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The isolation of bluetongue virus from field populations of the Obsoletus Complex in central Italy

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Summary

Between July and September 2002, bluetongue (BT) virus (BTV) serotypes 2 and 9 caused mortalities amongst sheep in the communities of San Gregorio Magno (Salerno, Campania), Laviano (Salerno, Campania) and Carpino (Foggia, Puglia), central Italy. On three of the affected farms, approximately 10 000 specimens of *Culicoides* were captured, representing fifteen species. Not a single specimen of the classical Afro-Asiatic BT vector, *C. imicola* Kieffer, was found; species of the Obsoletus Complex dominated the light-trap collections (90%) and included *C. obsoletus* (Meigen), *C. scoticus* Downes and Kettle and *C. dewulfi* Goetghebuer. Fifty-eight pools of the Obsoletus Complex (excluding *C. dewulfi*), each numbering 100 individuals per pool, and containing only parous and gravid females, were assayed for virus. BTV serotype 2 (BTV-2) was isolated from three pools (San Gregorio and Carpino) and BTV-9 from one (Laviano). These results indicate clearly that a species other than *C. imicola* is involved in the current re-emergence of BT in the Mediterranean Basin, but whether this is only *C. obsoletus sensu stricto*, or only *C. scoticus*, or both together, has yet to be established.

Keywords

Bluetongue – Bluetongue virus serotype 2 – Bluetongue virus serotype 9 – *Culicoides* – Italy – Obsoletus Complex – Virus isolation.

Introduction

The Mediterranean Basin is currently being affected by the most severe and long-lasting outbreak of bluetongue (BT) ever experienced. Since 1999, at least six countries that had never before reported BT have now reported outbreaks of the disease (12, 13, 14). In the outbreaks that affected Bulgaria in 1999 and 2002, entomological surveys confirmed *Culicoides imicola* to be absent. Instead, species of the Obsoletus Complex were found to dominate and represented up to 90% of all *Culicoides* captured (11). Although no field isolations of BT virus (BTV) have been made from the Obsoletus Complex in Bulgaria, it seems reasonable to assume that it was the vector of the virus as it has been isolated once before from this complex in Cyprus (10). Since August 2000 in Italy, the distribution of the many thousands of foci of BT has been found to overlap that of *C. imicola* (6). However, with each successive season, cases of BT have appeared in areas where *C. imicola* was either rare, or absent (7). The possibility that another species of *Culicoides* was involved in the transmission of BT in parts of Italy, prompted us to investigate entomologically outbreaks of BT in areas where *C. imicola* was not known to occur. Accordingly, three outbreak areas were visited during the 2002 season; the *Culicoides* captured were identified, age-graded and then assayed for the presence of BTV. The results are reported here.

Materials and methods

Bluetongue outbreaks

Three outbreaks of BT were investigated: two in Campania and one in Puglia (Fig. 1). To enhance the possibility of infected *Culicoides* being captured, the

selected flocks had to show severe clinical symptoms of BT. Disease symptoms and morbidity and mortality rates were recorded for each flock. Diagnosis was confirmed by virus isolation from spleen samples and from the blood of dead and infected animals. In addition, blood samples were collected twice, once while the animal presented clinical signs, and again a few weeks later to detect seroconversion.

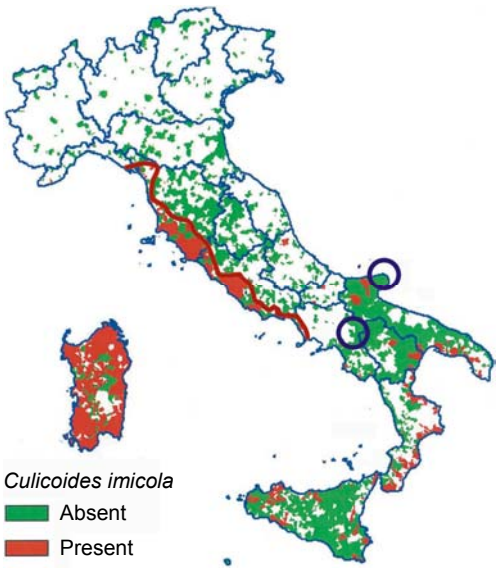

Figure 1
Distribution of *Culicoides imicola* in Italy, showing areas (blue circles) in which bluetongue virus was isolated from midges of the Obsoletus Complex
The red line delimits the area in which *C. imicola* has been found (8)

