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# FIRST DETECTION OF BLUETONGUE VIRUS GENOME IN *CULICOIDES* (DIPTERA: *CERATOPOGONIDAE*) FROM DISEASE OUTBREAKS IN ROMANIA

# PRIMA SEMNALARE A GENOMULUI VIRUSULUI BOLII LIMBII ALBASTRE ÎN CULICOIDE (DIPTERA: *CERATOPOGONIDAE*) CAPTURATE ÎN FOCARE DE BOALĂ DIN ROMÂNIA

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## ABSTRACT | REZUMAT

Biting midges (Diptera: *Ceratopogonidae*) are insects belonging to the genus *Culicoides*, involved in the transmission of several pathogens (bacteria, viruses, and parasites) that cause diseases to animals and humans. In Romania, the vectorial role of *Culicoides* species is mainly related to the transmission of bluetongue virus (BTV). In order to determine the vector competence of these insects, it is required to demonstrate their ability to ingest, replicate, and transmit the mentioned pathogens to an uninfected animal. The ability to take the pathogen from an infected animal can be demonstrated by isolating it from the vector or by the identification of the genome using molecular biology techniques. In this study, we evaluated the vector competence using the real-time RT-PCR technique on *Culicoides* species captured in 2014, in 6 counties where outbreaks of bluetongue were reported. 55 insect pools were tested and valid results were obtained for 41 pools. 16 pools, from three counties (Dolj, Gorj, and Vrancea), were positive for BTV genome. The real-time RT-PCR serotype test, performed on a positive pool for the BTV serogroup, demonstrated the presence of viral serotype 4. 15 of the 16 positive pools consisted of insects belonging to the Obsoletus complex. A single positive pool consisted of insects belonging to the Pulicaris complex.

**Keywords**: Biting midges, *Culicoides,* bluetongue

Insectele culicoide (Diptera:*Ceratopogonidae*) sunt diptere încadrate în genul *Culicoides*, implicate în transmiterea mai multor agenți patogeni (bacterii, virusuri, paraziți) care produc boli animalelor și oamenilor. În România, rolul vectorial al insectelor culicoide este legat, în principal, de transmiterea virusului bolii limbii albastre (BTV). Stabilirea competenței vectoriale a acestor insecte este legată de demonstrarea abilității acestora de a ingera, replica și transmite agenții patogeni menționați la un animal neinfectat. Capacitatea de a prelua agentul patogen de la un animal infectat, poate fi demonstrată prin izolarea acestuia din vector sau prin evidențierea genomului prin tehnici de biologie moleculară. În acest studiu am evaluat competența vectorială, utilizând tehnica Real Time RT-PCR, a insectelor culicoide capturate în anul 2014, în 6 județe unde au fost semnalate focare ale bolii limbii albastre. Au fost testate 55 pool-uri de insecte, obținându-se rezultate valide pentru 41 de pool-uri. 16 pool-uri (din trei județe: Dolj, Gorj și Vrancea) au fost pozitive pentru genomul BTV. Testul real-time RT-PCR de serotip, efectuat pe un pool pozitiv pentru serogrupul BTV, a demonstrat prezența serotipului viral 4. 15 din cele 16 pool-uri pozitive au fost constituite din insecte aparținând complexului Obsoletus. Un singur pool pozitiv a fost constituit din insecte aparținând complexului Pulicaris.

**Cuvinte cheie**: insecte culicoide, *Culicoides,* bluetongue

The first cases of bluetongue disease in Romania were reported in August 2014, when cattle with clinical signs from Buzău county were confirmed with infection (19). Bluetongue is an infectious, non-contagious disease specific to domestic and wild ruminants, maintained and transmitted by dipteran insects of the *Ceratopogonidae* family, genus *Culicoides*. Clinically it manifests by fever, facial and cavitary oedema, epithelial erosions, congestions, etc. (3, 5, 6). Bluetongue disease is introduced into a free area by means of infected vectors, which, through active or passive mechanisms, go beyond normal survival areas, or through infected receptive animals transported in the context of commercial activities. The geographical spread of the disease coincides with the growth and development areas of the competent *Culicoides* vectors (11, 17). The ability of the *Culicoides species* to manifest the role of biological vectors is characterized by two parameters: vector competence and vectorial capacity (2, 12, 15, 18). Vector competence is the ability of *Culicoides species* to ingest, replicate, and transmit the

