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# Entomological surveillance of bluetongue in Italy: methods of capture, catch analysis and identification of *Culicoides* biting midges

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## Summary

To elucidate the epidemiology of vector-borne diseases that can affect livestock in the Mediterranean Basin and elsewhere, it is essential to obtain a clear understanding of the life-cycle and habits of the vector insects involved. One purpose of such investigations is to provide data for an epidemiological surveillance system. As this depends heavily upon the collection of specimens in the field, it is necessary to establish the kinds of information required, and how it can be obtained. This requires, in turn, that the method (and instrument) of capture be standardised, so that all data are as complete as possible, are comparable, and are informative at many levels. Within the surveillance system for bluetongue (BT) in Italy, the National Reference Centre for Exotic Diseases (CESME: *Centro Studi Malattie Esotiche*) is leading an intensive and countrywide survey for *Culicoides* (Diptera: Ceratopogonidae) using standardised methods and protocols developed in collaboration with the Onderstepoort Veterinary Institute in South Africa. These methods have now also been implemented outside Italy in Malta, Croatia, Albania and Romania. This system includes the field protocols developed for the collection of *Culicoides*, the laboratory protocols developed around the insect analyses and the computer-based recording of all field data. Finally, the authors provide an 'Easy key' for the rapid identification of the principal BT vector *C. imicola*, and for grouping species that belong to the Obsoletus and Pulicaris vector complexes, and to the Nubeculosus and Schultzei potential vector complexes.

## Keywords

Bluetongue – *Culicoides* – *Culicoides imicola* – Entomology – Italy – Surveillance – Trap – Vector.

The entomological surveillance of bluetongue (BT) in Italy is based primarily on a net of permanently sited traps (at least one trap every 1 600 km², and operated weekly throughout the year). This survey is augmented by the random use of 'mobile traps' (to investigate more thoroughly outbreaks or any other kind of epidemiological situation such as the risks associated with annual transhumance movements). As hundreds of weekly collections are made throughout Italy, it is essential to conduct these surveys simply and in a standardised way, both in the field and in the laboratory. The light-trap collections are mostly undertaken by the local Veterinary Services, and sometimes also by the farmers; these activities are co-ordinated by the ten Istituti Zooprofilattici Sperimentali located across Italy. In each Institute, a veterinarian or biologist has been trained in the various field and laboratory protocols developed by the National Reference Centre for Exotic Diseases (CESME: *Centro Studi Malattie Esotiche*). The aims of the entomological surveillance system are as follows:

- to obtain field data for the continuous refinement of the distribution map of *Culicoides imicola* for Italy
- to detect and elucidate the prevalence of other suspected vector *Culicoides* in the absence of *C. imicola*
- to elucidate the seasonal dynamics of all vector *Culicoides*, in order to identify zones that are seasonally free of these vectors
- to collect samples for the detection of BT virus (BTV) in *Culicoides* during outbreaks of the disease.

Clearly these aims can be achieved only with data that are both comparable and sensitive.

## Field protocols for the collection of *Culicoides*

*Culicoides* can be captured using several methods. The adults are most easily captured using various light-trap models (these vary in power, in the use of different colours, with or without an additional attractant), truck traps, aspirators, bait-traps and also emergence traps for the sampling of larval habitats. In addition, the immature stages can be harvested from various moist habitats. Each method has specific advantages and disadvantages but when taken in combination, they are capable of yielding a wide range of valuable information on the biology of many species simultaneously. In any BT surveillance system, the principal aim is to capture adult *Culicoides* in the near vicinity of vertebrate hosts, and to employ a powerful trap (to enhance surveillance sensitivity at low *Culicoides* population levels and, furthermore, to increase the number of midges captured for virus isolation studies). Species identifications have to be performed by a specialised entomologist, but with a large influx of collections, it becomes important to carefully store the captured *Culicoides*, so that they can still be used for more detailed taxonomic studies months later.

### Equipment

Onderstepoort blacklight suction traps of the type described by Venter and Meiswinkel in 1994 (3) are used; blacklight has been demonstrated to be 8 to 10 times more attractive than white light (4) and will increase monitoring sensitivity in areas where vector abundances are low (and will also provide more midges for the isolation of BTV). The traps are of a robust design and can remain in the field for a number of years. Surrounding the blacklight tube is a netting (apertures of about 4 mm) to exclude larger insects, especially moths whose body scales can contaminate a collection and make the analysis of a sample more laborious. Below the suction fan of the light trap, there is a white fine-gauzed bag, which is linked to a white 500 ml collection beaker. White is the colour of preference for the beaker as it enables one to more easily see the tiny *Culicoides* with the naked eye and establish quickly whether a 'successful' collection has been made.

