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**Molecular epidemiology of Bluetongue virus in Portugal
during 2004-2006 outbreak.**

Sílvia C. Barros, Fernanda Ramos, Tiago M. Luís, Andreia Vaz, Margarida Duarte,
Margarida Henriques, Benedita Cruz, Miguel Fevereiro*.

**Laboratório Nacional de Investigação Veterinária, Estrada de Benfica 701
1549-011 Lisboa, Portugal**

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***Correspondence:**

Miguel Fevereiro

Laboratório Nacional de Investigação Veterinária, Estrada de Benfica 701, 1549-011, Lisboa – Portugal

Phone: +351 217115288

Fax: +351 217115387

E.mail: miguel.fevereiro@lniv.min-agricultura.pt

37 and four non-structural (NS1-NS3 and NS3A) proteins (Roy et al., 1990). VP2 induce
38 neutralising antibodies and is responsible for the serotype specificity (Gibbs and
39 Greiner, 1994). To date, twenty-four serotypes were identified, of which five (BTV-1,
40 BTV-2, BTV-4, BTV-9 and BTV-16) have been reported in the last years (1998-2005)
41 in Mediterranean countries (Mellor and Wittmann, 2002). Recently (August 2006) and
42 for the first time in Europe, BTV-8 was detected in northern European countries
43 (Netherlands, Belgium and Germany), where bluetongue (BT) had never been reported
44 before. An unusual higher incidence of infection in bovine than in sheep was observed
45 in these outbreaks where BTV-8 caused severe clinical signs and lesions in cattle
46 (Toussaint et al., 2006).

47 The first incursion of BTV in Portugal was reported in July 1956, mainly affecting
48 sheep flocks in the southern region of the country below Tagus River, excluding the
49 Algarve region (Silva, 1956). The virus was isolated in chicken embryonated eggs, and
50 identified as serotype 10 (Noronha, F. 1957, personal communication). The number of
51 outbreaks markedly decreased soon after a live attenuated vaccine was used and in 1958
52 the disease practically disappeared. The country was declared free of BTV in 1960.

53 A new introduction of the virus was registered in October 2004 in sheep flocks close to
54 the Spanish border, shortly after Spain notified an outbreak of BTV-4 in the Cadiz
55 region.

56 In this study the molecular epidemiology of BTV strains circulating in Portugal during
57 the 2004-2006 was analysed. For this purpose, the nucleotide sequences of genome
58 segments L2, S7 and S10, coding respectively for viral structural (VP2 and VP7) and
59 non-structural (NS3) proteins, were analysed to determine the serotype and explore the
60 origin of these viruses.

61

62 **2. Methods**

63 **2.1. Virus isolation**

64 For virus isolation, groups of twenty 11 day-old embryonated SPF chicken eggs (ECE)
65 were inoculated intravascularly with 100 µl of a 10⁻¹ dilution of washed and lysed
66 blood, from sheep or cattle. The eggs were incubated for five days at 35° C and
67 examined daily. Embryos that died between 36h and 5 days pi, showing hemorrhagic
68 lesions, were pooled and homogenized in Glasgow MEM BHK21 medium (Invitrogen).
69 The homogenates were clarified by centrifugation and were inoculated again in ECE.

70 Viruses isolated in egg embryos were propagated in baby hamster kidney-clone21
71 (BHK-21) or African green monkey (Vero) cell cultures.