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pathogen to an uninfected animal. One of the criteria that is considered in defining the vector competence is the demonstration of the ability of the vector to get infected by feeding on an infected host. Ideally, the ability to get infected can be directly demonstrated by isolating the pathogen from the vector that fed on an infected animal. When this criterion cannot be demonstrated, vector testing for the identification of the specific genome may be considered, in correlation with evidence of proximity to an outbreak. Positive results in PCR testing, correlated with epidemiological information, demonstrate the potential role of these insects in the transmission of the pathogen. To our knowledge, in Romania no research has been undertaken on the epidemiological role of *Culicoides species* in the transmission of bluetongue virus.

In the present study, we evaluated the vector competence using real time RT-PCR assay on *Culicoides species* captured in 2014, in 6 counties where outbreaks of bluetongue disease were reported.

## MATERIALS AND METHODS

The study was performed on insect samples taken from 22 villages in 6 counties: Bacău, Dolj, Gorj, Ialomița, Vâlcea, and Vrancea. The traps were placed in areas previously declared as outbreaks of bluetongue (backyards), following the detection of animals with clinical signs. The trapping period was 26.08.2014 - 24.09.2014 (Fig. 1).

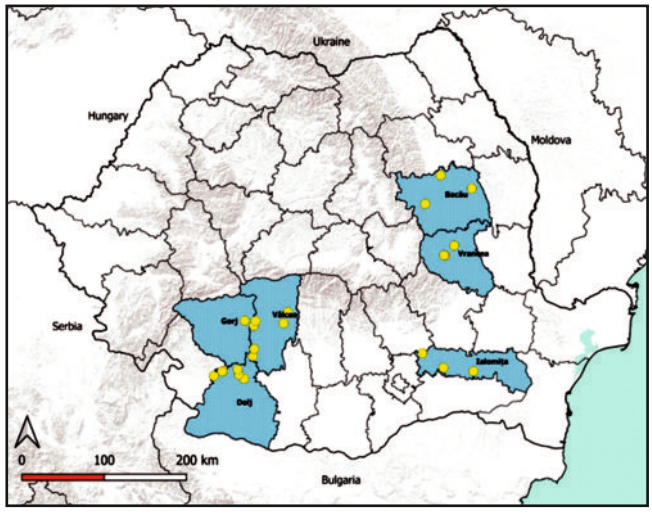


**Fig. 1.** Counties and locations where trapping activities were carried out in 2014

South Africa OVI type traps with UV light were used. One trap was placed in each location. Insect identification was based on the key presented by Goffredo and Meiswinkel, based on the wing model (9). The captured insects were classified into three species complexes: Obsoletus, Pulicaris and Nubeculosus.

Following identification, the insects were preserved in 1x Phosphate-Buffered Saline (PBS), given the intention to try to isolate bluetongue virus (BTV) from samples with positive PCR test results. Given the time period between capture (2014) and testing (2021), there were situations in which the abdominal content dissolved in the preservation medium, so that it was no longer possible to assess the feeding stage of those specimens. Those specimens were considered to be unfed. The identified insects were grouped in pools of 100 and 200 specimens, in Eppendorf tubes with 70% ethanol. A total of 55 pools were set up, out of which 19 had 200 specimens / pool and 36 had 100 specimens /pool.

The real-time RT-PCR assays for viral serogroup and serotype identification were performed using the universal nucleic acid extraction kit QIAmp cador Pathogen Minikit, qScript XLT 1-step RT PCR amplification kit, and the VetMAX European BTV Typing kit for BTV serotype 4 (BTV-4). The following primers and probes were used to determine the serogroup: Hofm_BTV_IVI_F2 – TGGAYAAAGCRATGTCAAA; Hofm_BTV_IVI_R2 – ACRTCATCACGAAACGCTTC; Hofm_BTV_IVI_P – FAM-ARGCTGCATTCGCATCGTACGC-Tamra (10).

