

The BTV segment sequences analysed have been extensively used to genetically characterize isolates around the world (Maan *et al.*, 2008, 2010; Shirafuji *et al.*, 2012; van Rijn *et al.*, 2012). Seg-2 analysis shows correlation between sequence and serological evidence, as well as some information regarding the geographical origin. Seg-6 partially correlates with the serological evidence (serotype) but serves as another molecular typing layer. Seg-3, Seg-7 and Seg-10 are useful for topotype characterization and to track the closest origin of an isolate.

Seg-2 sequences confirmed serological evidence available for Argentinian isolates which were detected as serotype 4 by SNT (Lager *et al.*, 2004). This is important due to the possible cross-reaction in serological tests (Maan *et al.*, 2007). In addition, the analysis resulted in a single cluster of Argentinian sequences into the serotype 4 clade and showed a high identity percentage in nucleotide and amino acid sequences, with a high bootstrap value, supporting that group in the whole analysis. Also, the Argentinian sequences grouped within the nucleotype A clade, along with the reference strains. The previously available isolates that were most closely related to the Argentinian isolates were from Turkey and Greece (4/TUR/1999 and 4/GRE/1999), and were almost as closely related to the reference BTV4 strain 4/RSArrrr. This may indicate that Argentinian, Turkish and Greek isolates have a common origin, but given the knowledge about the animal trade, the putative common ancestor would not be so recent.

The analysis of Seg-6 showed a high degree of conservation between the Argentinian isolates, as expected according to the results of Seg-2. All the sequences presented in this work grouped into the well-known Seg-6 nucleotype A (Maan *et al.*, 2007) along with isolates from Turkey (4/TUR, 4/TUR/1978), Greece (4/GRE/2000) and South Africa (4/SA/2002), as well as with reference strains (4/RSArrrr, 11/RSArrrr, 24/RSArrrr and 17/RSArrrr). Importantly, the Argentinian clade included the South American sequence previously reported by Lager *et al.* 2004. This sequence is highly similar not only to sequences obtained in 1999–2001, but also to the new sequences presented in this work (98.9–99.6 % nucleotide identity). This is particularly important because it demonstrates the genetic stability of Argentinian field isolates, which has been maintained from 1999 to 2010.

Table 1. Characteristics of Argentinian BTV field isolates

Abbreviated name*	BTV serotype	GenBank accession no.	Province of isolation	Year of isolation	Passage history†
4/ARG/2001/99	4	JX024940, JX024941, JX024942, JX024943, JX024944	Corrientes	2001	B3
4/ARG/2001/102	4	JX024945, JX024946, JX024947, JX024948, JX024949	Corrientes	2001	B3
4/ARG/2001/829	4	JX024950, JX024951, JX024952, JX024953, JX024954	Corrientes	2001	B3
4/ARG/2009	4	JX024955, JX024956, JX024957, JX024958, JX024959	Corrientes	2009	C2/B3
4/ARG/2010	4	JX024960, JX024961, JX024962, JX024963, JX024964	Corrientes	2010	C2/B3

*Named according to serotype/country of isolation/year of isolation/internal code.
†Number of passages in CRL1660 cells (expressed as C followed by number of passages) and/or BHK-21 cells (B followed by number of passages).

Phylogenetic analysis of Seg-3, Seg-7 and Seg-10 showed that Argentinian isolates grouped together and presented a high degree of conservation regardless of the year of isolation. These three segments grouped into the western topotype in the analysis of each segment, indicating that the circulating virus had an African/European origin, defined previously by others (Maan *et al.*, 2008, 2010; Nomikou *et al.*, 2009).

Seg-3 analysis showed a close relationship between the Argentinian isolates and the reference strains from South Africa reported previously as part of the main western topotype group (9/RSArrrr, 1/RSArrrr, 6/RSArrrr and 2/RSArrrr). The analysis of the Argentinian Seg-7 showed a high identity with the isolate from Brazil (12/BRA/2001) and other isolates from North and Central America and the Caribbean (2/USA/1982, 3/JAM/1989, 3/GUA/1991, 6/HOND/1990/02, 17/USA and 17/PR/1990), all belonging to the western topotype group (Maan *et al.*, 2008, 2010). Seg-10 analysis also showed a close relation with known western strains; the most closely related were isolates from Central America, the Caribbean and Europe (3/GUA/1990, 3/COS/1988, 1/HOND/1989, 12/JAM/1988 and 8/NET/2006).

The Argentinian sequences showed some South American genetic identity, assessed in the analysis of every segment, suggesting evolution of an independent lineage. In concordance, the sequences grouped together, forming an exclusive Argentinian cluster that represented a single virus lineage regardless of a small number of mutations between sequences. Seg-7 analysis showed that the sequences grouped along with the only Brazilian isolate (GenBank accession no. AY263377.1). Moreover, one of our samples (4/ARG/2001/829) shared particular mutations with the Brazilian sequence in Seg-7, indicating a close common origin because those mutations were not shared with other Argentinian sequences (data not shown). This genetic proximity between two segments belonging to different serotypes suggests a reassortment event in the evolution of the strains (Nomikou *et al.*, 2009). Seg-7 and Seg-10 analysis provide evidence for the genetic continuity of the region. We hypothesize that the circulating virus isolated in the north-east of Argentina had been introduced by a Central American–Brazilian path. In agreement with this hypothesis, BTV serological evidence has been reported in Mato Grosso do Sul, Brazil, a region close to the Paraguay–Argentina–Brazil border (Tomich *et al.*, 2009). On the other hand, Marcoppido *et al.* (2011) showed no serological evidence of BTV in South American camelids in the north-west of Argentina (Argentina–Bolivia border). This finding could be explained by the lack of detection of *Culicoides* spp., which cannot develop under the weather conditions present in the region.