72

73 **2.2. RNA isolation, real time RT-PCR and Sequencing**

74 Total RNA was extracted from BTV infected cells using the BioSprint96 workstation
75 (Qiagen) or RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions.
76 BTV was routinely screened by real-time RT-PCR using primers and probe specific for
77 NS1 gene; forward primer 5'-GTTCTCTAGTTGGCAACCACC-3' (BT-A, OIE),
78 reverse primer 5'-TTTAACACACATCCCATTGAATA-3' and Taqman probe FAM
79 5'-AGTGGGGATTATGCAAATGCCAC-3' TAMRA. The reactions were carried out
80 using the One-step RT-PCR Kit (Qiagen). The L2, S7 and S10 genes were amplified by
81 RT-PCR using group specific primers derived from the nucleotide sequences available
82 in GenBank (Table 1). Before cDNA synthesis, double-stranded RNA was denaturated
83 at 98 °C during 5 minutes and RT-PCR reactions were carried out using the Superscript
84 One-Step RT-PCR for Long Templates (Invitrogen). Usually, the RT reaction (30 min
85 at 50 °C) was followed by 40 cycles of the PCR amplification (15 sec at 94°C, 30 sec at
86 50°C, and 2 min at 68°C) with a final elongation step of 7 min at 68°C. The resulting
87 amplicons were cloned into pCR2.1 vector (Invitrogen) and were sequenced (Thermo
88 Sequenase Primer Cycle Sequencing Kit-Amersham Biosciences).

89

90 **2.3. Sequence and phylogenetic analysis**

91 The nucleotide sequences of L2, S7 and S10 genes of the isolates were submitted to
92 GenBank under the accession numbers EF434175 to EF434180. These sequences were
93 compared with sequences representative of serotypes most commonly detected in
94 Europe namely serotypes 1, 2, 4, 9 and 16 (Table 2). Multiple alignments of the
95 nucleotide sequences were generated by the CLUSTALW program, version 1.6
96 (Thompson et al., 1994) and phylogenetic analysis was carried out by maximum
97 likelihood analysis in TREE-PUZZLE program, version 5.1 (Strimmer and von
98 Haeseler, 1996), using a quartet puzzling algorithm to generate the tree. The analysis
99 was run with Hasegawa-Kishino-Yano (HKY-85) model of substitution (Hasegawa et
100 al., 1985) and quartet puzzling support values based on 1000 puzzling steps were
101 calculated. The overall tree topology was identical when the phylogenetic analysis was
102 repeated using the neighbor-joining method.

103

104 **3. Results**

105 **Virus and Phylogenetic analysis of the L2 gene segment**

106 BTV was not detected in Portugal for almost 50 years. To investigate the origin of the
107 viruses isolated during the recent outbreaks of 2004-2006, nucleotide sequence analysis
108 of the L2, S7 and S10 genes was performed.

109 At the beginning of the outbreak three strains of BTV of serotype 4 were isolated
110 (BTV4/22043/PT04, BTV4/22045/PT04, and BTV4/22126/PT04). These viruses were
111 obtained from sheep with clinical signs and lesions of BT disease that included fever,
112 submandibular oedema, hyperaemia and petechial haemorrhages in the oral cavity and
113 pulmonary artery. The affected sheep originated from neighbouring farms located in the
114 south of the country, close to the Spanish border (Fig. 1). Since then two more strains of
115 BTV-4 were isolated, one from an asymptomatic cow in 2005 (BTV4/28207/PT05) and
116 the other in 2006 (BTV4/37069/PT06) from sheep with symptoms and lesions of BT.
117 To date all the BTV-4 strains analysed showed nearly 100% nucleotide identity for the
118 L2, S7 and S10 gene segments and therefore strain BTV4/22045/PT04 from the initial
119 outbreak was used in this study as a prototype of BTV-4. In July 2005, one BTV of
120 serotype 2 (BTV2/26629/PT05) was isolated from the blood of an asymptomatic cow
121 belonging to a farm located in the southwest of Portugal (Fig. 1).

