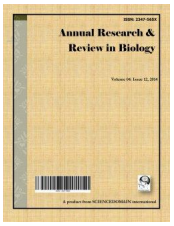

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New *Culicoides* Vector Species for BTV Transmission in Central and Central West of Anatolia

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SY and BD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OB, UU, OY, MK and OA managed the analyses of the study. Author OA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study was to investigate the presence of BTV infection and possible vector species in different regions of Turkey. In the study, blood samples taken from 666 Akkaraman sheep were examined. 2000 *Culicoides* specimens were captured by light traps from the same provinces and 20 *Culicoides* spp. were identified. Blood sera samples were investigated by c-ELISA and SNT for detecting Abs to BTV. Sera samples were detected as positive 67 (10.06%) and 160 (24.02%) by SNT and ELISA, respectively. SN_{50} values of the 67 positive sera samples by SNT were detected between 1/2.38 and 1/200. All sheep blood samples and pools became *Culicoides* spp. samples were examined for BTV Ag presence by BTACE. Thirty six (5.40%) blood samples were detected as positive, but no from *Culicoides* pools. In the meantime, all sheep blood samples and *Culicoides*

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samples were directly investigated for BTV genome by one step RT-PCR. Fourteen (2.10%) blood samples and 7 (11.11%) *Culicoides* species were detected as positive. Also, the blood samples and the *Culicoides* samples were inoculated into Vero cell culture and passaged 5 times. Twenty nine (4.35%) blood samples cultured in Vero cell culture lines showed CPE but non CPE was observed in *Culicoides* samples. While 5 (17.24%) of 29 CPE positive isolates were identified as BTV by One Step RT-PCR. Total 26 samples (14 blood samples, 7 *Culicoides* samples and 5 supernatants) which detected BTV genome positive by One Step RT-PCR were serotyped. At the end of the study, while 23 of 26 samples were serotyped as BTV-9, two samples were serotyped as BTV-4. One sample (*C. punctatus*) from *Culicoides* was not serotyped as none of serotypes of BTV. In the present study, BTV was isolated for the first time from *C. circumscriptus, C. kibunensis,* and *C. punctatus* in Turkey.

Keywords: Sheep; culicoides; bluetongue virus; one step RT-PCR.

1. INTRODUCTION

Bluetongue disease is one of the most serious viral diseases detected in sheep [1,2,3,4]. BTV causes Bluetongue disease mainly in sheep and less frequently in cattle, goats, deer, elks, camels and wild ruminants [5,6]. After introduction through the bite of an infected midge, the virus is transported by the host dendritic cells from the skin to the local lymph nodes [7], the sites of initial virus replication [8]. Subsequently, it spreads to the blood circulation inducing a primary viraemia which seeds secondary organs, i.e., lymph nodes, spleen and lungs [9]. The virus replicates in vascular endothelial cells, macrophages and lymphocytes [8]. In early viraemia virus is associated with all blood elements, while at later stages of viraemia it exclusively associates with erythrocytes [10]. Viraemia in infected animals has a prolonged course, but is not persistent [11]. Its duration depends on the longevity of erythrocytes to which virus is bound, in contrast to the other blood cells, even at the late stage of infection [10]. It is also related to the species and breed of the infected animal. Viraemia lasts 14 to 54 days in sheep and 19 to 54 days in goats [12]. In cattle, viraemia may last as long as 60 or, even 100 days [13], which makes this animal an important host, from the epidemiological point of view [14]. In the present study, we planned to determine the presence, serotypes, and potential vectors of BTV infections occurring time to time in Central and Central West of Turkey.

2. MATERIALS AND METHODS

2.1 Animals and Samples

The present study was performed between the months of June and September in 2007 in several provinces located in Central and Central West Anatolia. These provinces were located with their parallels and meridians bordering them as follows Konya 32º 31' E, 37º 52' N; Burdur 30º 17' E, 37º 43' N; Afyon 30º 33' E, 38º 45' N; Eskişehir 30º 32' E, 39º 46' N; Aksaray 33º 04' E, 37º 40' N; Nevşehir 34º 43' E, 38º 38' N; Kayseri 35º 30' E, 38º 43' N; Yozgat 34º 48' E, 39º 50' N; and Bursa 29º 04' E, 40º 11' N'. Each province was visited twice (June and September) to collect blood samples from different animal producers. During the study, 6000 sheep were screened in the private animal producers and among these unvaccinated 666 sheep showed clinical signs of the disease (elevated body temperature, running nose and eyes, and state of stagnation).

