Bluetongue virus in Oryx antelope (Oryx leucoryx) during the quarantine period in 2010 in Croatia

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Entomological survey,
Oryx antelope,
Quarantine.

Summary
Bluetongue (BT) is a viral infectious non-contagious disease of domestic and wild ruminants. Insect species of the genus Culicoides (Diptera: Ceratopogonidae) serve as biological vectors that transmit bluetongue virus (BTV) to susceptible hosts. The infection is present in the Mediterranean region. Recently, it has also been reported in Central, Western, and Northern Europe where BTV-8 was recognised as the causative serotype. In the meantime, BTV-14 has appeared in the North-Eastern part of Europe. In the present study, BTV serotype 16 (BTV-16) was detected by virus neutralisation (VNT)-assay and real-time reverse transcription-PCR (RT-PCR) in 1 antelope and BTV-1 in 3 of 10 Oryx antelopes (Oryx leucoryx) imported in Croatia from the Sultanate of Oman. No BTV vectors were collected during the antelope quarantine on the Veliki Brijun Island. Also, no BTV antibodies were detected in sheep, cattle, and deer on the Island. Entomological studies did not reveal any new vector species that may have been introduced with the infected antelopes on their transportation. It was the first time that BTV was demonstrated in animals imported in Croatia. It involved BTV-1, which had never been demonstrated before and BTV-16, which had been previously recorded in domestic ruminants.

Virus della Bluetongue rinvenuto in antilope (Oryx leucoryx) durante il periodo di quarantena nel 2010 in Croazia

Parole chiave
Antilope (Oryx leucoryx),
BTV-1,
BTV-16,
Croazia,
Entomologia,
Quarantena.

Riassunto
La Bluetongue (BT) è una malattia virale non contagiosa dei ruminanti domestici e selvatici. Gli insetti del genere Culicoides (Diptera: Ceratopogonidae) fungono da vettori biologi nella trasmissione del virus della Bluetongue (BTV) a ospiti sensibili. L'infezione è presente nella regione del Mediterraneo. Recentemente è stata anche documentata in Europa centrale, occidentale e settentrionale, dove BTV-8 è stato riconosciuto come sierotipo causale. In questo contesto è stata registrata la comparsa di BTV-14 in Europa nord-orientale. In questo studio, BTV sierotipo 16 (BTV-16) è stato rilevato in 1 antilope e BTV-1 in 3 di 10 Oryx antelopi (Oryx leucoryx) importati in Croazia dal Sultanato dell'Oman. Non è stato identificato BTV vettore durante la quarantena sull'isola di Veliki Brijun. Inoltre, non sono stati rilevati anticorpi per BTV negli ovini, bovini, e cervi presenti sull'isola. Gli studi entomologici non hanno evidenziato la presenza di nuovi vettori introdotti con le antilopi. Questo studio ha riportato il primo caso di BTV in animali importati in Croazia e dimostra, per la prima volta, la presenza nel Paese di BTV-1 e di BTV-16 che però è stato già in precedenza rilevato nei ruminanti domestici.
**Introduction**

Bluetongue (BT) is an insect-borne, infectious, non-contagious disease of ruminants transmitted among its hosts by *Culicoides* biting midges (Diptera: Ceratopogonidae) (Meiswinkel et al. 2004). Bluetongue virus (BTV) infection involves domestic and wild ruminants such as sheep, goats, cattle, buffaloes, deer, most species of African antelope, and various other Artiodactyla as vertebrate hosts (OIE 2009). About 30 *Culicoides* species play a role in BTV transmission worldwide (Meiswinkel et al. 2004). From 1998 to 2012, at least 6 serotypes (BTV-1, BTV-2, BTV-4, BTV-8; BTV-9, and BTV-16) were present in Southern Europe (Saegerman et al. 2008, Savini et al. 2009).

In the Mediterranean region, the most important vector is *Culicoides imicola*, which is probably responsible for 90% of disease transmission (Meiswinkel et al. 2004). The 3 remaining vectors are *Culicoides obsoletus* and *Culicoides scoticus* - also of the subgenus *Avaritia*, but placed within the Obsoletus Complex - and *Culicoides pulicaris* (subgenus *Culicoides*) (Meiswinkel et al. 2004). Earlier serological surveys have shown that many BTV serotypes, including those that have recently caused outbreaks in the Mediterranean region, have been present at the periphery of Europe for several decades, most notably in the sub-Saharan Africa, Cyprus, Turkey, and the Middle East (Mellor et al. 2009). Most commonly, transmission of BTV occurs by incursions from the wind-assisted movement of infectious *Culicoides* midges or imported viraemic livestock (Carpenter et al. 2013). Numerous wild ruminant species may serve as BTV reservoirs in Europe and are likely to play a role in the disease epidemiology (Falconi et al. 2011).

