

Serological evidence of bluetongue and a preliminary entomological study in southern Croatia

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Summary

In December 2001, bluetongue (BT) was confirmed serologically by the Croatian Veterinary Institute using the competitive enzyme-linked immunosorbent assay (c-ELISA). Results of the serological testing of blood samples from ruminants in the Dubrovacko-Neretvanska County are presented (3 318 sera of ruminants from 53 herds were examined). In total, 357 bovine sera (178 or 49.9% positive), 1 268 ovine sera (174 or 13.7% positive) and 1 693 caprine sera (270 or 15.9% positive) were tested. Antibodies to BT virus serotype 9 were detected in 212 of the positive sera by serum neutralisation. A preliminary light-trap survey for midges of the *Culicoides* genus was also performed in the Dubrovacko-Neretvanska County. Fourteen light-trap collections from seven locations were examined and yielded a total of 4 872 *Culicoides* of which 4 492 (92%) belonged to the *Obsoletus* Complex (including *C. obsoletus* and *C. scoticus*).

Keywords

Bluetongue – Competitive enzyme-linked immunosorbent assay – Croatia – *Culicoides* – Light trap – Ruminant – Serum neutralisation test.

Introduction

Bluetongue (BT) is a viral, non-contagious, arthropod-borne, infectious disease of domestic and wild ruminants caused by the BT virus (BTV) of the family *Reoviridae*, genus *Orbivirus* (5). To date, twenty-four virus serotypes have been identified worldwide (7). Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae), the biological vectors of BTV, play the principal role in disease spread. *Culicoides imicola*, subgenus *Avaritia*, which is the most widely spread species of bloodsucking *Culicoides*, acting as vectors of BT and African horse sickness, occurs in Africa and most countries of the Mediterranean Basin (8, 14). In central and southern Europe, *C. obsoletus* is one of the most common species encountered (11). More than 50 virus types have been isolated from *Culicoides* worldwide (8). For bluetongue, clinical symptoms vary from being subclinical to mild and acute, and can terminate in

the death of the animal. Symptoms are most pronounced in sheep. The disease can be seen in the acute or subacute form, depending on the pathogenicity of the viral strain and the sensitivity of the breed. In sheep, the predominant symptoms are elevated body temperature, oral lesions, lameness, catarrhal rhinitis, oedema of the tongue, lips, intermaxillary space and neck (4). In cattle and goats, the disease usually assumes a subclinical course without severe symptoms (4).

The disease was first identified and described in South Africa; however, with time, it has also been recorded from other regions of the world, i.e. in the Caribbean, Asia, Australia, North and South America, and Europe. BT is found only in regions where competent vectors occur, between latitudes of 35°S and 40°N. However, in the western areas of North America and in the People's Republic of China, the disease has spread to 50°N (11). In

Europe, the disease has previously been recorded in Cyprus (1924, 1943 and 1977), Portugal and Spain (1956-1957), and on the Greek islands of Rhodes and Lesbos (1979-1980).

Between 1998 and 2004, an epizootic of BT occurred in the Mediterranean Basin, also affecting countries where BT had never previously been recorded. At least four virus serotypes circulated during the epizootic, which progressed in two directions, with eastern and western fronts and with two sources of infection (11). The eastern front of the BT epizootic originated on the Greek islands (Rhodes, Leros, Kos and Samos) in October 1998 where serotype 9 was identified. During 1999, serotype 9 was confirmed in southern Bulgaria, the European part of Turkey and on mainland Greece, along the borders with Bulgaria and Turkey (the epizootic covering one third of Greece). In addition to serotype 9, serotypes 4 and 16 were also identified (11). During the years that followed, the disease was recorded for the first time in Kosovo, Serbia and Montenegro, the Republic of Macedonia and Croatia (11). In 2002, BT was recorded in Albania and Bosnia and Herzegovina for the first time. The western front of the epizootic originated in Tunisia in December 1999 where the virus was serotyped as BTV-2 which, in 2000, spread to Italy, Corsica (France) and the Balearic Islands (Spain) (11).

Results of serological studies to determine the prevalence and distribution of BT antibodies, and the conclusions of a preliminary study of the *Culicoides* species present in the Dubrovacko-Neretvanska County, are presented. The study was conducted after clinical symptoms of the disease were detected in September and November 2001.