Modified CDC type light traps were used to collect *Culicoides* samples. The light traps were set up 24 times in the barns where the sheep were sampled in stated provinces. The species identification for *Culicoides* was made according to distribution of light and dark spots on their wings. The samples containing light spots on the apical side of r-m cross vein and R2 cell or having no spots on their wings are designated as *Culicoides* sp. After determining the species, 63 *Culicoides* pools were established and each species included 1 to 250 samples. *Culicoides* samples were prepared as described by Caracappa et al. [15].

2.2 Serum Neutralization Test (SNT) and Competitive ELISA (cELISA)

Vero was used for the propagation and titration of BTV-4 and SNT. The presence of anti-BTV-4 antibodies in the serum samples collected from 666 clinically sick sheep was checked by SNT as described by Frey and Liess [16] and verified using commercially available cELISA kit[1]. The antibody titration of the blood samples turned out to be positive for anti-BTV-4 Abs in SNT was performed using serum neutralization 50 (SN_{50}).

2.3 Bluetongue Virus Ag Capture ELISA (BTACE)

All the erythrocyte samples prepared from the blood samples gathered from the sheep and entire of the *Culicoides* samples with established species were further studied for the presence of BTV Ag using BTACE[2], developed against whole serotypes of BTV.

2.4 One Step Reverse Transcriptase-Polymerase Chain Reaction (One Step RT-PCR)

All the leukocyte samples prepared from the blood samples gathered from the sheep and the *Culicoides* samples were directly tested by One Step RT-PCR. RNA extraction from leukocyte and *Culicoides* samples was carried out using commercial RNeasy Mini Kit[3]. Vero cell lines were used for virus isolation. For this, all blood leukocyte and *Culicoides* samples were passaged five times in Vero. The supernatants of the cell cultures visually observed cytopathogenic effect (CPE) at their 5th passage were collected and analyzed for the presence of viral genome with one Step RT-PCR. RNA extraction from supernatants was carried out using commercial QIAamp Viral RNA Mini Kit[4]. Viral RNA products obtained after extractions were verified using commercial One Step RT-PCR[5]. The primers prepared against BTV segment 7 were used for one step RT-PCR method [17]. Moreover, PCR products obtained from the cell culture isolates, the leukocyte and *Culicoides* samples were seroptyped using one step RT-PCR. For this stage, the primers specific for BTV serotype 2, 4, 8, 9, and 16 were employed [18].

3. RESULTS

In this study, total 2102 *Culicoides* samples, out of 1946 females and 156 males were captured and twelve species; *C. circumscriptus, C. festivipennis, C. gejgelensis, C. kibunensis, C. longipennis, C. newsteadi, C. nubeculosus comp.*, *C. picturatus, C. pulicaris, C. punctatus, C. shaklawensis* and *C. simulator* were identified (Table 1). As seen in Table 1, the most *Culicoides* specimens were caught in Afyon (1325 individuals) and in Burdur (694 individuals). Eleven *Culicoides* species were detected in Afyon and six species in Burdur. *C. picturatus* was found as dominant species, *C. nubeculosus comp.*, *C. kibunensis, C. simulator* and *C. festivipennis* were follow it. While *C. imicola comp., C. obsoletus comp.* and *C. schultzei comp.* vectors of BTV in Palearctic region were not detected, the other vector of BTV, *C. pulicaris complex* was captured in low numbers, especially in Burdur.

The results showed that while 67 (10.06%) out of 666 sheep were positive for the presence of anti-BTV-4 Abs using SNT, 160 (24.02%) out of 666 sheep were positive for the same antibody (Ab) using cELISA (Table 2). The distribution of SN_{50} of 67 sera samples detected positive by SNT was found between 1/2.38 and 1/200. While 36 (5.40%) erythrocyte samples were positive for the presence of BTV Ag by BTACE, none of the *Culicoides* samples were positive for the occurrence of the same Ag (Table 2). All leukocyte samples and *Culicoides* samples were directly investigated for BTV genome by One Step RT-PCR. 14 (2.10%) leukocyte samples and 7 (11.11%) *Culicoides* species were detected as positive. 29 (4.35%) of leukocyte samples cultured in Vero showed CPE but non CPE was observed in *Culicoides* samples. One step RT-PCR was used for the identification of 29 isolates. For this purpose, the supernatants collected from the 5th passage of Vero were used (Table 2). While 5 (17.24%) isolates were identified as BTV by one step RT-PCR. One step RT-PCR applied once again to 26 samples which detected BTV genome positive by one step RT-PCR for serotyping. At the end of the study while 23 (12 leukocytes, 5 supernatants and 6 *Culicoides*) of 26 samples were serotyped as BTV-9 and 2 (leukocytes) of 26 samples were serotyped as BTV-4. One sample (*C. punctatus*) from *Culicoides* was not serotyped as none of serotypes of BTV.