The PCR test was performed using Applied Biosystem 7900HT Fast Real Time PCR System. To identify the viral serogroup, the thermal profile of the reaction was: 50°C - 10 minutes - 1x; 95°C - 2 minutes - 1x; 95°C - 10 seconds/60°C - 60 seconds - 40x. To identify the serotype, the thermal profile of the reaction was as follows: 45°C - 10 minutes - 1x; 95°C - 10 minutes - 1x; 95°C - 15 seconds/60°C - 45 seconds - 40x.

## RESULTS AND DISCUSSIONS

During the study period, 8347 *Culicoides* insects were identified, classified into three species complexes: Obsoletus, Pulicaris and Nubeculosus. Captured specimens were divided into two categories: unfed and fed specimens (Table 1).

The category of unfed specimens also included specimens in which the contents of the abdominal segment were no longer detectable, being replaced with preservation liquid. Of the total specimens captured, the Obsoletus complex was represented in a proportion of 89.01% (n=7430), followed by the Pulicaris complex with 7.37% (n=616), and the Nubeculosus complex with 3.6% (n=301).

Most of the tested specimens were unfed: 98.82% (n=7343) of the Obsoletus complex, 93.99% (n=579) of the Pulicaris complex, and 66.44% (n=200) of the Nubeculosus complex. The total number of fed specimens was 225 (n=87 from the Obsoletus complex, n=37 from the Pulicaris complex, and n=201 from the Nubeculosus complex) (Fig. 2).

**Table 1**

***Culicoides* specimens tested within the study**

| County | Village | OU | OF | PU | PF | NU | NF |
|---|---|---|---|---|---|---|---|
| **Bacău** | Blăgești | 9 | | | | | |
| | Dărmănești | 94 | 5 | | | | |
| | Filipeni | 100 | | | | | |
| **Dolj** | Almăj | 14 | 2 | | | | |
| | Argetoaia | 47 | 4 | | | | |
| | Brădești | 76 | 7 | 9 | 2 | | |
| | Bușuleț u | 46 | 24 | 252 | 27 | | |
| | Pișcani | 162 | 5 | 21 | 2 | | |
| | Poiana Fântânii | 21 | 6 | | | | |
| **Gorj** | Prigoria | 1803 | | | | | |
| **Ialomița** | Adâncata | 2 | | 7 | 1 | 200 | 57 |
| | Axintele | 3 | | 7 | 1 | | 43 |
| | Misleanu | 2 | 1 | 7 | | | 1 |
| **Vâlcea** | Alunu | 5 | | 2 | | | |
| | Bogdănești | 29 | | 2 | | | |
| | Căzănești | 4 | 1 | 30 | 4 | | |
| | Ghioroiu | 31 | | | | | |
| | Mateești | 22 | | | | | |
| | Zătreni | 100 | | | | | |
| **Vrancea** | Naruja | 2400 | | | | | |
| | Podu Narujei | 2000 | | | | | |
| | Voloșcani | 373 | 32 | 242 | | | |

OU – unfed Obsoletus specimens; OF – fed Obsoletus specimens; PU – unfed Pulicaris specimens; PF – fed Pulicaris specimens; NU – unfed Nubeculosus specimens; NF – fed Nubeculosus specimens