The first flock selected (flock 1) was in the municipality of San Gregorio Magno, in the Province of Salerno, Campania. It comprised 124 sheep (118 adults and 6 lambs) and 20 goats (18 adults and 2 kids). Sheep began showing clinical signs on 1 July 2002 and ten animals died over the next ten days. Ethylene-diaminetetra-acetic acid (EDTA) blood and serum samples were collected from two sick animals and tested against BTV. On the night of 23 July, *Culicoides* were collected in the sheep paddock using Onderstepoort-type blacklight traps. Seventy days after the first clinical signs of disease were detected in the flock, serum samples were collected from eleven fully recovered animals.

The second flock (flock 2: 260 adults and 5 lambs) was located also in the Province of Salerno but in the municipality of Laviano. Here the first signs of clinical BT were reported on 9 July and by 22 July, ten animals had died. EDTA blood and serum samples were collected from each of four sick animals and tested against BTV. Light-trap collections were made for *Culicoides* on the night of the 25 July. On 10 September, 62 days after the first clinical appearance of disease, a post-mortem was performed on two adult sheep and the spleens and lymph-nodes collected for virus isolation.

The third flock (flock 3: 180 adult sheep) was located in the region of Puglia in the Province of Foggia near the village of Carpino on the Gargano Peninsula. Clinical signs typically associated with BT disease appeared in the middle of August and by 5 September, 26 animals had died. EDTA blood was collected from six animals that showed heightened temperatures and facial oedema. The spleen and lymph-nodes were obtained from one of the dead animals and serum samples were taken from 16 animals; all samples were treated as described above. Six days later, *Culicoides* were collected in association with this flock.

Laboratory studies

Virus isolations from blood and tissue samples were performed by intravenous inoculation of embryonating chicken eggs (ECE) followed by repeated passages in Vero cells (4, 17). A reverse transcriptase-polymerase chain reaction (RT-PCR) using group-specific and type-specific primers, was used for the detection of BTV nucleic acid in tissues and in blood samples according to described methods (15).

Serum samples were tested for the presence of BTV antibodies using a competitive ELISA (c-ELISA) (VMRD, USA) and virus neutralisation (VN) assays (5). Positive and negative controls for the VN assays were kindly provided by the OIE reference laboratory of the Onderstepoort Veterinary Institute (OVI) in South Africa.

Studies on insects

Onderstepoort-type blacklight traps were used to capture *Culicoides* according to the method developed by the National Reference Centre for Exotic Diseases (CESME: *Centro Studi Malattie Esotiche*) in Teramo, Italy, in collaboration with the OVI, South Africa (9). At each site, two to four traps were operated for one night. *Culicoides* were collected into 300 ml of phosphate-buffered saline (PBS) with antibiotics, to which 5 ml of soap detergent had been added. Collections were maintained on ice during transport to the laboratory of the Istituto Zooprofilattico Sperimentale in Teramo, and stored there at 4°C. All *Culicoides* were age-graded in chilled PBS according to the method of Dyce (3). Parous and gravid categories, which comprise the older individuals that had already had one or more blood-meals prior to capture, were separated out as these

are the only ones likely to yield BTV. Care was taken to not include freshly bloodfed individuals. Pools of 100 (mostly parous) individuals of species belonging to the Obsoletus Complex were sorted for virus isolation. These pools comprised a mixture of *C. obsoletus* and *C. scoticus*; specimens of *C. dewulfi* were excluded.