4. DISCUSSION

Transmission of BTV occurs when *Culicoides* suck blood from the infected animals during their viremia. In the present study, the first sampling was performed at the beginning of the summer and the second sampling was done at the end of the summer. After the first and the second sampling, 41 and 26 samples detected to be seropositive by SNT, respectively. When the samples were also checked by cELISA, 69 and 91 animals were detected to be seropositive atthe first and the second sampling, correspondingly. The ratio of seropositivity at first sampling to that of the second sampling tested using SNT was higher in Konya, Afyon, Bursa, Eskişehir, Burdur, and Yozgat. By contrast, the proportion of seropositivity at second sampling to that of the first sampling tested using SNT was higher in Aksaray, Kayseri, and Nevşehir.

Table 1. *Culicoides* species identified in Central and Inner Western Anatolia and distribution according to the collection centers

Species	Afyon		Bursa		Eskişehir		Burdur		Aksaray		Yozgat		Nevşehir		Kayseri	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
C. circumscriptus	13	2	-	-	-	-	44	7	-	-	8	2	30	3	3	-
C. festivipennis	81	19	-	-	-	-	-	-	-	-	1	-	-	-	-	-
C. gejgelensis	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
C. kibunensis	398	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. longipennis	6	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-
C. newsteadi	11	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-
C. nubeculosus comp	2	-	-	-	-	-	463	69	6	-	-	-	10	-	-	-
C. picturatus	558	5	3	-	-	-	-	-	2	-	-	-	1	-	-	-
C. pulicaris	2	2	-	-	-	-	45	4	1	-	-	-	-	-	-	-
C. punctatus	3	-	-	-	-	-	22	-	-	-	-	-	-	-	12	-
C. shaklawensis	24	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. simulator	134	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Culicoides sp.	22	34	-	-	-	-	7	-	-	-	-	-	-	-	-	-
Total	1254	71	3	-	1	-	614	80	9	-	9	2	41	3	15	-

Table 2. The distribution of sampling sheep and the results of tests

Provinces	Date	Sampled sheep		SNT Ab (+)	ELISA Ab (+)	ELISA Ag (+)	One step RT-PCR (Leukocyte)	Virus isolation (Leukocyte)	One step RT-PCR (Isolate)
Konya (Kadinhani)	1. Sampling	76	36	15	9	-	1	-	-
Konya (Centrum)	2. Sampling		40	6	7	-	-		-
Afyon	1. Sampling	76	36	10	-	5	-	5	-
	2. Sampling		40	1	7	-	-	-	-
Bursa	1. Sampling	80	40	2	4	2	2	2	-
	2. Sampling		40	1	5	1	-	-	-
Eskişehir	1. Sampling	69	34	2	2	4	3	2	-
	2. Sampling		35	1	-	-	5	-	-
Burdur	1. Sampling	75	40	6	-	4	5	4	3
	2. Sampling		35	4	-	3	4	-	1
Aksaray	1. Sampling	70	35	-	8	-	1	-	1
	2. Sampling		35	4	24	1	-	-	-
Nevşehir	1. Sampling	71	36	1	15	-	-	-	-
	2. Sampling		35	6	19	-	3	-	-
Kayseri	1. Sampling	69	39	-	4	1	-	-	-
	2. Sampling		30	3	22	-	5	1	-
Yozgat	1. Sampling	80	40	5	27	1	-	-	-
	2. Sampling		40	-	7	14	-	-	-
Total		666		67	160	36	14	29	5

No seropositive animals were noted by SNT at the first sampling, performed at the beginning of the summer, in Aksaray and Kayseri, and at the second sampling, done at the end of the summer, in Yozgat. These observations suggest that the animals come across *Culicoides* carrying viruses for the first time at the beginning of the summer and might catch infection; therefore, seropositive animals indicated that they were naturally infected with BTV and current samples were collected during acute phase of the viremia. When the non-inoculated animals sampled for the present study are considered, this consequence suggests that *Culicoides* are effective at the transmission of the virus and the presence of the reservoir animals in the region together can lead to natural infection of sheep, which then develop natural immunity against the viral infections. SN_{50} values of the first samples determined to be seropositive for BTV by SNT were between 1/2.51-1/89.1 and the highest value was 1/89.1 in only one animal. This result indicates that the animals might have encountered with the *Culicoides* during their flight period a year ago, and during this time antibody titration may have progressively decreased. Moreover, the number of the animals determined to be seropositive by cELISA at the first sampling was higher than that of the second sampling in Konya, Eskişehir, and Yozgat but lower than that of the second sampling in Afyon, Bursa, Aksaray, Nevşehir, and Kayseri. No animals were determined to be seropositive with cELISA at the first sampling in Afyon, at the second sampling in Eskişehir, and at the first and second samplings in Burdur. The results obtained using cELISA bring to mind similar interpretations made for those obtained using SNT. Therefore, significantly more sheep were noted to be seropositive for BTV using cELISA during the high flight activities of *Culicoides* at the end of the summer (*P: 0.0000; P<0.0001*).