In Croatia, the disease was first reported in 2001 in Dubrovnik-Neretva County, where the BTV-9 serotype was identified in domestic ruminants (Listeš et al. 2004, Listeš et al. 2011). Extensive serological studies of sentinel ruminants were carried out in the area, where the BTV-16 serotype was first demonstrated in 2004 (Listeš et al. 2009). Entomological survey of the blood-sucking genus *Culicoides* revealed the potential vectors of the *Culicoides obsoletus* group and *Culicoides pulicaris* group to be widely represented (Bosnić et al. 2011).

Here we describe the detection of the BTV-1 and BTV-16 serotypes in the Arabian Oryx antelope (*Oryx leucoryx*), imported from the Sultanate of Oman, during the quarantine period on the island of Veliki Brijun in Croatia. The results of testing for BTV accompanying the shipment were negative. In addition, comprehensive investigation for the presence of *Culicoides* vectors on the quarantine object was performed in order to assess the possibility of viral dissemination after departure of Oryx antelopes by serological testing of the sheep, cattle, and the wild population of fallow deer living on the island.

**Materials and methods**

**Case history and study area**

On March 4, 2010, 10 Oryx antelopes were imported to Croatia from the Sultanate of Oman. The animals were air transported, each in a separate box. The Oryx antelopes were placed into quarantine on the island of Veliki Brijun. As per protocol, they were meant to spend 6 months on the island before being transported to their final destination in the United Kingdom. The antelopes were accommodated in a vector-free facility to protect them from *Culicoides* attacks.

The Veliki Brijun National Park is located in the North of Adriatic Sea, 3 km from the South-West part of the Istra Peninsula. The island is characterized by mild Mediterranean climate, rather high air humidity, rich vegetation, with 3 ponds, and stagnant waters. The island has a considerable population of wild animals, predominantly fallow deer (*Dama dama*) and axis deer (*Axis axis*), mouflon (*Ovis musimon*), and exotic herbivores. Domestic animals, sheep, goats, cattle, horses, and donkeys, as well as autochthonous birds also live on the island.

**Serology and virology**

During quarantine, serum and ethylenediaminetetraacetic acid (EDTA) blood samples were collected from 10 Oryx antelopes. Serum samples were serologically tested at the Croatian Veterinary Institute using a commercial competitive enzyme linked assay (c-ELISA; INGEZIM BTV Compact 12.BTV.K.3, INGENASA, Madrid, Spain), which detects antibodies against the BTVVP7 protein. All serum and blood samples were also sent to the European Reference Laboratory for Bluetongue at The Pirbright Institute, UK, for further analyses including virus neutralization assay (VN-assay) (OIE 2009), real-time reverse transcription-PCR (rRT-PCR) (Shaw et al. 2007), and serotype specific rRT-PCR (Mertens et al. 2007). Immediately upon detection of the infection, in April 2010, blood samples were collected from 11 sheep accommodated some 30 metres from the quarantine stable. Then, 11 sheep and 13 cattle living nearby were blood sampled every 15 days (a total of 182 samples) from the end of June to the mid-October 2010, and 46 fallow deer sera were also collected once in August to monitor the possible BTV circulation by c-ELISA.
Entomological survey

Following the Oryx arrival, entomological survey of biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) was performed using the Onderstepoort-type blacklight suction traps (car battery) (Goffredo and Meiswinkel 2004). The traps were placed in the Oryx quarantine and in the sheep stable nearby the quarantine site. Insect samples were collected until April 5, when the animals were transported back to the Sultanate of Oman. Six samples were captured in the Oryx quarantine facility and another 6 in the sheep stable. Entomological survey was continued upon departure of Oryx antelopes. From March to October 2010, a total of 139 light trap collections and 90 *Culicoides* specimens were obtained from the empty Oryx quarantine facility, sheep stable, and ornithological reserve also inhabited by deer. All captured adult *Culicoides* were identified at the species level based on the wing pattern and sex (Delécolle 1985). Male *C. obsoletus/scoticus* genitals were differentiated by form and size, and were classified at the species level. All caught females were age-graded (Dyce 1969) as nulliparous, parous, and blood fed.