122 The phylogenetic analysis of L2 gene segment show that the BTV4/22045/PT04 and
123 BTV2/26629/PT05 strains clearly segregated according to serotype (Fig. 2).
124 BTV4/22045/PT04 strain showed the highest sequence identity (99%) with the Sardinia
125 and Corsica BTV-4 isolates of 2003. With respect to Portuguese BTV-2, isolate
126 BTV2/26629/PT05 clustered with the South Africa vaccine strain and its related field
127 strain isolated in Italy with a nucleotide sequence identity of 99.2% and 99.4%,
128 respectively. The diversity found among the L2 gene of the BTV serotypes analysed
129 ranged between 58%-97% (Table 3). Between strains of BTV-4 and BTV-2 serotypes
130 ranges of respectively 1%-11% and 0,5%-19% were observed.

131

132 **Phylogenetic analysis of the S10 gene segment**

133 The S10 gene was analysed since it has been suggested that meaningful phylogenetic
134 toptype inference can be obtained through the nucleotide variation of this gene
135 (Bonneau et al., 1999). Figure 3 shows that the various BTV serotypes clustered into
136 three different lineages, with high levels of bootstrap support. Lineage A includes the
137 four Chinese serotypes, two Italian serotypes (BTV-9 and BTV-16), the Australian

138 BTV-1, the Greek serotypes 1, 9 and 16, and the Israeli BTV-16. Lineage B groups
139 the majority of South African serotypes, BTV-1 from Honduras, the Portuguese isolate
140 BTV2/26629/PT05, the South Africa BTV-2 vaccine and its related Italian strain.
141 Lineage C includes mainly strains of BTV-2 and BTV-4 serotypes isolated in
142 Mediterranean countries, the Dominican Republic BTV-4 and South African BTV-1.
143 As with the L2, the S10 gene segment of BTV4/22045/PT04 and BTV2/26629/PT05
144 isolates show a high degree of identity (99% and 100%) respectively, with Corsica 2003
145 isolate and South Africa BTV-2 vaccine strain (Fig. 3).
146 The S10 phylogenetic tree revealed an overall branching pattern similar to that
147 previously described for some BTV isolates included in the present study (Nikolakaki et
148 al., 2005). According to our study, the lineages A and C correspond respectively to
149 Greek II and I clusters referred by these authors. Lineage A also corresponds to Asian
150 group as defined by Bonneau et al. (1999).

151

152 **Phylogenetic analysis of the S7 gene segment**

153 As with L2 and S10 genes, the phylogenetic analysis based on the S7 gene segment of
154 BTV4/22045/PT04 and BTV2/26629/PT05 isolates showed that they are more closely
155 related, respectively, to the Corsica 2003 (99.3%) and South Africa BTV-2 vaccine
156 (99.9%) strains (Fig. 4). A nucleotide sequence identity of 74% was observed between
157 the S7 genes of the two Portuguese strains.

158

159 **4. Discussion**

160 The present study reports on the outbreak of BTV occurring in Portugal since October
161 2004. The first clinical cases of BT were notified in sheep farms located in the
162 Southeast of Portugal, close to the Spanish border. The disease was confirmed by virus
163 isolation, detection of viral genome by RT-PCR and specific antibodies by ELISA and
164 seroneutralisation.

165 In 2004 three BTV of serotype 4 were isolated from blood or organs of sheep with
166 clinical signs or victimised by the disease in the initial outbreak. During 2005 the
167 disease was not reported in sheep but one BTV-4 and one BTV-2 were isolated from
168 asymptomatic cows. In November 2006 one outbreak of BTV4 was reported in sheep
169 located in the centre of the country.

170 The areas of the country affected by the present BT outbreak match with the predicted
171 geographic distribution of culicoides vectors (Tatem et al., 2003) and also with the areas
172 affected by the BT epizootics of 1956.