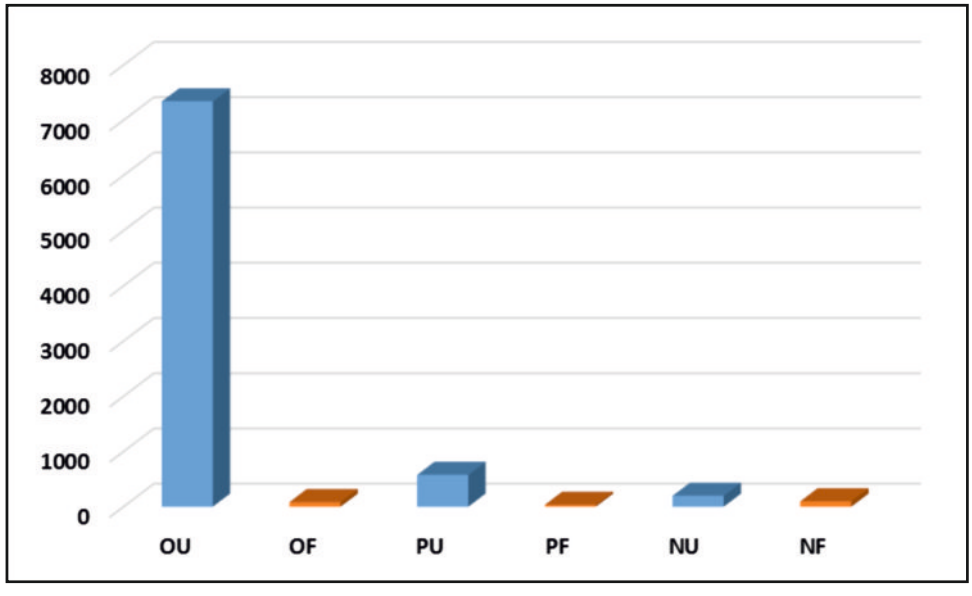


**Fig. 2.** Number of fed and unfed *Culicoides* specimens tested

Given the poor integrity of the samples, it was necessary to test the pools in two rounds: in the first round, invalid results were obtained for 14 of the 55 pools tested. A decision was made to retest the remaining 41 pools in a new round. The cycle threshold (Ct) values and the villages where the *Culicoides* species were collected are presented in table 2 (pools with negative result) and table 3 (pools with positive result).

16 of the 41 pools tested in the second round were positive, with Ct values between 32.36 and 37.14. 14 positive pools were made up of insects from a single county/pool. 2 positive pools (8 and 47) were constituted by merging *Culicoides* species from several villages in two counties (Ialomița and Dolj).

**Table 2**

**Negative results of real-time RT-PCR assays in 41 pools of *Culicoides* insects**

| Pool no. | Ct value | Species complex | County | Village |
|---|---|---|---|---|
| **1** | 0 | Obsoletus | Ialomița | Misleanu |
| | | | Dolj | Almăj, Argetoaia, Brădești, Busuleț u Pișcani, Poiana Fântânii |
| | | | Vâlcea | Căzănești |
| | | | Vrancea | Voloșcani |
| **2** | 0 | Pulicaris | Ialomița | Adâncata, Axintele |
| | | | Dolj | Brădești, Busuleț u, Pișcani |
| | | | Vâlcea | Căzănești |
| **3** | 0 | Nubeculosus | Ialomița | Adâncata, Axintele, Misleanu |
| **6** | 0 | Pulicaris | Sibiu | Orlat |
| | | | Neamț | Trifești |
| | | | Buzău | Stâlpu |
| | | | Giurgiu | Oncești |
| | | | Olt | Valea lui Alb |
| **9** | 0 | Obsoletus | Dolj | Brădești, Pișcani, Poiana Fântânii |
| **13** | 0 | Obsoletus | Vrancea | Voloșcani |
| | | | Gorj | Prigoria |
| **16** | 0 | Obsoletus | Gorj | Prigoria |
| **24** | 0 | Obsoletus | Vrancea | Podu Narujei |
| **25** | 0 | Obsoletus | Vrancea | Podu Narujei |
| **27** | 0 | Obsoletus | Vrancea | Podu Narujei |
| **28** | 0 | Obsoletus | Vrancea | Podu Narujei |
| **30** | 0 | Obsoletus | Vrancea | Naruja |
| **31** | 0 | Obsoletus | Vrancea | Naruja |
| **32** | 0 | Obsoletus | Vrancea | Naruja |
| **33** | 0 | Obsoletus | Vrancea | Naruja |
| **36** | 0 | Obsoletus | Vrancea | Naruja |
| **37** | 0 | Obsoletus | Vrancea | Naruja |
| **39** | 0 | Obsoletus | Vrancea | Naruja |
| **41** | 0 | Obsoletus | Vrancea | Naruja |
| **42** | 0 | Obsoletus | Vâlcea | Zătreni |
| **50** | 0 | Pulicaris | Vâlcea | Bogdănești, Căzănești |
| | | | Dolj | Brădești |
| | | | Vrancea | Voloșcani |
| **51** | 0 | Pulicaris | Vrancea | Voloșcani |
| **52** | 0 | Pulicaris | Vrancea | Podu Narujei |
| **53** | 0 | Nubeculosus | Ialomița | Adâncata |
| **54** | 0 | Obsoletus | Bacău | Blăgești, Dărmănești, Filipeni |