Material and methods

Competitive enzyme-linked immunosorbent assay

The testing of sera was performed by the competitive-enzyme-linked immunosorbent assay (c-ELISA) for detection of BT antibodies in ruminant sera using a commercial kit (VMRD Inc., USA) (1). Tests were conducted in accordance with the instructions of the manufacturer, with the known positive and negative controls tested on each plate. All serum samples were tested in duplicate. The optical density of each well was read at a wavelength of 620 nm on a microplate reader (Anthos II, Austria).

Microtitre serum neutralisation

The serum neutralisation (SN) test was used to type BTV in ruminant sera (6). Positive and negative controls for SN were kindly provided by the Onderstepoort Veterinary Institute (OVI) (OIE Reference Laboratory) in South Africa. The microtitre neutralisation method was used in this study. Fifty μ l of serum dilutions, from 1:10 to 1:1 280, were added to each well of flat-bottomed microtitre plates and mixed with an equal volume of OIE standard reference sera for BTV serotypes 2, 4, 9 and 16 (100 TCID₅₀). These were incubated at 37°C in 5% CO₂. After 1 h incubation, approximately 10⁴ Vero cells were added to each well at a volume of 100 μ l of minimum essential medium (MEM) containing antibiotics. After incubation for 4-6 days, the test was read using an inverted microscope. The wells were rated according to the degree of cytopathic effect (CPE) observed. A sample was considered positive when CPE neutralisation exceeded 50% at the lowest dilution (1:10). The serum titre represented the highest serum dilution capable of neutralising more than 50% of CPE in tissue culture.

Culicoides collections

Insect collections were made using three light traps (220V, 8W) placed close to the sheep, goats and cattle, and operated before dusk until after dawn in September 2002 at seven locations in the Dubrovacko-Neretvanska County. The insects were kept in 70% ethyl alcohol. The fourteen insect collections were made in accordance with the protocols of the National Reference Centre for Exotic Diseases (CESME: *Centro Studi Malattie Esotiche*) in Teramo, Italy.

Results

In October and November 2001, clinical symptoms indicative of BT were observed in sheep herds in Konavle (in the southernmost area of Croatia along the borders with Montenegro and Bosnia and Herzegovina) (Fig. 1). Symptoms included abundant salivation, oedema of the tongue, buccal and intermaxillary regions, erosive stomatitis, elevated body temperature (40.5°C-40.7°C), coronitis with resulting lameness and sporadic abortion during late gestation. Symptoms of the disease were not observed in cattle, whereas some sporadic cases were recorded in goats.



Figure 1
Clinical symptoms of bluetongue in a sheep from Konavle, southern Croatia, November 2001

A team from the Croatian Veterinary Institute visited the area to take serum samples from sheep herds that presented clinical symptoms of the disease. A sheep with very pronounced symptoms was sacrificed, and a gross pathological examination performed (Fig. 2); the liver, spleen and lymph nodes were despatched for virological testing.

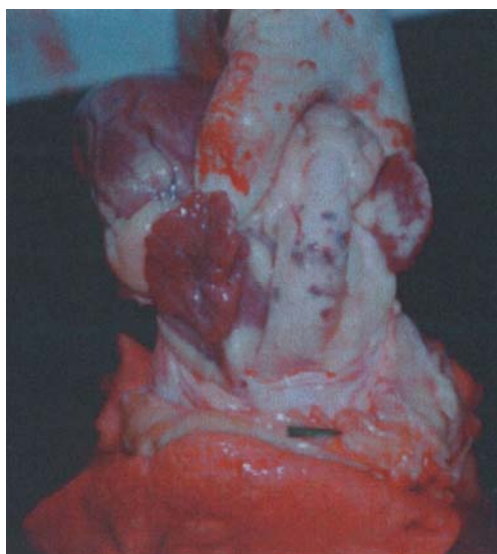


Figure 2
Haemorrhage in the tunica media at the base of the pulmonary artery of a sheep from Konavle, southern Croatia, November 2001

After the presence of BTV antibodies in the blood samples of the sheep was confirmed, serological testing was performed to determine the spread of the disease in Croatia. From the end of January to June 2002, blood samples were obtained from the majority of ruminants in the Konavle community, and randomly from the communities of Župa

Dubrovačka and along the Dubrovnik coast, and from one goat herd on the Pelješac Peninsula. Due to the small number of animals in individual herds, all ruminants from a village were considered a herd. All animals are usually kept indoors. A total of 3 318 serum samples from 53 herds were tested. These included 357 cattle sera, 178 (49.9%) of which gave positive results; of 1 268 sheep sera, 174 (13.7%) were positive; and of 1 693 goat sera, 270 (15.9%) were positive. The overall seroprevalence in all ruminants studied was 18.7%. The SN test revealed antibodies to serotype 9 in 212 of the positive sera. The geographic distribution of the animals tested is presented in Table I.