For the isolation of virus, insects were processed as described previously (16). Pools were homogenised in 100 µl of cold Dulbecco's minimum Eagle's medium (DMEM) using a battery powered microtissue grinder. Homogenised samples were centrifuged at 14 000 g for 10 min. and the supernatants collected into 1.5 ml microfuge tubes. One hundred µl of DMEM containing antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml, gentamycin 5 µg/ml and nystatin 50 IU/ml) and 20% foetal calf serum (FCS) were added. Ten µl of each insect pool was diluted 1:10, and then three additional ten-fold dilutions were prepared. Twenty-five µl of undiluted sample and 25 µl of each dilution were put into each of four wells of a 96 flat-bottomed well plate to which was added 100 µl of Vero cell suspension in DMEM containing approximately 2×10^5 cells/ml. The final concentration of FCS in the total volume (125 µl) was 5%. The inoculated microplates were incubated at 37°C in 5% CO_2 and observed microscopically for cytopathic effect (CPE) every day for 6 to 7 days post-inoculation. On day 7, the tissue culture fluid was collected from wells showing CPE and inoculated onto a two-day-old monolayer of Vero cells. The second passage of the virus was collected at a time when 90%-100% of the cells showed CPE and then used for virus identification. Immunofluorescence (IF) using BTV monoclonal antibodies (VMRD, USA) was used as the confirming assay. Virus characterised as BTV was subsequently typed by virus microneutralisation assays using type-specific antisera. In the absence of CPE, cultures were scraped, the cells centrifuged, and the supernatant re-passaged for two blind passages. At the end of the third passage, cells were checked by IF for the presence of BTV. The remaining volume of each individual insect homogenate was also tested using the intravenous inoculation of embryonating chicken eggs followed by two passages in Vero cells as previously described.

The BTV infection rate (and its confidence limits) in the midge populations collected were calculated using a Bayesian approach. The virus isolation data were analysed using the Beta (s+1, n-s+1) distribution where s, the number of successes, is the total number of positives and n, the number of trials, is the total number of tested individuals. As BTV was recovered from pools of 83 to 100 individuals, the minimum and maximum infection prevalence rates were determined.

Results

Clinical, serological and virological examinations

All flocks suffered clinical disease and deaths. Clinical signs persisting over two months in flock 2 included fever, depression, nasal discharge, facial oedema, hyperaemia and ulceration of the oral mucosa, coronitis, muscle weakness, diarrhoea and cachaessia. The mortality rate averaged 12% in the first two flocks (11.8% in flock 1 and 12.3% in flock 2), and approached 17% in the third flock. Subsequent virological and serological studies confirmed the presence of BTV in all three flocks (Tables I and II).

Table I
Bluetongue virus isolations from blood and organ samples from three infected sheep flocks in central Italy, July-September 2002

Flock No.	Sample	Method		Bluetongue virus serotype
		RT-PCR (positive/ No. tested)	ECE (positive/ No. tested)	
1	Blood	2/2	0/2	
2	Spleen	2/2	0/2	
	Blood	4/5	0/5	
3	Spleen	1/2	1/2	BTV-2
	Lymph-nodes	0/1	0/1	
	Blood	6/6	0/6	

RT-PCR reverse transcriptase-polymerase chain reaction
ECE embryonating chicken eggs

Culicoides identification

Approximately 10 000 midges, representing fifteen species, were captured on the affected farms. Not a single specimen of the classical Afro-Asiatic BT vector, *C. imicola* was found. Instead, other species of the subgenus *Avaritia* dominated the light-trap collections, and included *C. obsoletus* (Meigen), *C. scoticus* Downes and Kettle and *C. dewulfi* Goetghebuer, these comprising 90% of all *Culicoides* captured. In addition, more than 95% of the older parous and gravid females (these being the only ones likely to harbour virus) belonged to the Obsoletus Complex only. Male specimens indicated that two species of this complex were present: *C. obsoletus sensu stricto* and *C. scoticus*. Owing to the fact that the female of *C. scoticus* is inseparable from that of *C. obsoletus*, these two species were combined during