The collection kit (Fig. 1) comprises the following:

- blacklight trap (complete with net, white gauze bag and cord to suspend the trap). If electricity is not available, modified traps can be operated using a 12 V car battery (these need to be re-charged every three days)
- two white 500 ml collection beakers
- water and detergent (odourless commercial liquid soap)
- a cheap tea strainer with a separate square of fine white gauze (the size of half a handkerchief and of a smooth quality to which insects do not easily adhere)
- a labelled screw-lid container with 100-150 ml of 70% ethanol
- a pencil
- a maximum-minimum thermometer
- a global positioning system (GPS)
- 'SBT06' forms of the BT National Surveillance System
- phosphate-buffered saline (PBS) with antibiotics, refrigerated at 4°C, to be used instead of water and/or ethanol when collections are made for the isolation of virus.

Figure 1
*Culicoides* collection kit

### Choice of trapping sites

The choice of trapping sites is made as follows:

- either cattle, sheep, goats or horses must be present
- large livestock holdings are preferred, i.e. >10 animals
- livestock to be located in the near vicinity of the light trap all night; stabling can be of any type (but must be open)
- priority should be given to farms where conditions such as pools of water or mud are found, created either naturally (rain) or by irrigation or overflows

- it must be established whether electricity is available to operate the light trap
- it is essential to establish whether the farmer/owner is willing to collaborate.

## Catch procedure

The catch procedure should be conducted as follows:

- the trap must be suspended at a height of between 1.5-2 m and be as close to the animals as possible (i.e. in the stable entrance or on a nearby tree) (Fig. 2)

Figure 2
A blacklight trap suspended near sheep on a farm affected by bluetongue in Sardinia

- the minimum-maximum thermometer must be placed close to the trap
- use the GPS to take altitude, longitude and latitude, and record the data on the 'SBT06' form
- attach the white beaker to the gauze bag and fill the beaker (through the bag) with about 200 ml of water to which a few drops of detergent have been added (Fig. 3)
- activate the trap and the thermometer at least 1 h before sunset
- switch the trap off the following morning soon after daybreak
- place the gauze 'square' over the strainer and filter the insects from the white collection beaker through it; to prevent insects from adhering to the wall of the beaker, it should first be gently swirled a few times to get all the insects into suspension (Fig. 4)
- once all the insects have been trapped in the gauze 'square', it is folded and placed into 70% ethanol in a labelled bottle (Fig. 5), which is then sealed tightly
- if the collection is being made to attempt the isolation of virus, PBS with antibiotics must be used instead of water and/or ethanol; in addition, such collections must be sent immediately to the laboratory and must be refrigerated at 4°C during transit

Figure 3
The blacklight trap collecting beaker being half-filled with water and detergent through a gauze bag

Figure 4
An insect collection being concentrated into the gauze 'square' using a tea strainer

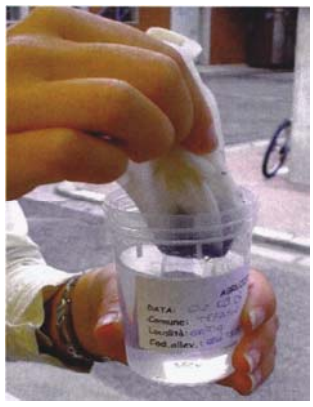

Figure 5
The 'concentrated' insect collection is then placed into a labelled screw-lid container with 70% ethanol

- complete labelling of the collection, using a pencil, indicating the trapping site and the date (that of the morning after the night of collection)
- store the catch in a cool place, out of direct sunlight, until transport to the laboratory
- complete all sections of the 'SBT06' form.

## Laboratory protocols for the analysis of catches

Each light-trap collection arrives in the laboratory with the attached 'SBT06' form; the collection is then registered with a code number (which is also recorded on the form and on the bottle label). This code number links all field and laboratory information associated to a particular collection.

