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Bluetongue virus in *Culicoides spp.* in Manabí province, Ecuador

Mariella Centeno [1], *Denisse Chliliquinga* [1], *José Velázquez* [4], *Euclides De La Torre* [2], *David Jarrín* [3], *Alex Maldonado* [2,5], *Jimmy Alava* [1], *Maritza Barrera* [1*]

[1.] *Veterinary Department. Faculty of Veterinary Sciences, Universidad Técnica de Manabí, Portoviejo 130105, Manabí, Ecuador.*
[2.] *Animal Diagnostic Laboratory. Agencia Ecuatoriana para el Control Fito y Zoosanitario (AGROCALIDAD), 170184 Quito, Pichincha, Ecuador.*
[3.] *Molecular Biology Laboratory. Agencia Ecuatoriana para el control Fito y Zoosanitario (AGROCALIDAD), 170184 Quito, Pichincha, Ecuador.*
[4] *Rapid Diagnostic Laboratotaory. Agencia Ecuatoriana para el Control Fito y Zoosanitario (AGROCALIDAD), 130802 Manta, Manabí, Ecuador.*
[5] *Alex Maldonado: Facultad de Ciencias de la Salud, Universidad Internacional SEK, Ecuador.*
**Corresponding Author: maritza.barrera.@utm.edu.ec*

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ABSTRACT

Bluetongue is a viral disease that affects sheep, cattle and other domestic or wild ruminants. Different species of Culicoides transmit the virus (BTV). High BTV seroprevalence was found in farms of Manabí and other two provinces of Ecuador, but the presence of the virus in the *Culicoides s*pp. vector has not been reported. In the current study, the main goal was to demonstrate the presence of BTV in C*ulicoides* in Ecuador for the first time and characterize the species of *Culicoides* collected in farms located in the central-east area of Manabí province. Six farms were selected to be monitored by BTV c-ELISA. All the 100 tested animals were positive. Using a CDC trap with ultraviolet light placed in three BTV-positive farms for three nights, 2240 specimens of Culicoides were collected. Six different *Culicoides* species have been identi-fied, which were presented in different abundance percent: 62% *C.insignis*; 7% *C. batesi*; 1.8% *C.foxi*; 1.8% *C.diabolicus;* 15.48% *C.crepuscularis*; 12% *C.antunesis*. These last two species have been identified for the first time in Ecuador. Q-PCR detected BTV RNA in the homogenates of female midges collected in each farm, so it was demonstrated that the epidemiological cycle of the virus is completed; since *female* midges infected with BTV were found, it is too a novel result for Ecuador.

Keywords: Bluetongue, BTV, Culicoides, cattle, real-time PCR, competitive ELISA, Ecuador, Manabi

INTRODUCTION

Bluetongue is a viral hemorrhagic fever that affects sheep, but cattle and other domestic or wild ruminants are susceptible. The etiologic agent of the disease is bluetongue virus (BTV) (genus *Orbivirus,* family *Reoviridae*). Currently, 27 serotypes have been widely recognized [1-4]. Depending on the BTV serotype, the clinical mani-festations of the disease vary between species, from being unapparent in the vast majority of infected animals to fatal [5-7]. In many countries, BTV infection is high in ruminants, although clinical disease is often not

recorded [8]. Bluetongue infection is included on the WHAO list of notifiable diseases as it infects multiple species[9]. It is a vector-borne virus whose biological vector is the biting midge of several species of the Culicoides (Diptera: Ceratopogonidae)[10-13].

Culicoides are nematocerous and hematophagous; some species are vectors for essential arboviruses. They inhabit warm and temperate zones, but the abundance of rainfall is a decisive factor in their life cycle[14].

The coastal region of Ecuador, where the province of Manabí is located, has climatic conditions that support the life cycle of Culicoides during the rainy season (December-July). Culicoides breed in various habitats and stay near their hosts, including in and around farms, decaying vegetation, manure, pond edges, and moist soils [15]. The geographic distribution of Culicoides species is variable, and each geographic zone has different species that act as BTV vectors. *C. insignis* is among the most common species in the southeastern USA, the Caribbean Basin, and Central and South America[16-18]. There are scattered investigations about the characterization of the Culicoides in Ecuador. Sixty-five species of Culicoides have been reported, and 16 were recently recorded. The *Hoffmania* subgenus is considered the most prevalent in the country. In addition, some anthropophilic species have been identified. [19]

Recently, we have reported a high BTV seroprevalence in farms north of Manabí province [20,] but the presence of the BTV virus in Culicoides had not been investigated. For effective risk management, we know the species composition and abundance of Culicoides populations in an area[15].