173 Phylogenetic analysis of L2, S7 or S10 genes showed that BTV4/22045/PT04 virus
174 clustered closely with the BTV-4 viruses circulating in Italy and Corsica in 2003. This
175 suggests that the Portuguese BTV-4 viruses have the same origin as the BTV-4 strains
176 circulating in these countries. In this study it was not possible to include sequences of
177 L2, S7 and S10 genes from Spanish BTV-4 strains since they are not yet available in the
178 GenBank database. However, due to the close geographic proximity and the time frame
179 between the occurrence of the Andalusia outbreak and the first episode of BT in
180 Portugal it is pertinent to consider that BTV4/22045/PT04 is most likely related to the
181 Spanish BTV-4 outbreak. This consideration is also supported by the absolute identity
182 found between the M6 gene of Portuguese and Spanish (SPA2004) isolates while when
183 compared with the Corsica BTV-4 the identity is lower (99%).

184 Beside Italy and Corsica, BTV of serotype 4 has been detected in Greece, Bulgaria and
185 Turkey, during the years 1998-1999 and in Spain and Morocco in 2004. Also BTV-4
186 has established both in South Africa and in the Caribbean area (Mo et al., 1994).
187 Previous studies indicated a western origin, probably North African, for Corsica and
188 The Balearics 2003 BTV-4 strains (Potgieter et al., 2005; Purse et al., 2005). According
189 to these authors, the European BTV serotypes have arrived from at least two sources.
190 The BTV-1, BTV-9 and BTV-16 serotypes belong to an eastern (Asian/Australian)
191 group of viruses, whereas BTV-2 and BTV-4 belong to a western (African) group. A
192 recent study suggest that BTV-4 strains of the Mediterranean basin have three different
193 lineages, the first includes the Greek isolates, the second groups strains from Italy,
194 Morocco, Spain, and Corsica, and the third lineage contains Turkish isolates (Breard et
195 al., 2007).

196 BTV-2 epidemics occurred in Tunisia in December 1999 and in the following summer
197 the virus was spread to northeast Algeria, Sicily and Calabria, Corsica and Balearic
198 Islands (Mellor and Wittmann, 2002). BTV-2 is also enzootic in the Southeast United
199 States and in some African countries (Aradaib et al., 2005; Mecham and Johnson,
200 2005).

201 The Scientific committee of European Union recommends the vaccination as an
202 emergency action, based on a cost/risk benefit analysis and the epidemiological
203 situation (Sanco/C3/AH/R19/2000). During 2000-2002 vaccination campaigns were

204 carried out in the Balearic Islands, Corsica and Italy, using the attenuated monovalent
205 serotype 2 vaccine produced by Onderstepoort Biological Products - South Africa
206 (Agüero et al., 2002; Breard et al., 2004; Ferrari et al., 2005). Attenuated vaccines have
207 been widely used to control the disease in southern Africa and USA. The circulation of
208 BTV vaccine strains in the field via active vectors among susceptible animals was
209 suggested before (Murray and Eaton, 1996). Nevertheless, only in 2005 the
210 transmission of BTV vaccine strain from vaccinated to non-vaccinated animals was
211 confirmed (Ferrari et al., 2005).

212 Regarding the genes analysed, the Portuguese BTV2/26629/PT05 showed the highest
213 nucleotide identity (>99%) with the South Africa BTV-2 vaccine strain and its related
214 field strain isolated in Italy (Ferrari et al. 2005) suggesting a common origin.
215 BTV2/26629/PT05 strain is apparently of low pathogenicity for sheep since it was
216 isolated from an asymptomatic cow belonging to a farm located in an area where the
217 disease in sheep had not been reported, and because in all the few clinical cases of BT
218 disease registered to date in the country only BTV-4 has been detected.

219 The BTV-2 vaccine strain used in the vaccination campaigns in Italy and Spain may
220 have been introduced in Portugal through imported viraemic animals or by aerial
221 dispersion of *Culicoides* from infected areas. Evidence of the role of *Culicoides* vectors
222 in spreading BTV-2 vaccine strain in areas where vaccination is not practised have been
223 reported before (Ferrari et al. 2005). BTV2/26629/PT05 could have also been
224 introduced as a contaminant of the BTV-4 live vaccine used in the vaccination
225 campaign in early 2005 in Portugal, though vaccine vials belonging to the same batches
226 of the BTV-4 vaccine tested negative for BTV-2 by RT-PCR.

