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Belén Rodríguez-Sánchez, Pedro J. Sánchez-Cordón, Verónica Molina, María A. Risalde, Ana Cristina Pérez De Diego, et al.. Detection of bluetongue serotype 4 in mouflons () from Spain. *Veterinary Microbiology*, 2010, 141 (1-2), pp.164. 10.1016/j.vetmic.2009.08.003 . hal-00560848

**HAL Id: hal-00560848**

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Submitted on 31 Jan 2011

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## Accepted Manuscript

Title: Detection of bluetongue serotype 4 in mouflons (*ovis aries musimon*) from Spain

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PII: S0378-1135(09)00360-5  
DOI: doi:10.1016/j.vetmic.2009.08.003  
Reference: VETMIC 4530

To appear in: *VETMIC*

Received date: 8-4-2009  
Revised date: 17-7-2009  
Accepted date: 3-8-2009

Please cite this article as: Rodríguez-Sánchez, B., Sánchez-Cordón, P.J., Molina, V., Risalde, M.A., Diego, A.C.P., Gómez-Villamandos, J.C., Sánchez-Vizcaíno, J.M., Detection of bluetongue serotype 4 in mouflons (*ovis aries musimon*) from Spain, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.08.003

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## 18 Abstract

19 Bluetongue serotype 4 (BTV4) has been detected for the first time in tissue  
20 samples from 2 mouflons (*Ovis aries musimon*) from the South of Spain, in a  
21 retrospective study. The samples included in this study had been fixed and paraffin-  
22 embedded for over a year prior to their analysis using a BTV group-specific and a  
23 BTV4 specific RT-PCR test. Lung and lymphatic nodes were found positive in both  
24 specimens. The amplified DNA was confirmed to be BTV4 by sequencing the RT-PCR  
25 products and comparing them with other sequences from GenBank. The combination of  
26 RNA extraction from paraffin-embedded samples and serotype-specific real-time RT-  
27 PCR assays provides the tools for the detection of BTV from samples stored for a long  
28 time. The results shown in this study set out the basis for a greater survey with fixed  
29 samples from different species of wild ruminants that the veterinary services have been  
30 collecting for years.

31  
32 **Keywords:** bluetongue, serotype-4, mouflons, wild ruminants, RT-PCR, paraffin-  
33 embedded tissues

## 36 Introduction

37 Bluetongue (BT) is a noncontagious, vector-borne disease of domestic and wild  
38 ruminants caused by an *Orbivirus* (Family *Reoviridae*) that includes at least 24 different  
39 serotypes (Mathews, 1982; Mertens & Diprose, 2004). Traditionally, sheep have been  
40 considered the most severely affected livestock species, whereas cattle and goats usually  
41 appeared as asymptomatic reservoir hosts (MacLachlan, 1994). However, the recent  
42 outbreaks of BT occurred in both southern and northern Europe caused by different

43 serotypes, some of them never described previously in these countries, displayed severe  
44 clinical signs in cattle, goats and different species of wild ruminants characterized by  
45 fever, depression, nasal discharge, respiratory distress, oral ulceration and  
46 haemorrhages, subcutaneous oedema and cyanotic tongue (MacLachlan *et al.*, 2008;  
47 Saegerman *et al.*, 2008; Backx *et al.*, 2007; Dercksen *et al.*, 2007). Wild ruminants have  
48 been considered for a long time as asymptomatic hosts. However, recent studies have  
49 reported BT characteristic symptoms (Mauroy *et al.*, 2008) and the presence of the virus  
50 (Fernandez-Pacheco *et al.*, 2008) or BT specific antibodies (Linden *et al.*, 2008; Ruiz-  
51 Fons *et al.*, 2008) in wild species like yaks (*Bos grunniens grunniens*), mouflons (*Ovis*  
52 *aries musimon*) or wild red deer (*Cervus elaphus*), respectively. The role of wildlife in  
53 the prevalence and spreading of BT is beginning to be elucidated (Ruiz-Fons *et al.*,  
54 2008).

55         The analysis of samples from wild ruminants is becoming increasingly important  
56 in order to develop control programs and to elucidate the role of wildlife in the  
57 spreading and persistence of the disease. Recently, a study on the presence of BTV  
58 antibodies in wild ruminants in Spain (Ruiz-Fons *et al.*, 2008) and the characterization  
59 of BTV1 in mouflons (*Ovis aries musimon*) (Fernandez-Pacheco *et al.*, 2008) have been  
60 published. The adaptation of rapid molecular techniques, such as real-time PCR, to  
61 these samples can provide valuable information about the BTV serotype present in it,  
62 just by sequencing the PCR product. The advantages provided by the PCR technique –  
63 rapidity, high through-put, robustness- could replace or accompany seroneutralization as  
64 a method to characterize BTV serotypes in wildlife species.