The main objective of the present study was to detect BTV-infected Culicoides for the first time in Ecuador and characterize the species of Culicoides collected in farms located in the central-east area of the province of Manabí. The study was conducted in this area because no cattle were introduced from other provinces, and the control of insect vectors was not applied.

Cross-sectional sampling was carried out during the rainy season of 2022 and in two stages: first, BTV-specific ELISA was used for screening of BTV sero-reactor cattle on six farms in central-Eastern Manabí province (Santa Ana and 24 de Mayo); second, Culicoides midges were collected from three farms for species identification and testing for BTV- RNA.

MATERIALS AND METHODS

Serologic screening for BTV seroreactors.

Six farms located in the cantons of Santa Ana and 24 de Mayo in the central-eastern area of Manabí province were selected. The selection criteria were based on the consent of the farmers, the size (no more than 300 animals) of their cattle population aged > 6 months and the environmental and climatic conditions during the rainy season that favor the presence of the vector (e.g., the presence of numerous bushes, many glens or natural wetlands, and the absence of any vector control measures. (Figure 1)

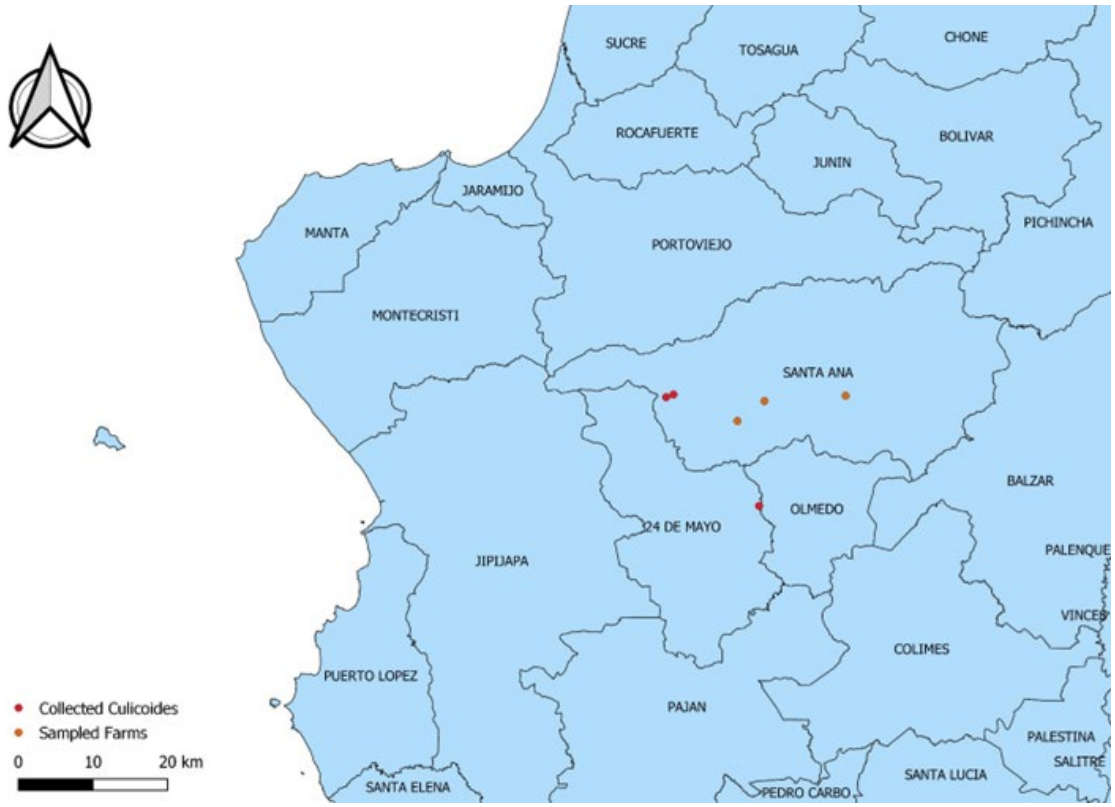

Figure 1. Farms sampled for serologic screening for BTV seroreactor (orange and red dots) and farms where Culicoides were collected (red dots).

