

An outbreak of bluetongue virus serotype 9 in Southern Croatia

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Abstract

The aim of this study was to provide a description of the first epidemic of bluetongue and the first survey on midges of the genus *Culicoides* in Croatia. Clinical signs were firstly observed on November 2001 in sheep in Konavle, Dubrovnik – Neretva County. During this epizootic the overall sheep morbidity and mortality were 5.2% (95% confidence interval (c.i.), 4.1–6.6%) and 2.29% (95% c.i., 1.6–3.3%), respectively. After the outbreak, 3,318 serum samples of ruminants from 53 villages of the Dubrovnik – Neretva County were examined for bluetongue virus (BTV) antibodies by competitive enzyme-linked immunosorbent assay (cELISA). In forty nine (92.45%, 95% c.i., 82.11–96.92%) of the 53 villages, animals with antibodies against bluetongue virus were found. In particular, a total of 178 cattle (49.86%, 95% c.i., 44.7–55.0%), 174 sheep (13.72%, 95% c.i., 11.9–15.7%) and 270 goats (15.95%, 95% c.i., 14.3–17.8%) were seropositive. Antibodies to bluetongue virus serotype 9 were detected in 212 positive sera by serum neutralization test. The percentage of positive animals decreased ($P > 0.05$) from the east to the west suggesting a possible east westward spreading of BTV infection. Fourteen light-trap midge collections from seven different sites were examined. Of the 4872 *Culicoides* spp. collected, 4,492 (92%, 95% c.i., 91.4–92.9%) of them belonged to the species of *Obsoletus complex*. This study showed for the first time that a pathogenic strain of BTV-9, probably from Montenegro, entered Croatia causing disease and death in local sheep and that *C. obsoletus* and *C. scoticus* were likely the major vectors of infection.

BTV-9, ruminants, cELISA, serum neutralization test, culicoides, light-trap

Bluetongue (BT) is a vector-borne viral non contagious disease of ruminants caused by bluetongue virus (BTV), an RNA virus which belongs to the *Reoviridae* family within the *Orbivirus* genus (Erasmus 1975). Twenty four serotypes have been formally identified (Gibbs and Greiner 1994) and other two serotypes have been recently suggested (Hofmann et al. 2008; Maan et al. 2011).

Midges of the genus *Culicoides* act as biological vectors. Bluetongue virus infection might cause disease in ruminants. Symptoms are more frequent and severe in sheep (Erasmus 1975); however, a clinical form of disease might occur in goats and cattle as recently observed in Northern Europe (Saegerman et al. 2008). The main symptoms of bluetongue include increased temperature, lesions in oral mucosa, lameness, catarrhal nasal discharge, and edema of the tongue, lips, maxillomandibular space and neck (Erasmus 1975).

Before 1998, BT was recorded only occasionally in Europe (Mellor and Wittmann 2002). Since 1998, 9 different BTV serotypes (BTV-1, BTV-2, BTV-4, BTV-6, BTV-8, BTV-9, BTV-11, BTV-16) have affected countries facing the Mediterranean region and

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then, from 2006, countries of the Central and Northern Europe (Mellor and Wittmann 2002; Listes et al. 2009).

The aim of this study was to describe the first occurrence of BTV-9 in Croatia.

Materials and Methods

Following the first confirmed BT case, several surveillance measures have been implemented to control and determine the extension of the exposed area. Extensive monitoring has been carried out in sheep, goats and cattle in the areas surrounding the outbreak of BT. In the Konavle county, blood samples were collected from all ruminants whereas in the Dubrovnik and Dubrovnik Littoral counties, samples were collected randomly. The same method was used for sampling in a goat flock from the Pelješac peninsula. Because of the limited number of animals per flock, the entire village was considered as a flock.

From the end of January till June 2002, a total of 3,318 serum samples from 53 villages were collected and examined for the presence of BT virus antibodies; the number of tested animals is presented in Table 1.