227 Preliminary sequence analysis revealed that BTV-4 viruses of 2006 do not differ
228 significantly from those isolated in 2004 therefore suggesting that the initial strains still
229 circulate in Portugal. In contrast, BTV-2 has not been detected since July 2005.

230 Concerning the genes analysed in this study, evidence of gene reassortment between
231 BTV-2 and BTV-4 was not found. However, it cannot be ruled out, as BTV genes may
232 reassort independently of serotype amongst field strains of BTV that co-circulate in the
233 same area (Bonneau et al., 2001).

234 Field strains of BTV from around the world vary markedly, as a consequence of both
235 genetic drift and reassortment (Heidner et al. 1991; de Mattos et al., 1994; Bonneau et
236 al., 1999). It has been suggested that genetic typing of BTV based on the genetic
237 diversity of the NS3 gene give meaningful phylogenetic topotype inference (Bonneau et

238 al., 1999). However, considering the reassortment probability, the definition of a
239 toptype based on any single genome segment might be inadequate (White et al., 2005).
240 This report presents the results of a study on the first wave of BTV-4 outbreak which
241 started in 2004 in Portugal. The prompt confirmation of the virus serotype at the
242 beginning of the outbreak allowed the Portuguese Veterinary Authorities to rapidly
243 implement a vaccination campaign in sheep using a BTV-4 live vaccine (Onderstepoort
244 Biological Products, South Africa) in the spring of 2005. Later this vaccine was
245 replaced by an inactivated vaccine (Merial) administered in both cattle and sheep in
246 winter of 2005.

247 In conclusion, assessment of the genetic variation of viruses in the field is therefore not
248 only useful for determining the origin of outbreaks, but also impacts the selection of
249 appropriate vaccine strains and the accurate design of molecular diagnostic tools.

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255

256

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258

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Fig. 1. Location of the farms and year where BTV-2 and BTV-4 were isolated. Alandroal, BTV4/22043/PT04 and BTV4/22045/PT04; Campo Maior, BTV4/22126/PT04; Barrancos, BTV4/28207/PT05; Grândola, BTV2/26629/PT05 and Alenquer, BTV4/37069/PT06.