Table II
Serological results from three bluetongue-infected sheep flocks in central Italy, July-September 2002

Flock No.	Date of sampling	c-ELISA (positive/No. tested)	Method Virus neutralisation (positive/No. tested)			
			BTV-2	BTV-4	BTV-9	BTV-16
1	July 2002	3/4	2/4	0/4	2/4	0/4
	September 2002	0/1	0/1	0/1	0/1	0/1
2	July 2002	2/2	0/2	0/2	2/2	0/2
	September 2002	9/11	5/11	0/11	4/11	0/11
3	September 2002	15/16	3/3	0/3	0/3	0/3

the selection of pools for BTV isolation. *C. dewulfi* comprised 2% of the collections; these were excluded from the pools assayed for virus.

Bluetongue virus isolation from insects

Fifty-six pools (of mostly 100 parous individuals each) were sorted for virus isolation. Twenty-eight pools derived from flock 1, 26 from flock 2 and 2 (each containing 83 individuals) from flock 3. Four isolations of BTV (three of BTV-2 and one of BTV-9) were made. All isolates were recovered by ECE (7.1%) but only two by tissue culture (TC) inoculation (3.6%) (Table III). BTV-2 was found in *Culicoides* collected around flocks 1 and 3, whereas BTV-9 was recovered from those collected around flock 2 (Table III). The estimated BTV infection prevalence rates in midges are shown in Table IV.

Discussion

To determine the vector status of any arthropod for an arbovirus, it has to be demonstrated that it can become infected with the virus after feeding on a viraemic host (or on an artificial substitute) and that it is able to then transmit the virus biologically to a healthy host by bite after an extrinsic viral incubation period of one week or more. Furthermore, the virus should be recovered from wild-caught specimens whose abdomens are free of visible fresh blood; also, there should be field evidence confirming the association between the arthropod and the disease-infected vertebrate host (1). However, before it can be classified as a proven vector of BTV, conclusive proof of the ability of the arthropod to transmit BTV to a vertebrate host is required also via the establishment of an infection by bite.

Whilst the latter requirement is imperative towards establishing the vector competency of species of the Obsoletus Complex, it remains indisputable that in this study most of the above vector status criteria were fulfilled, i.e. BTV was recovered from parous individuals belonging to species of the Obsoletus Complex collected in the vicinity of infected flocks (that had received no new animals during the preceding year) and in which BTV infection had been demonstrated clinically, serologically and virologically. Furthermore, almost half the specimens of the Obsoletus Complex captured in the outbreaks were parous, demonstrating that a significant number of midges were surviving sufficiently long enough to ingest, replicate and then transmit BTV some 7 to 14 days later. As 97% of all the parous midges captured belonged to one or two species of the Obsoletus Complex, it is almost certain that they were the only species transmitting the virus locally.

Table III
Bluetongue virus isolations from 56 pools of *Culicoides* of the Obsoletus Complex in central Italy, July-September 2002

Flock No.	Method (positive/No. pool tested)		Bluetongue virus serotype			
	ECE	TC	BTV-2	BTV-4	BTV-9	BTV-16
1	2/28	0/28	2	–	–	–
2	1/26	1/26	–	–	1	–
3	1/2	1/2	1	–	–	–
Total	4/56	2/56	3	–	1	–

ECE embryonating chicken eggs
TC tissue culture

Table IV
Estimated bluetongue infection prevalence rates in *Culicoides* of the Obsoletus Complex captured on affected farms in central Italy, July-September 2002