### Analysis of the catches

The following procedure should be used when analysing catches:

- rinse the insects from the gauze 'square' (by using a gentle but persistent spray) and place them back into the same labelled bottle using fresh 70% ethanol
- first separate all *Culicoides* midges from other insects; this usually is done under a stereo-microscope at low magnifications (6-10×)
- sort into separate Petri dishes the species *C. imicola*, Obsoletus Complex, Pulicaris Complex and other *Culicoides* spp. and count the number of specimens in each category
- count all insects other than *Culicoides* to obtain an insect:*Culicoides* ratio and then dispose of the non-*Culicoides* insects
- for large collections containing over 500 *Culicoides*, a subsample is analysed as described previously (2), and recorded (Fig. 6)
- the sorted *Culicoides* are stored in fresh 70% ethanol, in glass bottles, each labelled with the code number, the region of capture and the species or species complex
- these collections are stored in a cool, dark place to preserve the midges for further investigations (i.e. for the mounting of slides, and for the detection of virus using PCR).

The results obtained following this protocol are both qualitative (positive/negative for *C. imicola*) and quantitative: total insects (other insects + *Culicoides*), total *Culicoides*, number of *C. imicola*, number of Obsoletus Complex, and number of Pulicaris Complex.

At the end of each working day, all these collection data are inserted into a database designed to automatically upgrade the distribution maps (and/or graphs) of *C. imicola* daily; these can be accessed on the website (www.izs.it). Similarly, the abundance maps (or graphs) of the *Culicoides* captured at the permanent sites are updated daily.

| | REGION Abruzzo | | Municipality Teramo | | | |
|---|---|---|---|---|---|---|
| | No. | no. examined pipettes | no. total pipettes | total | Total *Culicoides* | Total insects |
| *C. imicola* | 310 | 3 | 15 | 1550 | | |
| *Culicoides* spp | 243 | 3 | 15 | 1215 | 2765 | |
| Other insects | 440 | 1 | 15 | 6600 | | 9365 |
| Code number 80000/03 | | DATE 15/08/2003 | | | Signature M. G. | |

Figure 6
A subsample analysis form

### Samples for virus detection

Virus detection can be performed by PCR or by isolation. In the first instance, midges stored in ethanol can be used and the sample can be screened months after it was collected. However, if virus isolation is to be attempted, the sample must be analysed immediately and the sorting of midges (not stored in ethanol) should be done on a chill table using only PBS with antibiotics.

The kind of sample to be sorted for virus detection is dependent upon the aim of the analysis, as follows:

1. To detect the presence of BTV circulation in an area, pools of engorged and parous females of *Culicoides* numbering 100 individuals (or less) and of mixed species can be submitted for isolation, or PCR. Such pools can be prepared rapidly, but if positive for BTV the infected species cannot be determined.
2. To identify the vector in areas where BTV has been shown to circulate in the vertebrate host, pools of parous females of a single species or species complex are submitted for virus isolation. Maximum pool size should be 100 individuals, but single individuals can be submitted (such as is done in vector competence studies). These investigations require expertise in the accurate identification and age-grading of *Culicoides* (as only parous and not bloodfed females are required).

## Identification of *Culicoides*

Within a surveillance system, the identification of insects of epidemiological interest has to be precise but also rapid; therefore, it is important to know which morphological characters to search for. The first step is to separate the genus *Culicoides* from the 'rest of the insect world'. Within the family

Ceratopogonidae there are more than 100 genera, but only four genera feed on warm-blooded vertebrate hosts: *Leptoconops*, *Austroconops*, *Forcipomyia* (subgenus *Lasiohelea)* and *Culicoides*. The body-shape of *Culicoides* is highly characteristic, but is roughly similar to other ceratopogonid genera. However, a distinguishing feature is that *Culicoides* usually have spotted wings (all species implicated to date in the transmission of BTV possess a wing pattern); however, some species of *Culicoides* lack a pattern, and, also, a wing pattern is not exclusive to *Culicoides*. If one relies only on body-shape and on a patterned wing (at low magnification under the stereoscope), it becomes possible to identify *Culicoides* with 80% specificity and 80% sensitivity; the 20% 'false positives' will be other insects with spotted wings (e.g. *Chironomidae*) and the 20% 'false negatives' will be plain-winged *Culicoides*. Identification of the latter group is best achieved by careful examination of the wing venation (Fig. 7), using higher magnification. Regarding identification to species, not all species of *Culicoides* can be reliably identified based solely upon wing pattern. Accurate identification relies upon highly developed taxonomic expertise; at best the majority of species, especially those of the vector complexes, can only be identified to 'species complex' level; fortunately this is achieved with relative ease. Within the Obsoletus and Pulicaris Complexes (which, after *C. imicola*, contain the most important species involved in the transmission of BTV in Europe), there are at least six and twelve species respectively in Italy alone (R. Meiswinkel, personal observation).