Results

Four of 10 Oryx sera were positive to c-ELISA. In 3 of them, BTV-1 neutralising antibodies were detected, whereas 1 had antibodies against BTV-16. Specific antibodies titre ranged from 1:80 to ≥ 1:1280 (Table I). The real time rRT-PCR also identified 4 positive samples from the same 4 seropositive animals. Serotype-specific rRT-PCR showed that 1 blood sample contained BTV-16 and 2 blood samples tested positive for BTV-1. In the fourth rRT-PCR-positive sample, the attempt to identify serotype failed. All 182 sheep and cattle, and 46 fallow deer serum samples tested with ELISA were negative.

The predominant species collected at the 2 sites were *C. obsoletus/scoticus* (subgenus *Avaritia*) belonging to the Obsoletus complex (43%, n = 39), followed by *Culicoides newsteadi* (subgenus *Culicoides*) of the Pulicaris complex (40%, n = 36), *Culicoides circumscriptus* (subgenus *Beltranmya*) (13%, n = 12) and *Culicoides maritimus* (subgenus *Oecacta*) (3%, n = 3) (Table II). None of the 6 catches in the Oryx quarantine facility included species of the genus *Culicoides*. During the quarantine period, adult females of *C. obsoletus/scoticus* (10 nulliparous) were collected in the trap in the sheep stable. One *C. obsoletus/scoticus* (1 nulliparous) was caught in the facility 4 months after departure of the antelopes. The highest number of the BTV potential vector (*C. obsoletus/scoticus*) was detected in sheep stable, i.e. in 12 of 48 light trap collections. According to age grade, 18 of 32 adult females were nulliparous, 11 parous, and 3 blood fed; whereas males belonged to the *C. obsoletus* (n = 2) and *C. scoticus* (n = 1) species. Accordingly, only *C. obsoletus/scoticus* (n = 35) were detected in the catches placed in the sheep stable. Out of 43 catches in the ornithological reserve, 3 *C. obsoletus/scoticus* (1 nulliparous, 1 parous and 1 male belonging to *C. obsoletus*), 36 *C. newsteadi* (17 nulliparous, 11 parous, 2 blood fed and 6 males), 12 *C. circumscriptus* (7 nulliparous and 5 parous) and 3 *C. maritimus* (2 nulliparous and 1 parous) were collected in 9 samples.

Discussion

At the beginning of March 2010, 10 Oryx antelopes were imported to Croatia from the Sultanate of Oman. BTV-1 and BTV-16 were detected in 4 animals housed in the quarantine facility on the Veliki Brijun Island. We found that the antelopes

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**Table I.** Results of serological tests for Bluetongue virus (BTV) antibodies performed on antelope sera.

<table>
<thead>
<tr>
<th>Antelope sera</th>
<th>ELISA</th>
<th>VN-test antibody titre</th>
<th>BTV-serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1715/1</td>
<td>positive</td>
<td>1:640</td>
<td>BTV-16</td>
</tr>
<tr>
<td>1715/3</td>
<td>positive</td>
<td>1:80</td>
<td>BTV-1</td>
</tr>
<tr>
<td>1715/4</td>
<td>positive</td>
<td>≥1:1280</td>
<td>BTV-1</td>
</tr>
<tr>
<td>1715/8</td>
<td>positive</td>
<td>≥1:1280</td>
<td>BTV-1</td>
</tr>
</tbody>
</table>

**Table II.** Culicoides adults collected at 3 sites in Veliki Brijun island (Croatia).

<table>
<thead>
<tr>
<th>Culicoides species</th>
<th>Quarantin facility</th>
<th>Empty stable</th>
<th>Sheep stable</th>
<th>Ornithological reserve</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. obsoletus/scoticus</em></td>
<td>0</td>
<td>1</td>
<td>35</td>
<td>3</td>
<td>39 (43.4%)</td>
</tr>
<tr>
<td><em>C. newsteadi</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>36</td>
<td>36 (40.0%)</td>
</tr>
<tr>
<td><em>C. circumscriptus</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>12 (13.3%)</td>
</tr>
<tr>
<td><em>C. maritimus</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3 (33.3%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0</td>
<td>1</td>
<td>35</td>
<td>54</td>
<td>90 (100%)</td>
</tr>
</tbody>
</table>

*after departure of antelope.
had already been infected when transported from the Sultanate of Oman. While BTV-16 had been previously detected in Dubrovnik-Neretva County (Listeš et al. 2004), BTV-1 was identified for the first time in Croatia, as confirmed by the VN-assay and the RT-PCRs used in the study.