Flock No.	BTV serotype	No. of pools* positive/examined	No. of midges examined	No. of possible positive individuals (min./max.)	95% confidence limits of prevalence estimate (%)
1	2	2/28	2 800	2	0.02-0.26
				200	6.25-8.16
2	9	1/26	2 600	1	0.01-0.21
				100	3,17-4.66
3	2	1/2**	166	1	0.15-3.29
				83	42.47-57.53
	2	3/56	5 566	3	0.02-0.16
				283	4.54-5.69
Total	9	1/56	5 566	1	0.004-0.10
				100	1.48-2.18
	2 and 9	4/56	5 566	4	0.029-0.18
				383	6.26-7.59

* each pool comprises 100 individuals

** two pools of 83 individuals

Observations in Bulgaria and Croatia indicate *C. obsoletus* to be the principal or only vector of BTV-9 in this part of Europe (14). Our study suggests that species of the Obsoletus Complex are able to transmit more than one serotype of BTV in Europe, and, furthermore, that it may involve more than one species of the complex.

As to the epidemiology of BT in Europe, the isolation of the virus from the Obsoletus Complex should be viewed with some concern. *Culicoides imicola*, the proven classical vector of BTV, is restricted to the southern parts (mostly) of only some countries that adjoin the northern border of the Mediterranean Sea. The converse is true for species of the Obsoletus Complex containing *C. obsoletus*, one of the commonest *Culicoides* to occur across central and northern Europe and where it is known to attack both livestock and man (2). Essentially its distribution only seldom penetrates into that of *C. imicola* but this area of overlap may enable species of the Obsoletus Complex to spread BTV further north. Stated simply, *C. imicola* remains the principal vector involved in the rapid and explosive incursions of BT that periodically affect the Mediterranean Basin but in 'carrying' the virus northwards 'hands it over' to a member of the Obsoletus Complex: the 'baton effect' (11). Italy falls within this zone of vector overlap and thus is one of the Mediterranean countries most at risk to incursions by BT as both vectors are now known to occur widely there and in occasional sympatry (Figs 1 and 2). This implies that in areas of intense infection, both vectors may have been involved and there may have been many instances in which the

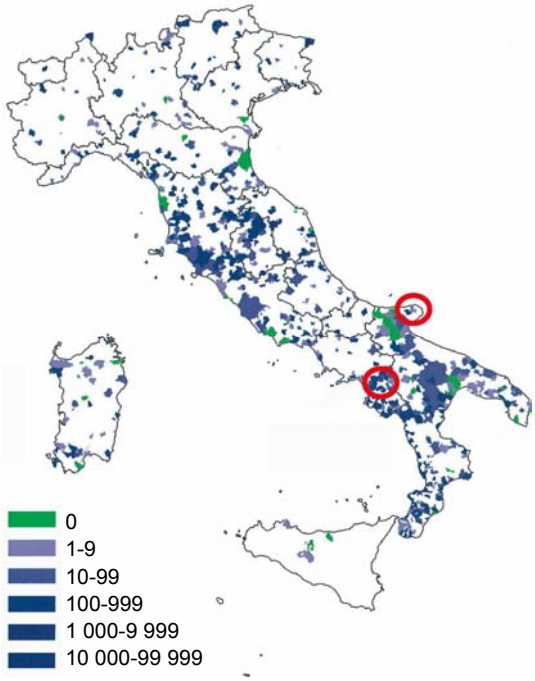

Figure 2
Distribution of *Culicoides obsoletus* in Italy, showing areas (red circles) in which bluetongue virus has been recovered from pools of midges comprising both *C. obsoletus* and *C. scoticus*

'baton effect' could have occurred (6, 8). It has not been established whether this spread occurs only from areas of overlap or whether a species of the Obsoletus Complex, in the absence of *C. imicola*, is able to carry the virus northwards and to then sustain outbreaks across a season or more. In view of the location of the affected farms (Figs 1 and 2) it would appear that a species of the Obsoletus

Complex was indeed able to 'carry' the virus and to sustain outbreaks in the absence of *C. imicola*.

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