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VP2

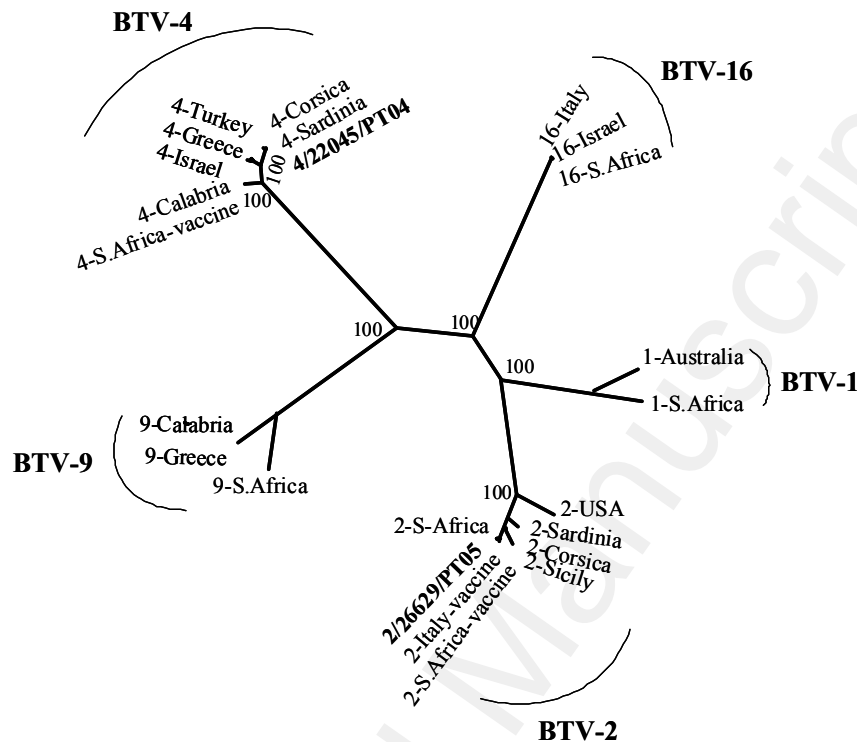


Fig. 2. Phylogenetic analysis of the nucleotide sequences of the L2 gene segment. Maximum likelihood tree performed by PUZZLE, version 5.1 with the HKY-85 model was generated by using a transition/transversion of 1.57, a nucleotide frequency of A =0.308; C =0.172; G =0.246; T =0.274. Log Likelihood = -21544.30. Branch lengths represent genetic distances between sequences. The branch values represent the support based on quartet-puzzling steps of 1000 replicates. The GenBank accession numbers of the published sequences of the BTV isolates are listed in Table 2. Only a single representative of 100% identical isolates was included in the tree.

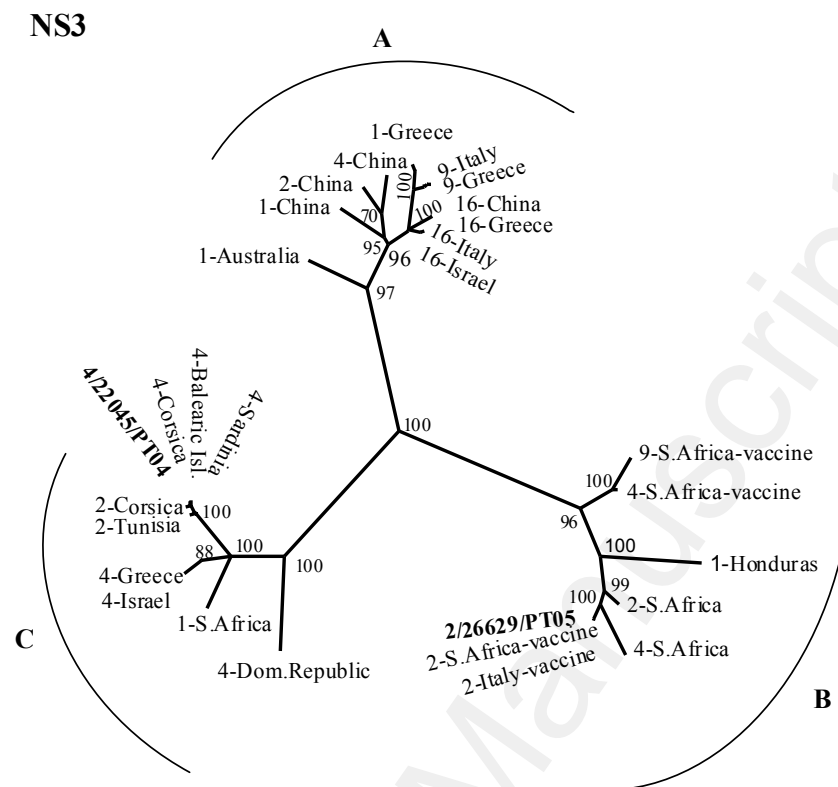


Fig. 3. Phylogenetic analysis of the nucleotide sequences of the S10 gene segment. Maximum likelihood tree performed by PUZZLE, version 5.1 with the HKY-85 model was generated by using a transition/transversion of 4.75, a nucleotide frequency of A =0.318; C =0.185; G =0.252; T =0.246. Log Likelihood = -3467.74. Branch lengths represent genetic distances between sequences. The branch values represent the support based on quartet-puzzling steps of 1000 replicates. The GenBank accession numbers of the published sequences of the BTV isolates are listed in Table 2.