Table 1. Total number of tested animals according to species and geographic area

Geographic area	Cattle	Goat	Sheep	Total
	Positive/total (%)	Positive/total (%)	Positive/total (%)	Positive/total (%)
Dubrovnik Littoral	11/60 (18%)	13/203 (6.4%)	9/179 (5%)	33/442 (7.5%)
Dubrovnik	15/20 (75%)	8/109 (7.3%)	19/266 (7.1%)	42/395 (10.6%)
Konavle	152/277 (54.9%)	245/1295 (18.9%)	146/823 (17.7%)	543/2395 (22.7%)
Pelješac peninsula	0	4/86 (4.6%)	0	4/86 (4.6%)
Total	178/357 (49.8%)	270/1693 (15.9%)	174/1268 (13.7%)	622/3318 (18.7%)

A commercial competitive enzyme-linked immunosorbent assay (cELISA, Veterinary Medical Research & Development, Inc., Pullman, WA, USA) was used to detect BT antibodies in serum samples. To identify the BT virus serotype involved and determine the titres of BT virus antibodies, c-ELISA positive serum samples were further tested by the serum-neutralisation assay (Listes et al. 2009) at the Virology Department, Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise (IZSAM) "G. Caporale", Teramo, Italy, OIE Reference Laboratory for BT. Positive and negative controls for serum neutralization were provided by the OIE Reference Laboratory of the Onderstepoort Veterinary Institute, South Africa.

One sheep from the village Gruda, Konavle area, with severe signs of the disease was euthanised. At necropsy, liver, spleen and lymph-node samples were collected, stored in 10% buffered formalin solution and subsequently processed for BTV detection by using the reverse-transcriptase polymerase chain reaction (RT-PCR) (Parsonson and McColl 1995).

Spleen and mesenteric lymph-node samples were stored for 4 years in 10% formalin before examination. The samples were first washed three times in PBS to remove formalin, then 1 g of spleen and 1 g of lymph-node were homogenized by adding 1 ml of PBS and using a sterile mortar and pestle. The two tissue samples were analyzed in separate. Then, 500 ml of each minced organ were transferred to 1.5-ml Eppendorf tubes and frozen at -20 °C. A homogeneous portion of each tissue was thawed and incubated with protease K (final concentration of 50 ml/ml) and Sodium Dodecyl Sulphate (SDS) (final concentration of 0.1%). Viral RNA extraction was performed by use of TRIzol reagent according to the method described elsewhere (Chomczynski and Sacchi 1987).

One-step RT-PCR with BT9R and BTB primers, as described by Katz et al. (1993) were used in the study. The primers amplify a part of the region encoding non-structured NS1 in viral RNA segment 5. The primers were

Table 2. Sites of collection of *Culicoides* made in September 2002

County	Location	Animal hosts in the stables	Number of collection
Split-Dalmatia	Brštanovo	sheep	1
Split-Dalmatia	Čiovo	sheep	1
Dubrovnik-Neretva	Brsečine	sheep, goats, cattle	4
Dubrovnik-Neretva	Komolac	goats, cattle	5
Dubrovnik-Neretva	Pridvorje	sheep	1
Dubrovnik-Neretva	Čilipi	goats	1
Dubrovnik-Neretva	Molunat	sheep	1
Total			14

used in reaction concentration of 0.6 pmol/ml. RNA was denatured by methyl-mercury hydroxide, as described by Wade-Evans et al. (1990) and used as a basis for cDNA synthesis. The One-Step RT-PCR kit (QIAGEN, Germany) was used for the reaction of reverse transcription and amplification. The temperature setting was as follows: 30 min at 48 °C, 10 min at 95°C, 40 cycles at 94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 7 min. Electrophoresis was performed on 1.5% agarose gel.

The entomological survey was carried out in collecting sites of two counties of southern Dalmatia: Split – Dalmatia and Dubrovnik – Neretva (Table 2).

Insect collection was performed by using Onderstepoort black light-traps (220 V, 8 W) placed close to the sheep, goats and cattle farms. In September 2002, the traps were active from the sunset till the sunrise at seven locations in Dubrovnik – Neretva County. In 14 cases midges were collected. They were then identified according to the protocols of the OIE Reference Laboratory for BT at IZSA&M and kept in 70% ethanol.

The prevalence levels in animals were calculated through a Bayesian approach using the Beta (s+1, n-s+1) distribution where s is the total number of positives and n is the total number of tested animals (Sivia 1996). The probability distribution of the percentage of positive animals shows not only the most probable value of prevalence, but also the level of uncertainty due to sample size. Upper and lower interval limits at 95% confidence level were also calculated.