Between April 11 and May 1, 2022, samples of blood (without anticoagulant) were taken from 100 bovines aged > 6 months and distributed across the different selected farms (Table 1). All serum samples were analyzed using a c-ELISA to measure antibodies specific for BTV (Kit ID Screen Bluetongue Competition IDVET).

Canton	Farms	Cattle population	No. Samples
Santa Ana	Bonce	50	6
Santa Ana	Monte Oscuro	20	5
Santa Ana	La Uniòn	300	33
Santa Ana	Visquije	100	14
Santa Ana	Tierras Negras	70	9
24 de mayo	Bella Vista	300	33
Total		840	100

Table 1. Number of total and sampled bovines in the farms of the center-east of the province of Manabi during the serologic screening (April 11 to May 1, 2022).

Entomological collections in selected farms

The insects were collected in May 2022 on three BTV seropositive farms (Bonce, Monte Oscuro and Bellavista), signaled as red dots (Figure 1). A battery-powered UV light trap (miniature CDC Light Trap with UV light) was set inside each livestock farm, next to the cattle barn, and at a height of 2 meters, the lamp was left in place for 3 nights (6 pm to 6 am).

Specimens were collected in a flask containing 70% ethanol, although some felt dry, into mesh bags and then transferred to 70% ethanol immediately after clearing the trap. The insects were taken to the Entomological Laboratory (Rapid Diagnostic Laboratory of Manta) for taxonomic identification.
Culicoides were separated from other arthropods, based on morphological keys (observed under stereomicroscope) and stored in 70% Ethanol.

Identification of Culicoides species
Females and males were separated, and their wings, head, alter, mesonotum, legs and the last three segments of the female's abdomen were mounted on slides with Hoyer solution. They were then examined under a stereo microscope. Morphological and morphometrical patterns described by various authors were used to identify *Culicoides* at the species level. Calculation of relative abundance was performed.

Detection of BTV- RNA by Q-PCR
Female midgets containing blood were selected for RNA extraction by observation under a stereoscope of the reddish or brown col-oration of the abdomen. They comprised 50 midges per pool (three pools from each of the three farms) and were placed in 1.5 mL microtubes containing 70% ethanol. One pool from each farm was processed for RNA extraction, followed by one-step BTV qPCR.
Briefly: RNA was extracted with TRIZOL[21-22] with agitation in Cryomil (RETSCH) 20 Hz, 2 min and 25 Hz, 1 min, followed by Chloroform extraction and Ethanol addition. According to the manufacturer's instructions, the total RNA from each microtube was finally purified with High Pure Viral RNA Kit, Roche, S.A. One-step real-time RT-PCR assay was performed, using a primer set and TaqMan® Probe, which is specific for leading BTV serotype (serotypes 1-26) and targets BTV segment 10, which codes for the NS3 protein [23,24,9] and the One-Step RT-q-PCR enzyme kit (GoTaq® 1-Step RT-qPCR System, Promega, Corp.) using the Light Cycler (Roche). Positive control was BTV-2 strain, BHK passage. The results were expressed on the Threshold cycle Value (Ct)

RESULTS

Culicoides collected from selected farms.
Several entomological samples were collected in microtubes: 14 from Bellavista, eight from Bonce, and six from Monte Oscuro (80 specimens of Culicoides were present in each sample). Thus, 2240 specimens of the genus *Culicoides* were collected.
According to the taxonomic classification work carried out, six different species were identified; these were (in decreasing order of abundance): 62% *C.insignis;* 15.48% *C.crepuscularis;* 12% *C.antunesis;* 7% *C. batesi;* 1.8% *C.foxi;* 1.8% *C.diabolicus.* In the figures, there is showing the wing pattern of C. insignis (Figure 2), *C.crepuscularis* (Figure 3) *and C.antunesis* (Figure 4), with the morphologic characteristics that allowed the specific identification.