65         In October 2007, two mouflons (*Ovis aries musimon*) -one male and one female-  
66 were found dead in the North of the Córdoba province (Spain). The animals were  
67 referred to the Diagnostic Services of the Veterinary Faculty (Córdoba University) for

68 routine necropsy. Tissue samples from these two animals have been analyzed in a  
69 retrospective study in order to detect the serotype of BTV that caused their death.

70

## 71 **Materials and Methods**

72 The samples analyzed in this study belong to two mouflons found dead in the  
73 field and transported to the Diagnostic Services of the Veterinary Faculty (Córdoba  
74 University, Spain) in order to determine the cause of their death. Macroscopical  
75 examination revealed the presence of subcutaneous oedema of the head and neck,  
76 hydrothorax and hydropericardium. Lungs appeared pale and non-collapsed, showing  
77 alveolar oedema. Subintimal petechial hemorrhages at the base of the pulmonary artery  
78 were also observed, together with the presence of diffuse hemorrhages in  
79 subendocardial and subepicardial locations of the heart. During necropsy, tissue  
80 samples from lung, heart, pulmonary artery, trachea, lymph nodes (submandibular and  
81 tracheobronchial), tongue, spleen, liver, small intestine, rumen, kidney, urinary bladder,  
82 adrenal gland, ovary and uterus were taken and fixed in 10% buffered formalin solution  
83 during 24h. After that, samples were routinely processed and embedded in paraffin wax.  
84 4 µm sections were cut and stained with haematoxylin and eosin (H&E) for  
85 histopathological study. Microscopical examination (Table 1) confirmed the lesions  
86 observed at necropsy. Both, histopathological and macroscopical studies were  
87 consistent with BTV infection.

88 Paraffin-embedded samples that remained after the study carried out by the  
89 Diagnostic Services of the Veterinary Faculty (Córdoba University, Spain) in order to  
90 determine the cause of death of the mouflons, were kept at RT and further analyzed in  
91 this study. One year after they were processed, the slides were included in a  
92 retrospective study that tentatively tried to identify the BTV serotype that caused the

93 death of the mouflons. Paraffin-embedded tissues from sheep experimentally infected  
94 with BTV1 and BTV4 were included in this study as positive control. These two  
95 serotypes were circulating in Spain at the time the mouflons were found dead, while  
96 BTV 8 was not detected in the country until January 2008, i. e. three months later  
97 ([http://www.oie.int/wahis/public.php?page=single\\_report&pop=1&reportid=668](http://www.oie.int/wahis/public.php?page=single_report&pop=1&reportid=668)). As  
98 negative controls As negative controls, paraffin-embedded tissue samples from BTV-  
99 free sheep and from a PRRS-infected pig were included in this study.

100 In order to avoid RNA contamination, disposable razors and the microtome  
101 components in contact with the samples were treated with RNaseZap (Ambion) prior to  
102 cutting each paraffin block. Furthermore, 10 µm sections were obtained by cutting a  
103 paraffin block from pig tissues inoculated with PRRS after each paraffin block from  
104 mouflons. Pig tissues were included as RNA extraction controls and negative controls  
105 for BTV. After trimming the excess of paraffin, tissues were scrapped off from the  
106 slides and their RNA was extracted using RNeasy<sup>®</sup> FFPE kit (Qiagen) following the  
107 manufacturer's instructions. Briefly, tissues were incubated for 10 sec at RT with  
108 xylene (Sigma-Aldrich) and ethanol 100% (Panreac). After centrifuging for 2 min at full  
109 speed, the supernatants were removed by pipetting and the tubes remained with the  
110 lids open until all residual ethanol evaporated. Then, the samples were incubated for 15  
111 min at 55°C and then 15 min at 80°C with Proteinase K and a buffer that reverses  
112 formaldehyde modification of nucleic acids. The mixture was transferred to a silicon  
113 column where the genomic DNA present in the sample was bound. After a short  
114 centrifugation, the flow-through was collected and mixed with 100% Ethanol in order to  
115 precipitate RNA. From this step on, the protocol proceeded as a regular RNA extraction  
116 protocol using spin columns. In the final step, RNA was resuspended in 20µl of PCR-  
117 quality water and frozen at -80°C. 3µl of the RNA obtained were amplified by RT-PCR.