**Table 3**

**Positive results of real-time RT-PCR assays in 41 pools of *Culicoides* insects**

| Pool no. | Ct value | Species complex | County | Village |
|---|---|---|---|---|
| **8** | 33.94 | Obsoletus | Ialomița | Adâncata, Axintele, Misleanu |
| | | | Dolj | Brădești Busuleț, Pișcani |
| **11** | 36.35 | Obsoletus | Dolj | Argetoaia, Brădești |
| **12** | 33.92 | Obsoletus | Vrancea | Voloșcani |
| **14** | 33.51 | Obsoletus | Gorj | Prigoria |
| **15** | 32.36 | Obsoletus | Gorj | Prigoria |
| **17** | 34.68 | Obsoletus | Gorj | Prigoria |
| **18** | 34.46 | Obsoletus | Gorj | Prigoria |
| **19** | 33.73 | Obsoletus | Gorj | Prigoria |
| **20** | 34.47 | Obsoletus | Gorj | Prigoria |
| **21** | 35.65 | Obsoletus | Gorj | Prigoria |
| **22** | 34.89 | Obsoletus | Gorj | Prigoria |
| **26** | 37.14 | Obsoletus | Vrancea | Podu Narujei |
| **29** | 37.06 | Obsoletus | Vrancea | Podu Narujei |
| **44** | 32.73 | Obsoletus | Vrancea | Podu Narujei |
| **46** | 36.89 | Obsoletus | Vrancea | Podu Narujei |
| **47** | 35.93 | Pulicaris | Ialomița | Adâncata, Axintele, Misleanu, |
| | | | Dolj | Busuleț, Pișcani |

This is also the case for other pools under study. In these cases, due to the small number of specimens identified per village, it was necessary, for reasons related to the cost of testing, to combine the specimens so that a sample with a sufficient number of specimens could be constituted.

The presence of *Culicoides* species positive for BTV genome was confirmed in Dolj, Gorj, and Vrancea counties. We also consider possible the presence of positive specimens in Ialomița county, as pools 8 and 47 consisted of insects from this county and Dolj (with a proportion of 7/93 specimens in pool 8 and 21/78 specimens in pool 47).

15 of the 16 positive pools consisted of specimens of the Obsoletus complex. 1 pool consisted of specimens of the Pulicaris complex.

In order to determine the viral serotype, the pool with the lowest Ct value was selected, consisting of insects captured in Prigoria village from Gorj county (n=15), Following testing by real-time RT-PCR, using the Vetmax European BTV 4 Typing Kit, the BTV-4 viral serotype was identified, with a Ct value of 32.86, comparable to that obtained for bluetongue serogroup.

*Culicoides* vector competence represents the ability of the insects to maintain the biological cycle of replication/dissemination of the pathogen from/to the host species. Goffredo et al. (2015) mentions four criteria that are taken into account in determining the competence of a vector:

- the vector species is found in geographical areas where the disease is recorded;
- the pathogen was isolated from females that did not feed recently;
- after feeding, the species is able to multiply the vector;
- the species is capable of transmitting the pathogen by feeding on an uninfected animal.

If all four criteria are met, then the *Culicoides* species is considered competent for the transmission of a pathogen. If at least one of the criteria is not met, then the vector competence is partial. However, in the absence of concrete results from appropriate research, all vectors are considered potentially competent in the transmission of pathogens (8).