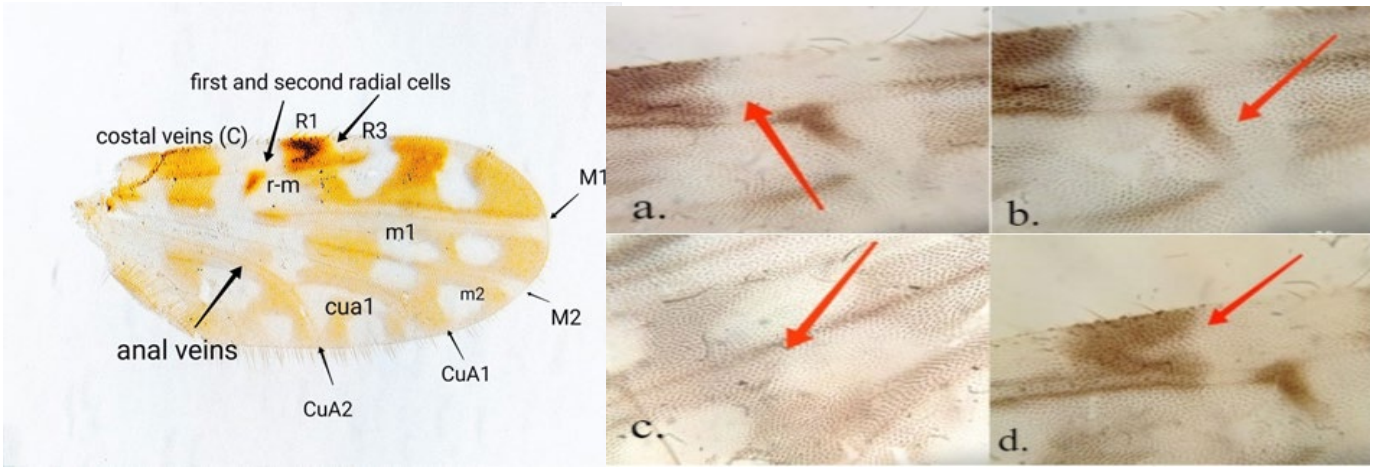

Figure 2. Wing pattern of *C. insignis.* a. Second radial cell wholly or mainly included in a depleted spot; b. Dark rm transverse vein, extending on both sides of the continuous M2 vein, ninth male tergite without mesal cleft, apicolateral; c. cell m1 with a pale distal spot; d. vein R3 dark to the point where it turns to meet the costal vein.

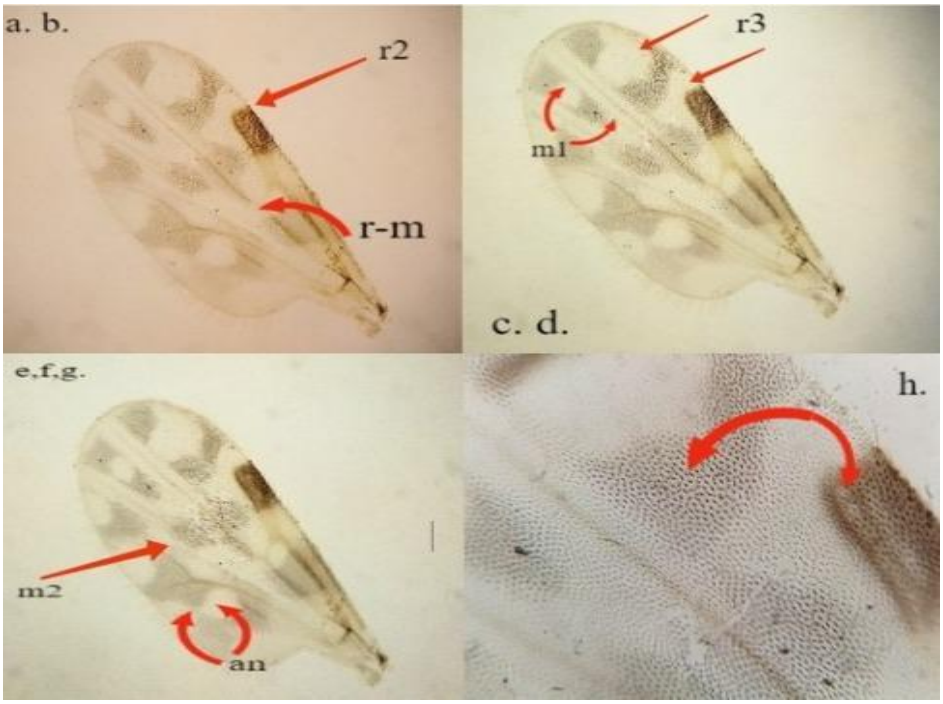

Figure 3. Wing pattern of *C. crepuscularis.* a. No pigmented vein r-m; b. Second radial cell in dark macula; c. Cell r3 with two clear areas; d. Cell m1 more than one white spot; and. M2 vein not embraced by clear area; F. Anal cell with a white spot; g. Wings with abundant microtrichia.