118 Three RT-PCR assays were applied to the RNA samples: 1) a gel-based, group-specific  
119 RT-PCR that amplifies a common sequence -274bp- within BTV segment 5 (Agüero *et*  
120 *al*, 2002), 2) a gel-based, serotype specific RT-PCR using a set of primers that flank a  
121 765bp sequence within the segment 2 of BTV1 (Mertens, *et al* 2007) and 3) a serotype-  
122 specific real-time RT-PCR using a TaqMan<sup>®</sup> probe that overlaps a unique sequence of  
123 124 nucleotides within BTV segment 2, that is only present in BTV serotype 4  
124 (Rodriguez-Sanchez *et al.*, 2008). RNA extracted from purified BTV4 (SPA-1/2004)  
125 and BTV1 (ALG2006/01) and paraffin-embedded tissues from sheep experimentally  
126 infected with BTV1 and BTV4 were used as positive controls. The experiment was  
127 carried out blindly, using a code to identify each sample. Positive RT-PCR products  
128 obtained were sequenced with QIAquick PCR Purification Kit (Qiagen) and sequenced  
129 in an ABI 377 DNA sequencer and subsequently compared with other published BTV  
130 sequences using BLAST software (<http://blast.ncbi.nlm.nih.gov/>)

131

## 132 **Results**

133 Tissue samples from BTV-free sheep and PRRS infected pigs were negative to  
134 the three BTV RT-PCR assays.

135 Fixed and fresh tissues from sheep experimentally infected with BTV1 and  
136 BTV4 were also included in the RT-PCR assays. Tissues from sheep infected with  
137 BTV1 –mainly lung- were positive to the group-specific RT-PCR and to the BTV1  
138 specific RT-PCR, but all the tissues from the mouflons were negative to this assay (data  
139 not shown). Lung and tracheobronchial nodes were the samples that gave positive  
140 results for the group-specific RT-PCR and for BTV4-specific RT-PCR assay, while  
141 BTV-free tissues were always negative for these two tests (Fig 1). Two samples from  
142 lung tissue and lymph node, one from each mouflon, gave a positive plot for BTV4 real-



143 time RT-PCR (Fig 1). The same samples were also positive to the group-specific RT-  
144 PCR assay (data not shown).

145

146 BTV4-specific RT-PCR products were purified and sequenced. When these  
147 sequences were compared with other sequences from GenBank using BLAST software,  
148 the homology with other BTV4 segment 2 sequences ranged between 90% and 100%  
149 (Fig 2). The homology variation was due to the length of the RT-PCR product and to  
150 the genome variability in BTV segment 2. The sequences from the dead mouflons were  
151 100% homologous to BTV4 circulating in Portugal - EF434176-, Corsica - AY839945-  
152 and Turkey - DQ825670- or with BTV4 reference strains - AJ585125- and 90%  
153 homologous to other BTV4 strains - DQ191281, DQ191280-. No homology was found  
154 with other BTV serotypes.

155

## 156 **Discussion**

157 Identification of BTV serotypes has been previously carried out in mouflons  
158 (Fernandez-Pacheco *et al.*, 2008). In their study, the authors identified BTV1 in blood  
159 samples from animals that had been affected by BTV during the outbreak that took  
160 place in the south of Spain in 2007. In October 2004, a BTV4 outbreak occurred in  
161 southern Spain. The virus was detected for the last time one year later, in October 2005  
162 ([http://www.oie.int/wahis/public.php?page=event\\_summary&this\\_country\\_code=ESP&](http://www.oie.int/wahis/public.php?page=event_summary&this_country_code=ESP&reportid=5615)  
163 [reportid=5615](http://www.oie.int/wahis/public.php?page=event_summary&this_country_code=ESP&reportid=5615)). Domestic ruminants were vaccinated with the appropriate BTV vaccine  
164 -a live attenuated BTV4 vaccine in 2005 and an inactivated BTV4 from 2006 to 2008  
165 (Savine *et al.*, 2008)-. This control measure avoided the subsequent presence of both  
166 serotypes in the locations where they were first detected. However, the results of this  
167 study show the presence of BTV4 in wild ruminants from the same geographical area, at

168 a time when BTV4 was not detected in livestock anymore. Two hypotheses are possible:  
169 1) the presence of BTV4 in wild ruminants, as it has been described in this study, may  
170 be due to the arrival of infected *Culicoides* in Spain –in this case, the vaccination  
171 campaign would have proved to be highly effective- or 2) the persistence of infected  
172 vectors in the area due to milder temperatures. In the latter case, wild ruminants could  
173 have a role as a reservoir for BT. Thus, efforts should be focussed on controlling and  
174 surveying the wildlife, as well as improving the methods to detect and eliminate the  
175 vector.