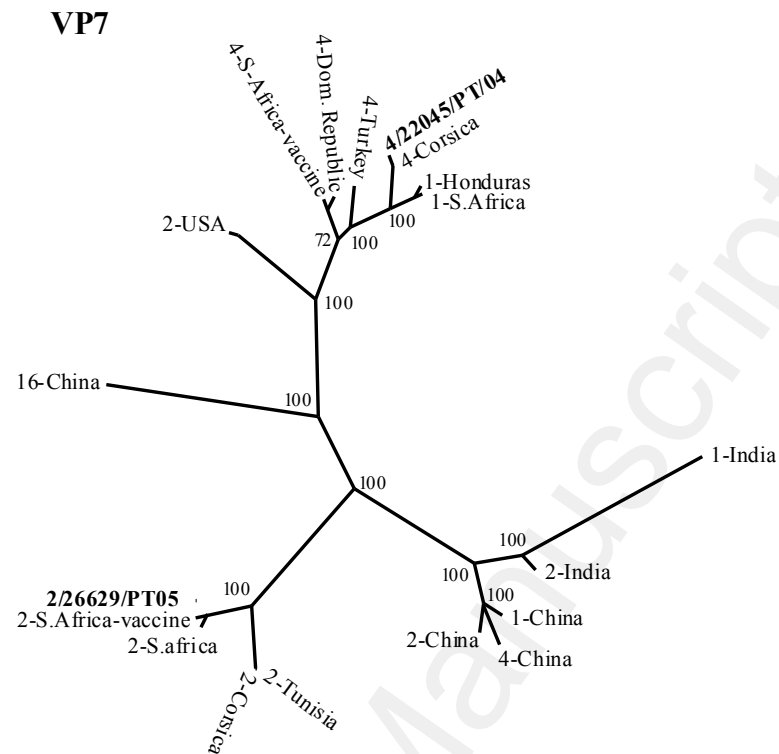


Fig. 4. Phylogenetic analysis of the nucleotide sequences of the S7 gene segment. Maximum likelihood tree performed by PUZZLE, version 5.1 with the HKY-85 model was generated by using a transition/transversion of 2.54, a nucleotide frequency of A =0.273; C =0.212; G =0.26; T =0.256. Log Likelihood = -5779.45. Branch lengths represent genetic distances between sequences. The branch values represent the support based on quartet-puzzling steps of 1000 replicates. The GenBank accession numbers of the published sequences of the BTV isolates are listed in Table 2. Only a single representative of 100% identical isolates was included in the tree.

Table 1: Location and sequence of the primers used in this study.

Serotype	Gene	Primers	Location	Sequence 5'-3'	Size (bp)
4	L2	BTV4-L2-F	1-22	GTAAAAAGAGTGTCTACCATG	1054
		BTV4-2-856R	1054-1033	AGCTATCGCGATAAGCGCATAG	
		BTV4-2-520F	719-740	ATCTCTAGCATGGTGCAAGTGG	1101
		BTV4-2R	1819-1799	GTCAATGCAGGGAAGTCATC	
		BTV4-2F	1700-1719	ACGCTTGGGAAGTACTACGA	1227
2	L2	BTV4-L2R	2926-2907	TAAGTGTAAGAGGCTACCG	
		BTV2-F	1-23	GTAAAAACAGGATCGCGATGGAT	1102
		BTV2-1100R	1102-1081	GATATGGATTCGACCACCAA	
		BTV2-1000F	1019-1039	AGATGAAGATAGATTCTATCG	903
		BTV2-1900R	1921-1899	ATTAGAAGTAAAGAATATTCTC	
4	S7	BTV2-1850F	1851-1870	AAGAAACCGACAAGACCTTG	1089
		BTV2-(Term3)R	2939-2924	GTAAGTTGAACAGATCGCGG	
		VP7(F)	1-17	GTAAAAAATCTATAGAG	1156
		VP7(R)	1156-1140	GTAAGTGTAATCTAAGA	
		VP7-2-F	1-20	GTAAAAAATCTATAGAGATG	1153
2	S7	VP7-2-R	1153-1134	GTAAGTGTAATCTAAGACAC	
		NS3(F)	1-19	GTAAAAAAGTGTCGCTGCC	822
2 and 4	S10	NS3(R)	822-804	GTAAGTGATAGCGCCGCA	

Table 2. BTV strains, GenBank accession numbers, origin, serotype and year.