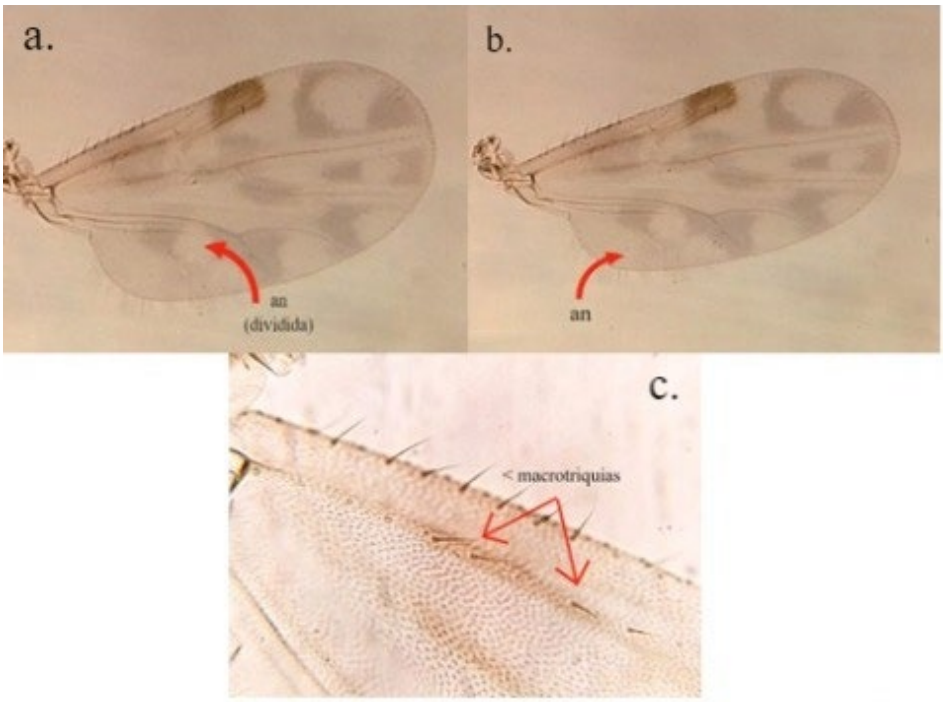

Figure.4. Wing pattern of *C. antunesi.* a. Divided anal cell clear area, b. Anal cell with a white spot; c. Wings with few microtrichia

Detection of the BTV RNA in pools of midges collected on each farm

According to the q-PCR results for the RNA extracted from Culicoides collected from each farm, three samples and the positive control yielded a characteristic fluorescence/cycles curve, with Ct (threshold cycle) values ≥ 35 (Table 2), which corresponds to a positive result. The Culicoides collected from these three farms (Bellavista, Bonce and Monte Oscuro) contained BTV- RNA.

Farms	Threshold cycle	Result
Bella Vista	33.11	+
Bonce	35.53	+
Monte Oscuro	35.22	+

Table 2. Results of the real-time RT-PCR of bluetongue virus I ARN purified from a pool of culicoides collected in each of three farms of Santa Ana y 24 de Mayo

DISCUSSION

Bluetongue is an endemic disease limited to tropical and subtropical latitudes [5] at which the vector finds optimal conditions for its life cycle development. The eastern part of Manabí province has a humid megathermal climate. There are two seasons: rainy and dry. During the rainy season (December to July), the climate pro-vides suitable environmental conditions that support the life cycle of the Culicoides vector[25].

Tabachnik [26] reviewed the global epidemiology of bluetongue virus infection and applied the concept of an ecosystem to BTV; this considers the virus to be part of a complex system in which the host, vector, and virus species, along with environmental aspects, form a particular ecosystem that affects the distribution and dynamics of a pathogen and disease. As a result of this interaction, the major Culicoides species that transmit BTV are different for each geographic region. Two BTV-competent vector species have been reported in the Americas: *C. sonorensis* in North America and *C. insignis* in Central and South America. *C. imicola* in Africa and *C. wadai* and *C. brevitarsis* in Australia play a role in BTV transmission within specific regions and at specific times.