The second of the mentioned criteria is the isolation of the pathogen from specimens that have not fed recently (considered as unfed). In Europe, in the context of bluetongue evolution, several studies were conducted to demonstrate this criterion. Caracappa et al. (2003) reported the isolation of BTV-2 serotype in unfed specimens of *Culicoides pulicaris* by using BHK21 cell lines and propagation on embryonated eggs (1).

Savini et al. (2004) tested 58 pools consisting of 100 parous (which laid eggs at least once) and gravid females of the Obsoletus complex, successfully isolating BTV-2 (from three pools) and BTV-9 (from one pool) (16). Similarly, De Liberato et al. (2005) managed to isolate BTV-2 from a pool of unfed females of *Culicoides obsoletus*, using BHK21 cell line (4). In an experiment on vector competence,Paslaru et al. (2018) succeeded to isolate BTV-1 on Vero cell line, from females of *Culicoides scoticus* captured in Switzerland and artificially fed with spiked blood (15).

Although the success of isolating the pathogen from an unfed vector is directly related to the demonstration of vector competence, many other studies have focused only on the identification of the BTV genome from insects captured in epidemiological contexts favourable to vector-virus interaction.

Mehlhorn et al. (2007) carried out capture and identification activities of *Culicoides* species, between August 2006 and January 2007, in two farms with seropositive cattle. The specimens were separated into two categories (fed – unfed) and were investigated by real-time RT-PCR for BTV-8. Positive results were obtained for both fed and unfed individuals (13). Research conducted by Meiswinkel et al. (2007) on *Culicoides* captured in the Netherlands, indicated *Culicoides dewulfi* as a potential vector for bluetongue, following the positive results obtained by the in-house PCR assay based on segment 10, which synthesizes the non-structural protein NS3 (14). Vanbinst et al. (2009) tested 130 pools with unfed females, using a PCR protocol based on segment 5. Positive results were obtained on pools consisting of specimens of the Obsoletus and Pulicaris complexes (20). In 2019, Foxi et al. published the results of a research on *Culicoides* species trapped in Sardinia from sheep farms. 68 of the 77 pools were positive in the PCR assay. Vector competence for *Culicodes imicola*, *Culicoides newsteadi,* and Obsoletus complex was confirmed. The role as a potential vector was proposed for the species *Culicoides paolae* and *Culicoides circumscriptus* (7).

The aspects presented in the previous paragraph validate our approach, both in terms of the test used (real-time RT-PCR) and in terms of the pre-analytical stage (capture, identification, pooling, and separation of fed/unfed individuals).

In our study, following PCR testing of 55 insect pools, validated results were obtained for 41 pools. 16 pools, from three counties (Dolj, Gorj, and Vrancea) were positive for the BTV genome. The PCR test performed on one serogroup positive pool, demonstrated the presence of the BTV-4 viral serotype. 15 of the 16 positive pools were constituted of *Culicoides* species within the Obsoletus complex. A single positive pool consisted of insects within the Pulicaris complex.

These results represent, to our knowledge, the first record of the presence of BTV in populations of *Culicoides* species, in Romania. Identification of the BTV-4 serotype in *Culicoides* species, has an epidemiological significance regarding the evolution of bluetongue in Romania, being in accordance with the tests performed in 2014 at the Institute of Diagnosis and Animal Health on blood samples taken from ruminants with clinical signs. At that time, the results for the viral serogroup and serotype (BTV-4) were confirmed by the European Union Reference Laboratory (Pirbright, UK).

## CONCLUSIONS

Studies on the vector competence of *Culicoides* species are important in the context of the evolution, at European level, of diseases with significant economic impact, such as bluetongue and African horse sickness. Identification of species that are involved in the transmission of viral pathogens, in correlation with the preparation of distribution maps and the determination of seasonal abundance, are relevant tools that can be considered as part of control strategies for these diseases. Identification of the BTV-4 genome in *Culicoides* species trapped in disease outbreaks in Romania, has an important epidemiological significance regarding the evolution of bluetongue in this country, by demonstrating the presence, in the same epidemiological context, of the three main elements of an arboviral disease: animal with clinical signs, the vector, and the pathogen.

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