Richards et al. [19] showed that the lambs inoculated with BTV developed humoral immune response against to BTV; however, this immune response failed to immediately neutralize the virus. Except for the results of the study of Burgu et al. [20], the seropositivity rate determined with SNT and cELISA in the present study was lower than the previous studies carried out in Turkey [21,22,23]. This lower antibody prevalence might be due to the use of Akkaraman sheep strain whose antibody prevalence is shown to be lesser in local sheep strains in comparison to foreign sheep strain [24]. The reason for this is reported to be the migration of *Culicoides imicola comp.*, vector for the spread of the virus and distributed throughout Southern Europe, changes in climate conditions, and global warming. In addition, new vector species (*Culicoides imicola comp.* and *Culicoides obsoletus comp.*, particularly, *Culicoides dewulfi*) are reported to contribute the spread of viral infection toward Northern Europe [25,26,27].

In early viraemia virus is associated with all blood elements, while at later stages of viraemia it exclusively associates with erythrocytes [8,10]. Virus particles appear to be sequestered in invaginations of the erythrocyte membrane [8], allowing prolonged viraemia in the presence of neutralizing Abs [14,19]. In this study, blood erytrocyte samples were examined by BTACE that detectable all serotypes of BTV. While 36 (5.40%) erytrocyte samples were detected as positive for antigen presence. Directly leukocyte samples were investigated for BTV genome by One Step RT-PCR. Fourteen (2.10%) leukocyte samples were detected as positive. Excluding one (Bursa 40), antibodies were not detected in 13 of the animals either with ELISA (Ab) or with SNT. The presence of viral Ag and genome in all blood cells in these 13 animals demonstrated that they were in the early stage of viremia.

In the present study, 8 samples were detected for both BTV antibodies and antigens. Five samples by ELISA and 3 samples by SNT were determined presence of BTV antibodies, while all viral antigens were detected by BTACE. One sample (Burdur 15) observed CPE in Vero were also detected for viral Ag by BTACE and for viral genome by one step RT-PCR. But, blood serum sample from the same sheep were not detected the presence of BTV Abs by SNT and ELISA. It might indicate early stage (prior to 14 days) of the infection as reported by Verwoerd and Erasmus [28]. Viraemia in infected animals has a prolonged course, but is not persistent [11]. Its duration depends on the longevity of erythrocytes to which virus is bound, in contrast to the other blood cells, even at the late stage of infection [10]. It is also related to the species and breed of the infected animal. Viraemia lasts 14 to 54 days in sheep [12]. In view of the possible co-existence of neutralizing Abs and Ags in the body in BTV infection, in the Bursa 40 blood sample, in addition to the presence of viral Ag and genome, the ELISA (Ab) test having produced positive results were considered to be related to the viremia having advanced and the Abs having been produced as from day 14 onwards. In 22 of the 36 samples, the presence of viral Ag was

determined by BTACE, but viral genome was not detected in the leukocytes. 18 out of the 22 samples having negative for Abs, demonstrated them to belong to the early stage of viremia (period before the 14th day of infection). 4 samples were found to be positive by ELISA (Ab). It was considered that these 4 animals were in the last stage of viremia and that the production of Abs had already started. Also, blood leukocyte samples were inoculated onto Vero and then passaged 5 times for virus isolation. CPE was observed on 29 (4.35%) leukocyte samples. For identification of isolates, 29 CPE positive leukocyte samples were controlled for viral genome by one step RT-PCR and 5 (17.24%) of them were identified as BTV by one step RT-PCR. In 5 samples CPE was detected in the Vero at the end of the 5th passage, and using the supernatant fluid of the cell culture, the serotype was determined as BTV-9 with one step RT-PCR. However, the viral Ag was not detected in the erythrocytes of these animals by BTACE (Ag). These animals were also considered to be in the early stage of viremia.