Results

In November 2001, clinical symptoms indicative of BT were observed in sheep flocks in Konavle, the southernmost area of Croatia bordering on Montenegro and Bosnia and Herzegovina. The symptoms included excessive drooling, oedema of the maxillomandibular space, tongue and cheek, oral mucosa erosions, and elevated body temperature of up to 40.7 °C. Reddened coronary hoof band with consequential lameness and sporadic abortion in late gestation sheep were also recorded. In the Konavle area, 51 sheep showed BT clinical signs. The morbidity was 6.2% (51/823, 95% confidence interval (c.i.), 4.7-8.1%) whereas the mortality 2.79% (23/823, 95% c.i., 2.1-4.4%). In the Dubrovnik area 15 sheep were sick, in this county the morbidity was 5.64% (15/266, 95% c.i., 3.5-9.1%) and the mortality 2.25% (6/266, 95% c.i., 1.1-4.8%). The overall sheep morbidity and mortality during BT epizootic was 5.2% (66/1268, 95% c.i., 4.1-6.6%) and 2.29% (29/1268, 95% c.i., 1.6-3.3%), respectively. No symptoms were observed in cattle whereas three cases were reported in goats. In these animals the symptoms were similar to those observed in sheep but much less severe.

Seropositive animals were detected in 49 flocks, yielding 92.45% (95% c.i., 82.11-96.92%), of infected flocks. Of the 3,318 serum samples tested, 622 (18.7%, 95% c.i., 17.5-20.1%) were positive to BTV c-ELISA, namely 178 cattle (49.86%, 95% c.i., 44.7-55.0%), 174 sheep (13.72%, 95% c.i., 11.9-15.7%) and 270 goats (15.95%, 95% c.i., 14.3-17.8%). Antibodies to serotype 9 were detected by MTSN in 212 positive sera. Comparison of the species seroprevalence revealed a higher ($P < 0.05$) seroprevalence in cattle (49.86%) compared to that observed in goats (15.95%) and sheep (13.72%). A higher prevalence of BTV was found in cattle (54.87% and 75%) and sheep (17.74% and 7.34%) of Konavle and Dubrovnik, respectively. In contrast, more ($P < 0.05$) BTV positive goats (18.92%) were observed in Konavle compared to the other three areas included in the study (Fig. 1).

The PCR method performed on tissue sections, detected presence of BTV RNA. However, the very small amount of the amplified product did not allow any sequencing and molecular characterization.

Culicoides were identified on the basis of their wing pattern, and the findings were confirmed at the IZSA&M. Fourteen insect catches were obtained from the entomological survey. Of a total of 44,008 insects collected, 4,872 were identified as *Culicoides* spp. and 4,492 (92%) of them belonged to the species of the *Obsoletus complex*: *C. obsoletus*, *C. scoticus*. Other *Culicoides* species such as *C. circumscriptus*, *C. paolae*, *C. punctatus*, species of the *Pulicaris complex*, *C. seavanicus*, *C. fascipennis*, *C. haranti* and *C. fagineus* were also identified. *Culicoides C. obsoletus* and *C. scoticus* were the most widespread

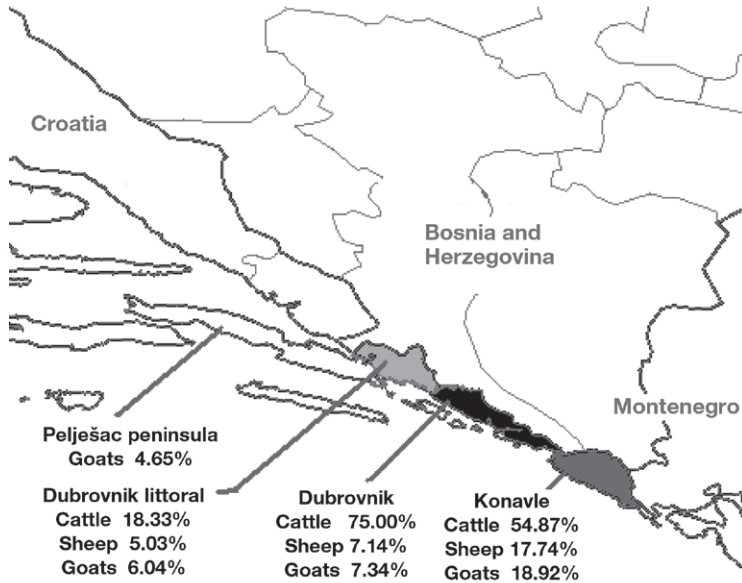


Fig. 1. Bluetongue prevalence in ruminants in Dubrovnik - Neretva County.