Because of the global distribution and the surveillance previously conducted (Lager *et al.*, 2004), Argentina was considered as a random incursion country. However, here we showed that the sequences from isolates between 2001 and 2010 are 99 % identical. This indicates a genetic stability explained by a putative initial strain that is still circulating in the region. White *et al.* (2006) suggested that genetic stability could be explained by the presence of immunologically naïve animals, which can become infected every year by the same virus strain, and by some undefined factors (host, viral or environmental), which can probably act as tools for purifying selection (White *et al.*, 2006). In addition, it has been reported that BTV can persist in asymptomatic cattle for years (Bréard *et al.*, 2003). In summary, the presence of the viral reservoir could explain the lack of variation in the Argentinian sequences between 2001 and 2010.

The distribution and the proposed introduction pathway of the disease in the region could be explained by animal trade, climatic factors and the presence of a competent vector. According to previous studies, the dominant *Culicoides* species in the north-east of Argentina are *Culicoides insignis*, followed by *Culicoides paraensis* and *Culicoides venezuelensis* (Ronderos *et al.*, 2003). Other reports also point to *C. insignis* and *Culicoides pusillus* as the main representatives of the genus in the Americas in which the virus has been detected and isolated (Lager, 2004).

The results presented here represent the first phylogenetic analysis including Argentinian sequences and is the first contribution to databases of Seg-2, Seg-3, Seg-6, Seg-7 and Seg-10 of Argentinian BTV4 field isolates from 2001 to 2010. More new and old South American isolate sequences are needed to track the origin of our isolates and to broaden the understanding of the biology behind the disease spread in the subcontinent.

METHODS

Virus detection, isolation and propagation. The BTV strains named 99, 102 and 829 (isolated during the surveillance conducted in 1999–2001), which were available at our laboratory (Lager *et al.*, 2004), were amplified by three passages in baby hamster kidney (BHK)-21 cells. The 2009 and 2010 BTV isolates were obtained from asymptomatic infected animals detected in our diagnostic service of the Virology Institute at INTA, Buenos Aires, Argentina. Seroconversion was detected by ELISA and agar gel immunodiffusion (AGID) test. Subsequent viral genome detection was carried out by RT-nPCR (reverse transcriptase-nested PCR).

Isolates 4/ARG/2009 and 4/ARG/2010 (named according to serotype/country of isolation/year of isolation) were obtained by inoculating 500 µl blood on a CRL1660 (C6/36) cell monolayer. Two passages were carried out (7 days of incubation without cytopathic effect, CPE) followed by three passages in BHK-21 cells, detecting CPE after 7 days of incubation. The presence of BTV in BHK-21 cultures was further assessed by diagnostic Seg-6-based nRT-PCR (OIE, 2012).

Molecular characterization. Total RNA was extracted from the supernatant of infected BHK-21 cells using Trizol reagent (Invitrogen), according to the manufacturer's procedures. Segments that codified for L2 (Seg-2), L3 (Seg-3), M6 (Seg-6), S7 (Seg-7) and S10 (Seg-10) were obtained and amplified by RT-PCR using specific laboratory-designed primers derived from consensus sequences available online (for primer sequences, see Table S2). Amplicons were purified from agarose gels with the GE Amersham Gel Extraction kit. The purified amplicons were directly sequenced, using 30 ng template per reaction, according to the instructions of the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), as described by the manufacturer. Sequencing reactions were run in an ABI Prism 3730 Genetic Analyzer (Applied Biosystems). The sequencing processes were carried out using the same primers mentioned above and others designed by primer walking. For all segments, both direction primers were used. Ambiguities were resolved by manually checking the chromatograms. In each segment, the first and last 20 bp were not included in the data for the analysis because primers annealed at those positions in the first round of amplification. The information regarding the Argentinian field isolates is summarized in Table 1.

Phylogenetic analysis. The sequences were analysed with BioEdit 7.0.9.0 software and then aligned using ClustalX2, 2.0 software. The Argentinian isolates were aligned with sequences available online at GenBank (Table S1). The phylogenetic analysis was carried out using the Neighbour-joining method in MEGA5 software (Tamura *et al.*, 2011) and evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2011). As branch support, bootstrap analysis was conducted for 1000 replicates.

Submitted sequences. GenBank accession numbers for the submitted sequences (Seg-2, Seg-3, Seg-6, Seg-7 and Seg-10 sequences of Argentinian BTV isolates) are JX024940–JX024964.

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