Table I
Distribution of animals tested for bluetongue in Croatia, January-June 2002

Area	Cattle	Goats	Sheep	Total
Dubrovnik coast	60	203	179	442
Dubrovnik	20	109	266	395
Konavle	277	1 295	823	2 395
Pelješac	0	86	0	86
Total	357	1 693	1 268	3 318

Culicoides were identified on the basis of their wing pattern and findings were verified by the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' in Teramo, Italy. A total of 4 872 *Culicoides* were collected; of these, 4 492 (92%) belonged to the Obsoletus Complex and included *C. obsoletus* and *C. scoticus*. The other *Culicoides* species identified were *C. circumscriptus*, *C. paolae*, *C. pulicaris*, *C. punctatus*, *C. seavanicus*, *C. fascipennis*, *C. baranti* and *C. fagineus*. The Obsoletus Complex was represented in 13 of the 14 catches. The mean air temperature at the time of the study was 20°C.

Discussion

Serological testing and the preliminary survey for the presence of *Culicoides* species, following the occurrence of clinical symptoms characteristic of BT in the Dubrovacko-Neretvanska County (situated in the southernmost area of Croatia), are presented. The area is 52 m above sea level and lies at 42°N, corresponding to the latitude at which BTV and BTV vectors have been recorded in other parts of the world (11). Between 1993 and 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.

Croatia had never previously recorded the presence of BT and the present study revealed an overall seroprevalence of 18.7%. Of the ruminants examined, the highest total seroprevalence was recorded in cattle (49.9%); this is consistent with the opinion that due to protracted viraemia, cattle enable viral replication and thus become virus reservoirs (2). In sheep, seroprevalence was 13.7%, whereas in goats the percentage was 15.9%. Sheep presented very pronounced symptoms of the disease, whereas in goats the infection developed without notice showing only sporadic symptoms of excessive salivation and intermaxillary oedema.

Highest seroprevalences occurred in cattle (54.87%), goats (18.92%) and sheep (17.74%) in Konavle (Table II). Seroprevalence was much lower in other parts of the region, decreasing from the south-east (Konavle) to the north-west including Dubrovnik where the seroprevalence rate in cattle reached 75% (but only 20 animals were tested), in goats 7.34% and in sheep 7.14%. Along the Dubrovnik coast, a seroprevalence rate of 18.33% was recorded in cattle, 6.4% in goats and 5.03% in sheep. In the Pelješac Peninsula, seroprevalence in goats was 4.65% (Fig. 3). The differences observed between geographic areas are highly significant ($\chi^2 = 89.4$, $P < 0.0001$).

Conclusions

The predominant species of *Culicoides* captured in Croatia were those belonging to the Obsoletus Complex, and included *C. obsoletus* and *C. scoticus* (92%). However, the following species were also

Table II
Percentage prevalence of bluetongue antibodies in livestock in the bluetongue-affected areas of Croatia, January-June 2002

Area	Cattle	Goats	Sheep	Total
Dubrovnik coast	18.33	6.40	5.03	7.47
Dubrovnik	75.00	7.34	7.14	10.63
Konavle	54.87	18.92	17.74	22.67
Pelješac	0	4.65	0	4.65
Total	49.90	15.90	13.70	18.75

identified: *C. circumscriptus*, *C. paolae*, *C. pulicaris*, *C. punctatus*, *C. seavanicus*, *C. fascipennis*, *C. baranti* and *C. fagineus*.