176 The fact that the samples were not taken right after the death of the animals and,  
177 moreover, they were fixed and paraffin-embedded prior to RNA extraction and  
178 amplification by RT-PCR, shows that this method preserves the tissues and allows the  
179 detection of BTV by RT-PCR (Parsonson & McColl, 1995). Thus, the implementation  
180 of specific RT-PCR tests to fixed samples facilitates the development of retrospective  
181 studies. This methodology provides an efficient and rapid tool for surveillance and  
182 epidemiological studies.

183

## 184 **Conclusions**

185 To the authors' knowledge, this is the first identification of BTV4 in wild  
186 ruminants. The fact that the analysed samples from which BTV4 was detected had been  
187 fixed and paraffin-embedded for over one year prior to the isolation of BTV RNA  
188 proves that this method preserves enough nucleic acids to allow the detection of  
189 putative pathogens present in tissue samples.

190

191

192 **Acknowledgements**

193 This work was supported by grants from the Consejería de Innovación, Ciencia y  
194 Empresa, Junta de Andalucía (P05-AGR-00132). P. J. Sánchez-Cordón holds a contract  
195 of the “Ramón y Cajal programme” (Ministry of Education and Science, Spain). The  
196 authors want to thank the Spanish Ministry of Natural, Rural and Marine Environment  
197 for funding this project (RASVE 274/2007), Dr. Marisa Arias, Technical Director of the  
198 CISA-INIA laboratory (Madrid, Spain) for kindly providing us with BTV1 and BTV4  
199 positive controls, and the lab technicians from the Animal Health Department –Belen  
200 Rivera & Rocio Sanchez- for their enthusiasm and dedication to this study.

201

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- 246
- 247

248 **Figure legends**

249

250 **Table 1.** Summary of BTV4 detection by RT-PCR in paraffin-embedded tissue samples

251 and histopathological study.

252

253 **Figure 1.** Amplification plots obtained from the specific BTV4 real time RT-PCR on

254 different tissue samples. Fluorescence (on the Y axis) is represented against the

255 PCR cycle (on the X axis). 1) purified BTV4 (SPA-1/2004), 2) lung from the

256 female mouflon, 3) lymph nodes from the male mouflon, 4) lymph nodes from

257 female mouflon 5) lung from the male mouflon 6) fresh lung from a sheep

258 experimentally infected with BTV4, 7) formalin-fixed, paraffin-embedded lung

259 from the same sheep infected with BTV4 and 8) negative plots obtained from

260 fresh and paraffin-embedded lung of a sheep experimentally infected with BTV1,

261 from fresh lung belonging to a BTV-free sheep and from pig tissues inoculated

262 with PRRS.

263

264 **Figure 2.** DNA sequence from the RT-PCR product. The sequence from the amplified

265 mouflon samples is shown on the lower line and compared with GenBank

266 available sequences, with which it shares between 90-100% homology in the 3'

267 region. \* indicates perfect match with published BTV4 sequences.

268

Animal	Tissue samples	Results for the specific RT-PCR For BTV4	<i>Histopathological lesions</i>
Male mouflon	Lung	+	Alveolar oedema
	Heart	-	Diffuse haemorrhages in the myocardium
	Pulmonary artery	-	Subintimal petechial haemorrhages
	Tongue	-	No histopathological changes
	Lymph nodes	+	Intense lymphoid depletion
	Spleen	-	No histopathological changes
	Liver	-	Congestion
Female mouflon	Lung	+	Alveolar oedema
	Heart	-	Diffuse haemorrhages in the myocardium
	Pulmonary artery	-	No histopathological changes
	Tongue	-	Subepithelial and intermuscular haemorrhages
	Lymph nodes	+	Intense lymphoid depletion
	Spleen	-	Moderate lymphoid depletion
	Liver	-	Perivascular haemorrhages in portal spaces

Table 1