The first European isolates of BTV-1 GRE2001 proved to be related to viruses from India and Malaysia (Mellor et al. 2009). The virus is thought to have entered Europe from the East, possibly via Turkey, even though the serological survey conducted in Turkey in the early 1980s did not find any BTV-1 positive animals. Instead, a Western corridor was hypothesised for the BTV-1 2006-2007 incursions in Sardinia (Italy), Spain, Portugal, Gibraltar, and France (Mellor et al. 2009). New BTV-1 incursions have recently been observed in Sardinia and mainland Italy (Lorusso et al. 2013, Lorusso et al. 2014). Bluetongue virus has been reported in the Arabian Peninsula, as well as in the Sultanate of Oman, Saudi Arabia, and the United Arab Emirates (Al-Busaidy and Mellor 1991, Frölich et al. 2005). In Oman, BTV (serotypes 3, 4, 17, 20 and 22) was detected in 1987-1988, having become enzootic in the Northern part of the country (Al-Busaidy and Mellor 1991). In another study, Taylor and colleagues (Taylor et al. 1991) indicated that BTV was present throughout Oman and that domestic ruminants were involved to a varying extent in its maintenance. In 2009, BTV-1, BTV-4, BTV-8, and BTV-16 were also isolated in Oman. Concerning the Arabian Oryx, Frölich and colleagues (Frölich et al. 2005) found a high prevalence of antibodies against BTV and epizootic hemorrhagic disease virus in Saudi Arabia and the United Arab Emirates during the 1999-2001 period. These findings indicate that Arabian Oryx antelopes are likely to be susceptible to both viruses, yet saying nothing about the possible epidemiological role played by this species.

Based on these data, the possibilities that infected Culicoides might have been carried with antelopes on their transportation and disseminated over the island as well as viraemic animals infected by local vectors were investigated during and after the quarantine period. Serological testing did not demonstrate the presence of BTV in the sheep, cattle, and fallow deer living near the quarantine facility. No potential Culicoides vectors were captured in the Oryx antelope facility during the quarantine period. Moreover, no other potential vectors that are not normally present in Croatia were captured at any other study site. Finally, after 33 days of quarantine, the antelopes were removed from the island and transported back to the Sultanate of Oman.

According to the results of this study, BTV was not disseminated via vectors to domestic and wild animals on the island. Previous entomological studies have demonstrated that the species belonging to the Obsoletus and Pulicaris complexes were likely to play an important role in transmitting BTV in Croatia (Bosnić 2011). In the present study, C. obsoletus/scoticus were the Culicoides species most often detected in the sheep stable. Only 1 C. obsoletus/scoticus (1 nulliparous) was captured in the quarantine facility after departure of the antelopes; the mites may have entered the facility upon door opening. As the trap was placed in the ornithological reserve near the marshy lake inhabited by wild ducks and in the reserve inhabited by fallow deer, the mammalophilic species Culicoides newsteadi predominated among the captured species, followed by the ornithophilic species Culicoides circumscriptus and Culicoides maritimus favouring saline areas and occurring mostly along the coastlines of Europe (Meiswinkel, personal communication).

The peak catches occurred in the second half of April and in May 2010. In contrast to the results of a previous monitoring in Croatia reporting the peak catches occurred in September, October and November (Bosnić 2011). Obviously, ecological conditions and heterogeneous animal population on the island proved to be favourable for the development of certain Culicoides species.

According to the Decree Prohibiting the Importation and Transit into the Republic of Croatia Territory of Domestic and Wild Ruminants and Genetic Material Derived from Ruminants in Order to Prevent the Introduction of the Bluetongue Disease (NN 66/08), no ban was in force on the import of wild ruminants from the Sultanate of Oman at the time. However, a ban has been enforced after this incident. Based on the measures taken and the results of this study, it was very important that no BTV vectors were identified in the quarantine during the stay of the Oryx antelopes, and that serology tests in the sheep, cattle, and fallow deer were negative for BTV. Accordingly, the risk of BTV circulation could be considered negligible.

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References