species, being recorded in all sites except on the island of Čiovo (Split – Dalmatia county). The mean air temperature at the time of the study was 20 °C.

Discussion

We reported the first occurrence of BT in Dubrovnik – Neretva County in the southernmost part of Croatia. The area lies at 52 m above sea level, 42 °N. During the years 1993–2002, the mean daily air temperature in the area in September was 21.3 °C, mean air humidity 61.4%, and mean monthly precipitation 90.8 mm. Bluetongue virus was demonstrated clinically, and confirmed by serology and virology. The Croatian strain was pathogenic and capable of causing disease and death in sheep, mild sporadic symptoms in goats but resulting completely asymptomatic in cattle. The clinical signs were in line with those described in sheep by Erasmus (1975).

The infection which involved most (92.45%) of the Southern Croatian flocks was not uniformly spread. More cattle (49.86%) were found infected compared to goats (15.95%) and sheep (13.72%) This supports the hypothesis that insects prefer to feed on cattle (Bowne et al. 1966) which, in other words could mean that BTV multiplied initially in cattle and subsequently it was transmitted to sheep and goats (Parsonson 1992). The presence of BTV-9 neutralising antibodies in 212 c-ELISA positive sera proved that BTV-9 was the serotype responsible for the Croatian outbreaks. The fact that the BTV-9 infection was reported in Turkey, Greece, Bulgaria, Macedonia, Serbia and Montenegro (Mellor and Wittmann 2002), and later also in Albania, and Bosnia and Herzegovina suggests that BTV-9 reached Croatia *via* eastern arm of the epizootic circulating in the Mediterranean basin.

It appears quite conceivable that BT was transferred by infected *Culicoides* or animals from the neighbouring Serbia and Montenegro as the prevalence of seropositive animals

in this study declined from the east westwards. The highest proportion of seropositive animals were recorded in Konavle, followed by Dubrovnik, Dubrovnik Littoral and Pelješac peninsula. The significant difference in the prevalence of seropositive animals between particular areas suggests that the infection likely spread westwards.

Among the *Culicoides* caught, the predominant species were those of the Obsoletus complex, in particular *C. obsoletus* and *C. scoticus* (92%). Species of the Obsoletus complex have already been associated to BTV transmission. Bluetongue virus was isolated from *C. obsoletus* in Cyprus (Mellor and Pitzolis 1979) and in Italy (Savini et al. 2005). Other species were also identified in the outbreak areas: *C. circumscriptus*, *C. paolae*, *C. punctatus*, *C. seavanicus*, *C. fascipennis*, *C. haranti* and *C. fagineus*, and *C. pulicaris*, a species belonging to the Pulicaris complex. Among these species, *C. pulicaris* could be a possible BTV vector (Caracappa et al. 2003). The identified species *C. circumscriptus*, *C. pulicaris*, *C. seavanicus*, *C. fascipennis* and *C. fagineus* were also found in Greece in 1983 (Mellor et al. 1984), whereas *C. paolae* is a new species recently recorded in Southern Italy (Boorman et al. 1996).

Analysis of climate data has been used to predict the potential distribution of *C. imicola* in Europe, revealing the possibility for *C. imicola* to spread from Greece and Italy to some parts of the Croatian Littoral as well as to the littoral areas of Albania, Serbia, Montenegro, and Bosnia and Herzegovina (Wittmann et al. 2001), which was not demonstrated in the present study. In conclusion, a pathogenic strain of BTV-9, probably from Montenegro, entered Croatia infecting ruminants and causing disease and death in local sheep. As no *C. imicola* was caught, the major role of transmitting the infection might have been played by *C. obsoletus* and *C. scoticus*.

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