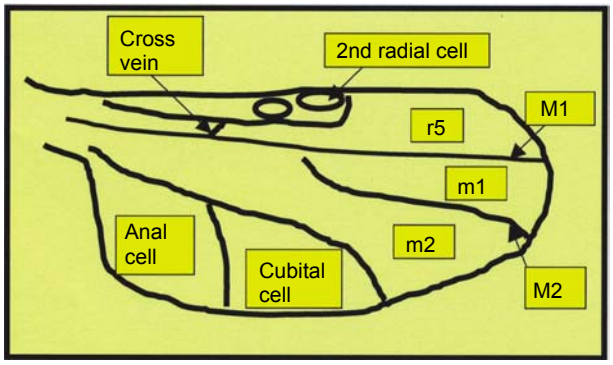


Figure 7
A diagrammatic representation of the *Culicoides* wing showing the principal nomenclature of the various veins (e.g. M1, M2) and cells (e.g. r5, m2)

## 'Easy key' for the rapid identification of *C. imicola* and species of other vector complexes

Ultimately, the correct identification of *Culicoides* is reliant upon a highly developed taxonomic expertise. This can require that many specimens be dissected and slide-mounted, but this procedure is impracticable in those field and laboratory situations where large numbers of captures have to be identified. Thus, the majority of species can be identified to the species complex level only using a stereo-microscope at low power when the identification is based almost solely upon wing pattern. Adding to the confusion is the fact that some species complexes are poorly defined; for example, species of the Grisescens Complex (subgenus *Silvicola*) and the Fagineus Complex (subgenus unknown) are currently 'lumped' (incorrectly) in the Pulicaris Complex (subgenus *Culicoides*). Although most of the species within these possess characteristic wing patterns, the variations are so subtle that one is not able to select simple key characters that are infallibly diagnostic. Thus the 'Easy key' presented below has limitations and consequently it is advised that identifications be cross-referenced against published species descriptions or a taxonomist be consulted (Fig. 8).

| | | |
|---|---|---|
| 1. | Wing with a distinct pattern of light and dark spots | 2 |
| | Wing without a pattern | Ignore |
| 2. | Wing: second radial cell entirely dark | 3 |
| | Second radial cell partly or wholly pale | 5 |
| 3. | Wing with the pale spot in the centre of cell r5, irregularly shaped (this spot not to be confused with pale spot at apex which may extend to the centre of r5) | 4 |
| | Pale spot in centre r5 round or absent | Ignore |
| 4. | Dark spot in centre of cubital cell eccentrically-shaped | **Nubeculosus Complex** |
| | No dark spot in centre of the cubital cell | 8 |
| 5. | Wing with a neatly rounded dark spot in the centre of the cubital cell | **Pulicaris Complex** |
| | No dark spot in centre of the cubital cell | 6 |
| 6. | Wing mostly or entirely hairy including anal cell | Ignore |
| | Wing hairy in apical ⅓-½ only and excluding anal cell | 7 |
| 7. | Pale spot in the apex of r5 sometimes absent; if present, it ranges from being small/weakly defined to large/better defined; this spot either ovoid, round or square in shape; vein M2 without a distinct preapical excision | **Obsoletus Complex** |
| | Pale spot in apex of r5 large/well-defined, its anterior margin distinctly pointed; vein M2 with a distinct preapical excision | ***C. imicola*** |
| 8. | Wing hairy, with a small distinct round spot straddling vein M1 medianally | ***C. paolae*** |
| | Wing distinctly less hairy, without a round spot straddling vein M1 medianally | **Schultzei Complex** |

Figure 8
Easy key for the rapid identification of *Culicoides imicola* and species of other vector complexes found in the Mediterranean Region

The key deals mostly with species and species complexes known, or suspected, to be involved in the transmission of bluetongue and African horse sickness (AHS) in the Mediterranean Region. It facilitates the identification of *C. imicola sensu stricto*, and the correct placement of species into the Obsoletus, Pulicaris, Schultzei and Nubeculosus vector complexes. Although rarely captured in the western half of the Mediterranean, the Schultzei Complex has been included as it is considered a potential vector and because it may be confused with the superficially similar (and relatively prevalent) *C. paolae* Boorman (1). The Nubeculosus Complex is included as orbiviruses replicate in this species under laboratory conditions in Europe, and because it is closely related to *C. sonorensis*, the most important vector of BTV in North America.

## References


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