Strains	GenBank accession number			Origin	Serotype	Year
	S10	L2	S7			
SG93	D00253			Australia	1	
Clones AG[44, 303]		M21844		Australia	1	
Y863	AF135223		AF172825	China	1	
GR1472/01LS	AY677628			Greece	1	2000
BT1-2172	AY426598		AF188670	Honduras	1	1989
Hisar			AY789967	India	1	
S1REF	AF512911		AY776331	South Africa	1	
RSArrrr/01		AJ585122		South Africa	1	
V440	AF135224		AF172826	China	2	
Corsica2000	AF481092	AF356601	AF346302	France: Corsica	2	2000
Corsica2001			AY079124	France: Corsica	2	2001
Chittoor 2/1995			DQ399837	India	2	1995
8341/00				Italy: Sardinia	2	2000
14643-2808		DQ191261		Italy: Sardinia	2	2001
10500-3		DQ191262		Italy- Sicily	2	2000
81640-01-vaccine	AY823221	DQ191270		Italy- Sicily	2	2001
Onders tepoort-Vaccine	AF481094	AF481096	AF481095	South Africa	2	
557 SA		AY855264	AF188672	South Africa	2	
S2REF	AF512916			South Africa	2	
-	AF469114		AF469115	Tunisia	2	
FL99 13406-2		AY855267	AY855282	USA	2	1999
V442			AF172828	China	4	
V413	AF135226			China	4	
BT4-2227	AY426602		AF188667	Dominican Republic	4	1990
Corsica2003	AY857503	AY839945	AY839949	France	4	2003
Mandiko/1999		DQ191277		Greece	4	1999
2002	AY775159			Greece	4	2002
3222222	AY775157	DQ191279		Israel	4	2001
82027		DQ191276		Italy: Calabria	4	2002
10353	AY775155	DQ191280		Italy: Sardinia	4	2003
IAH SPA2003/02	AJ783911			Spain:Balearic Isl.	4	2002
P524/1999	AF512907			South Africa	4	1999
vaccine	AY857506	AY839948	AY841353	South Africa	4	
-	-	DQ825670	DQ825672	Turkey	4	1999
Afandou/1998		DQ191283		Greece	9	1998
GR408/99ChS	AY449651			Greece	9	
12217/00	AY775161			Italy	9	2000
8368		DQ191285		Italy: Calabria	9	2001
vaccine	AY823224			South Africa	9	
RSArrrr/09		AJ585130		South Africa	9	
SW	AF135229		AF172831	China	16	
GR308/99RS	AY449652			Greece	16	
243277	AY775163	DQ191259		Israel	16	
8054	AY775162	DQ191260		Italy	16	2001
RSArrrr/16		AJ585137		South Africa	16	

Note: Only available complete L2, S7 and S10 genes sequences were used for phylogenetic analysis.

Table 3: Average percentage diversity between L2 gene segment of different serotypes used in this study.

	Serotype 1	Serotype 2	Serotype 4	Serotype 9	Serotype 16
Serotype 1	-	58%	93%	90%	73%
Serotype 2		(0.5%-19%) ^a	88%	87%	80%
Serotype 4			(1%-11%) ^a	86%	97%
Serotype 9				-	96%
Serotype 16					-

Note: The genetic distances were calculated by Maximum Likelihood. (^a) Numbers between brackets represent the variation within the serotype. This value was only calculated for serotypes 2 and 4 due to the small numbers of representative strains available from others serotypes.