BTV has been isolated from *C. obsoletus* in Cyprus (9). African horse sickness virus was found in mixed pools of *C. obsoletus* and *C. pulicaris* in Spain; both species are widespread in Europe (14). *Culicoides obsoletus* comprises a complex of at least four species with almost identical wing patterns (12). The remaining species identified (*C. circumscriptus*, *C. pulicaris*, *C. seavanicus*, *C. fascipennis* and *C. fagineus*) are also found in Greece (10), whereas *C. paolae* is a new species recently discovered in southern Italy (3).

Climatic factors play an important role in the occurrence of BTV infection in animals and also influence the size of vector populations and periods of their seasonal activity. *Culicoides* activity has not been recorded below approximately 13°C and above 35°C (13). An analysis of climatic data was used to model the potential distribution of *C. imicola* in

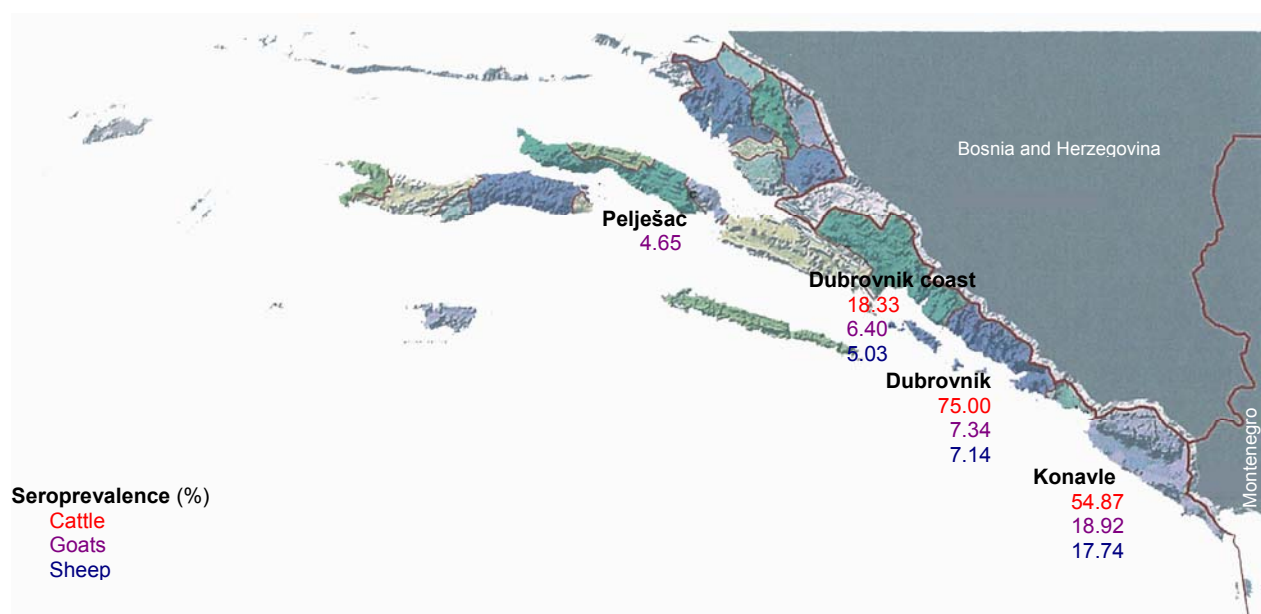


Figure 3
The areas of Croatia affected by bluetongue, showing the seroprevalence rates (%) in cattle, goats and sheep, January-June 2002

Europe, predicting that *C. imicola* might have spread from Spain, Greece and Italy to some areas along the Croatian coast as well as to the coastal areas of Albania, Serbia and Montenegro, and Bosnia and Herzegovina (15). However, *C. imicola* has not yet been reported from these latter Balkan states.

It appears quite conceivable that BT was spread by infected *Culicoides* or by infected animals from neighbouring Serbia and Montenegro. This presumption is supported by disease reports from Kosovo in October and from Serbia and Montenegro in November 2002 (11).

The Istituto Zooprofilattico Sperimentale in Teramo screened a total of 212 sera using the SN test. The presence of BT antibodies in these sera had previously been demonstrated by the c-ELISA. Antibodies to BTV serotype 9 were identified by the SN test, confirming the presumption that BT had spread to Croatia along the eastern front of the epizootic in the Mediterranean Basin, since the infection with BTV serotype 9 had previously been confirmed in Turkey, Greece and Bulgaria (11).

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