With the use of a CDC trap with ultraviolet light placed in each farm for three nights, 2240 specimens of Culicoides were collected from 2 farms in Santa Ana canton (Bonce, Monte Oscuro) and one in 24 de Mayo canton (Bellavista), at the center-east area of Manabí province. Six species were identified in decreasing abundance*: C. insignis, C. crepuscularis, C. antunesis, C. batesi, C. foxi and C. diabolicus.*

Similar studies in Venezuela [27], in which 2610 specimens of Culicoides were collected, identified *C. insignis* and *C. foxi*. In Brazil [28], 1718 specimens, including C. batesi and C. foxi, were collected. Navarro [29] collected three species of Culicoides (*C. insignis, C. foxi,* and *C. diabolicus*) from sheep farms in Peru.

In Ecuador, Gualapuro [30] recorded 65 species of the genus Culicoides. In addition, 3317 species of Culicoides were collected in five provinces of Ecuador (Esmeraldas, Pichincha, Bolívar, Santo Domingo and the northern part of Manabí) by Torres[19]. The species *C. insignis, C. batesi, C. foxi*, and *C. diabolicus* were identified in previous studies, but *C. crepuscularis* and *C. antunesis* were reported for the first time in Manabí province and Ecuador. These species are also not found within the updated list of Mosquera et al. [31], who collected culicoids from Junin in the center of Manabi province. Santa Ana and 24 de Mayo are more in the south of the Manabi province.

The present study was conducted on six cattle farms located in the central-eastern area of Manabí province. However, it is planning to extend to the central livestock-producing regions of the country, with serological evidence for BT virus exposure derived from the passive surveillance system of national veterinary services, as reported by Acosta et al. [32].

Culicoides were collected only during the rainy season (May 2022), when there are moist soils and high temperatures that favor the life cycle of cullicoides, and there is the most significant number of potentially infected females because they need to ingest blood from cattle, to achieve maturation and development of the eggs. The temperature has an essential effect on the rate of BTV virogenesis in the vector, with higher temperatures being associated with more rapid BTV replication [33]. The rainy season was selected to collect the insects because the dry and less warm season might not reveal BTV RNA in the female midges.

Some reports in America identified *C. insignis* as the most abundant in the investigated areas, indicating that it is a potential vector of BTV. Navarro [29] found that this species had an abundance of 94.8% in Peruvian sheep farms. Perruolo [27] in Venezuela have collected *C.insignis* with a variable abundance of 74 % in Guayabo, 69.7% in Machiques and 85% in Villa del Rosario. It is also predominant in Barbados (26%) [34]; Brazil (61.6%) [35]; Colombia (98.6%) [36]; and Puerto Rico, Costa Rica, Honduras and Panama (95%)[37]. Thus, only *Culicoides*

insignis has been reported as a competent species for BTV in the Caribbean basin, Central and South America [13]. Therefore, it remains unclear whether other species of Culicoides are involved in transmitting BTV. For example, we identified *C.crepuscularis* with an abundance of 15.48%. To demonstrate vector competence, studies should determine the intrinsic potential of BTV to enter and replicate within the new culicoidea species and then disseminate to, replicate within, and be released from the vector's salivary glands into the saliva at sufficiently high concentration to initiate infection in the next host[38].

Before the first OIE report of the bluetongue outbreak in cattle in Ecuador in 2015, the disease in cattle had been unknown by Ecuadorian owners and veterinary health personnel because the clinical signs are very mild and transient, and hemoparasite infections overlap the diagnosis. Thus, our primary goal was to report the presence of female culicoides with BTV RNA for the first time in Ecuador as evidence of the transmission of BTV in farms with seroreactor bovines without evident clinical signs. The present study's design did not include the objective of finding the vector-competent species; we collected insects for three nights on each farm, so we could not collect enough complete females to obtain pools of each species and each farm. In the future research project, we must demonstrate that *Culicoides* species reported in the present study would be BTV-competent vectors in Manabí and other endemic areas of Ecuador.

Real-time RT-PCR analysis of RNA extracted from each pool of 50 specimens from three farms (Bellavista, Bonce, Monte Oscuro) identified BTV RNA. To our knowledge, this has not been reported at the province or country level in Ecuador. We did not determine the infection rate within the Culicoides population or the serotype by sequencing the Seg-2 region of the BTV genome because we used pools of female culicoides. The study's goal was limited by the funding to demonstrate the presence of BTV in the farms, as we discussed above.