Previous studies carried out in Turkey indicate that the primary vector transmitting BTV is *C. imicola*, which is primarily seen in Aegean and Mediterranean regions but no other parts of Turkey. While *C. obsoletus s.s* is reported to be encountered in very low numbers in general, *C. obsoletus comp*. (*C. scoticus* and *C. montanus*) are rarely seen [29]. Nevertheless, only a male sample of *C. dewulfi* [30], argued to be a potential vector of BTV, was encountered in Adana province [31]; however, *C. chiopterus,* classified in the same group, has never been reported, suggesting that other species may play a role in the transmission of BTV as well. In Emirdag district of Afyon province, 1325 *Culicoides* samples (1254 females and 71 males) were collected and 11 species were achieved from the analyses of the collected *Culicoides* samples. The assessments of these samples showed that while *C. imicola comp.* and *C. obsoletus comp.,* the vectors of BTV, were not present, *C. pulicaris comp.* (*C. pulicaris s.s., C. punctatus, C. newsteadi*) were encountered at lower rates (1.36%); however, no viral genome presence was found among the collected samples using one step RT-PCR. The most common (42.5%) species was *C. picturatus*; *C. kibunensis* was second most frequent species among the collected samples.

We detected viral genome with one step RT-PCR in one of the samples pulled from the *C. kibunensis* pool. Furthermore, while 10 out of 75 sheep sera samples collected from Burdur were detected to be positive for BTV Abs using SNT, 7 of them were noted to be positive for the presence of BTV Ag by BTACE. The virus was isolated from the leukocyte samples of 9 sheep; however, only four of the isolates were detected to have viral genome presence using one step RT-PCR. Although no *C. imicola comp.* and *C. obsoletus comp.* were encountered, *C. pulicaris comp.* was detected in this region. We discovered BTV viral genome in *C. circumscriptus* (Table 1) samples, collected from Aşağımüslimler and Yassıgüme villages and in *C. nubeculosus comp.* samples gathered from Yassıgüme village using one step RT-PCR. Meiswinkel et al. [32] reported that 30 *Culicoides* spp. played a role more or less in spread of BTV infections. While *C. nubeculosus comp.* (*C. nubeculosus s.s* and *C. puncticollis*) are among the 30 *Culicoides* spp., *C. circumscriptus* and *C. punctatus* are not found among those *Culicoides* spp. Therefore, BTV virus was isolated from the *C. circumscriptus* and *C. punctatus* for the first time in the present study.

Carpenter et al. [33] indicate that real time RT-PCR is equally sensitive in the detection of infectious RNA and non-infectious RNA. In the present study, the same reason might explain the inability to get positive results using BTACE in leukocyte and *Culicoides* samples cultured in Vero, and it may also elucidate failure to observe positivity using one step RT-PCR in *Culicoides* samples (cell culture supernatants) but obtaining low (17.24%) positive results from the supernatants of leukocyte samples. The correlation coefficient detected between the virological tests (one step RT-PCR and BTACE or BTACE and one step RT-PCR, Table 2) was positive (r: + 0.613) and the P-value was 0.000. The correlation coefficient between BTV (+) cell culture and one step RT-PCR or between One step RT-PCR and BTV (+) cell culture was positive (r: + 0.594) and the P-value was 0.000. On the other hand, the correlation coefficient between BTV (+) cell culture and BTACE or between BTACE and BTV (+) cell culture was positive (r: + 0.362) and the P-value was 0.000. The positive correlation between the tests was strong and statistically significant. The test results (virus isolation, BTACE and one step RT-PCR) of the sheep leukocyte samples belonging to the animals used in this study were analyzed both comparatively and statistically. To determine the difference between the tests, the 2-sample t test (Minitab 11.12 version) was used.

Accordingly; statistically significant differences were determined to exist between BTACE and one step RT-PCR (*P: 0.0015; P<0.01*) and between virus isolation and one step RT-PCR (*P: 0.020; P<0.05*). On the other hand, no statistically significant difference was determined between virus isolation and BTACE (*P: 0.37; NS*).

5. CONCLUSION

Recurrent viral infections in Turkey are present. Therefore, as in the world the diagnoses of the etiologic agent, determination and serotyping of field isolates are essential to properly struggle with viral infections. Consequently, wide ranging studies in the future should be carried out using advanced techniques such as real time RT-PCR for getting rapid and effective results.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

SOURCES AND MANUFACTURERS

[1]cELISA kit Veterinary Medical Research Development Inc., Pullman, USA.
[2]BTACE, Narrabri, Australia.
[3]RNeasy Mini Kit, Qiagen, Catalog No: 74106, Hilden, Germany.
[4]QIAamp Viral RNA Mini Kit, Qiagen, Catalog No: 52906, Hilden, Germany.
[5]One Step RT-PCR, Qiagen, Catalog No: 210212, Hilden, Germany.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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