The VP2 protein of the bluetongue virus shows high antigenic variation, resulting in 28 serotypes. New BTV serotypes are being introduced globally due to the rapid evolutionary changes in the BTV genome through reassortment, mutations, and intragenic recombination [39]; in addition, virulence and susceptible species are different for BTV strains, phenomena thought to be related to the serotype [40].

Verdezoto et al.[41] reported different serotypes of three viruses isolated from the blood samples of cattle in different places: serotype BTV -18 from Cotundo (Napo province), BTV-9 and BTV-13 from Alleurquin (Santo Domingo province). Because the circulating BTV serotypes in Manabí, which has such a different climate and geographical relief, may be different, viral isolation and subsequent serotyping of isolated BTV from Manabí is a necessity if we are going to apply control measures based on the vaccination with a homologous serotype. The main goal of future investigations should be to determine the BTV serotypes circulating among the different livestock areas, not only in Manabí but also in other areas with the appropriate climatic conditions to support the life cycle of *Culicoides.*

All the animals tested by c-ELISA have shown anti-BTV – VP7 antibodies, indicating a high prevalence of infection in these farms. The BTV competitive ELISA (c- ELISA) using a monoclonal antibody specific for group antigen VP7 is both sensitive and specific, and the results for the number of seroreactors correlate very well with the number of infected animals [42-43]. The c-ELISA is BTV-specific because it does not cross-react with other orbiviruses [9].

Verdezotto et al.[41] reported a high prevalence (almost 100%) in cattle in other Ecuador provinces (Pichincha, Santo Domingo and Napo). In the north part of Manabí, De La Torre et al.[20] found a seroprevalence of 99.1% in 430 bovines on 37 farms in nine Chone parishes. High BTV seroprevalence was reported in the Americas: 99.7% in Cuba [44] and 100% in Sao Paulo[45]. However, older reports such as that by Gibbs et al.[16] found a BTV seroprevalence of 70% in cattle from seven countries in the Americas. These high prevalence rates indicate that the disease has become endemic in Manabí, as in Brazil and Cuba. This may be due to the development of livestock production and the absence of suitable control measures.

The rate and extent of BTV dissemination depends on the presence and abundance of competent Culicoides populations [22], so it is essential to investigate the different species of the vector that resides throughout the Ecuadorian territory to identify the different BTV serotypes circulating in Ecuador and to design a control program based on vaccination and vector control measures.

CONCLUSIONS

A very high positive rate of BTV seroreactor cattle was found in the six farms studied in the central-east area of Manabi province, suggesting that BTV is endemic without clinical signs. There was characterized the population of Culicoides living in the studied area and six species of Culicoides were identified: *C. insignis, C. crepuscularis, C. antunesis, C. batesi, C. foxi and C. diabolicus; C. crepuscularis* and *C. antunesis* has not been reported in Ecuador. For the first time in Ecuador, it was demonstrated that the epidemiological cycle of the virus is fulfilled since BTV RNA was detected in female Culicoides collected in farms of Santa Ana and one in 24 de Mayo.

We recommend the collection of Culicoides from different regions of Ecuador and its analysis by molecular methods to identify the serotypes prevalent in Ecuador. Also, it will study which species act as competent vectors.

Author Contributions: Initials of contributors: Mariella Centeno: MC; Denisse Chliliquinga: DC; José Velázquez: JV; Euclides De la Torre: ED; Alex Maldonado: A.M.; David Jarrín: DJ; Jimmy Alava: JA; Maritza Barrera: MB MD and DC collected the samples, processed the insects, and drafted the first version of the manuscript; JV performs the taxonomic identification; A.M. and DJ conducted the laboratory analyses; MB, J. A: and ED contributed to the study design; MB wrote the final version.

All authors approved the final version of the manuscript. All authors contributed equally to this work.

Institutional Review Board Statement: The study was approved by the Ethical Committee of the Universidad Técnica de Manabí (reference number: Tomo 0213 Folio 2139). The authorization to publish was obtained after assessment by AGROCALIDAD. This organization is an agency that regulates phyto- and zoosanitary control (https://www.agrocalidad.gob.ec/).

Informed Consent Statement: The farmers verbally consented to implement the cross-sectional and longitudinal surveys.

Data Availability Statement: Not applicable

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Conflicts of Interest: The authors declare no conflict of interest.

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Additional information Correspondence should be addressed to *maritza.barrera.@utm.edu.ec*

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