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Molecular detection of bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) in captured *Culicoides* spp. in the northeastern regions of Mexico

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Midges of *Culicoides* spp. (Diptera: Ceratopogonidae) captured in 2013 (February to November) in four micro-regions located in the northeast of Mexico were analyzed to determine the presence of genetic sequences of the viruses of Bluetongue (BT) and Epizootic Hemorrhagic Disease (EHD) by reverse transcription–polymerase chain reaction (RT-PCR). In all the micro-regions sampled, it was possible to detect the presence of culicoides biting midges, along with other insects. The basic phenological identification of midges showed that all specimens corresponded to the *Culicoides variipennis* complex (Wirth & Jones). The amount of captured midges was associated with the annual mean temperature, altitude and time of year at the site of capture ($p < 0.01$). A total of 51 pools, each one of 3 to 15 female midges, were analyzed for genetic sequences for BTV (part of NS3 / NS3A gene segment 10) and EHDV (part of the NS1 gene segment 5) using oligonucleotides reported in previous studies. Except for the micro-region located in the municipality of Zaragoza, Nuevo Leon (23 ° 57'10.24''N, 99 ° 46'12.24''O; 1454 m), where it only was possible to capture one midge, midges groups captured at all other sites were RT-PCR positive for BTV and EHDV. The highest proportion of positive midges groups to RT-PCR was found in spring and summer. The presence of genetic sequences of BTV and EHDV in captured *Culicoides* spp. may eventually be associated with outbreaks of diseases caused by these viruses in wild and domestic ruminants in the northeastern part of Mexico. The information on the movement of midges carrying both viruses could be taken into account for the management of animal populations, and further studies are needed to confirm the presence of the mentioned viruses.

Key words: Bluetongue virus (BTV), Epizootic Hemorrhagic Disease virus (EHDV), *Culicoides*, RT-PCR, Ruminants, northeast Mexico.

INTRODUCTION

Bluetongue virus (BTV) and Epizootic Hemorrhagic Disease virus (EHDV) (Family Reoviridae, Genus Orbivirus) are two highly related viruses which are considered emerging infectious agents globally

(Maclachlan and Guthrie, 2010; Maclachlan and Mayo, 2013). Both Orbivirus are able to affect a large variety of wild and domestic ruminants (Legisa et al., 2014; MacLachlan and Guthrie, 2010). Due of their negative effects on the economy of livestock systems, and the difficulties in their control and eradication, the appearance and movement of such Orbivirus in ruminants are a required declaration in the countries members of the World Organization for Animal Health (OIE) (Wittmann and Baylis, 2000), (OIE, 2015). Both viruses are transmitted, by primary form, between susceptible animals by the species of midges of the *Culicoides* genus (Diptera: Ceratopogonidae) (Searle et al., 2014; Mellor et al., 2000). The *Culicoides* genus groups are the smallest and most abundant flying blood-sucking arthropods, and more than 1,400 species are reported worldwide (Mellor et al., 2000); of these, more than 39 species have been implicated as suspicious or confirmed as biological vectors of BTV and EHDV (Savini et al., 2011). The identification of different species of *Culicoides* can be accomplished by phenological and molecular methods (Meiswinkel et al., 2004; Mathieu et al., 2007; Werner and Kampen, 2007). The ability of these vectors to transmit BTV and EHDV lies in its ability to increase the viral load in the environment (Mellor, 2000; Ruder et al., 2012). It implies the recognition of the virus receptors in the intestinal cells of midges, subsequent internalization and replication, as well as the spreading from this site to other tissues that allow the dispersion to salivary glands and ovaries of the insect (Mellor, 2000).

The presence of these viruses in the tissues of their insect vectors is indicative of their movement in certain geographic region (Hoffmann et al., 2009); therefore, the distribution of arboviral diseases caused by these viruses depends on the presence and distribution in the environment of such insect vectors (Wittmann and Baylis, 2000). The epidemiology and outbreaks of infections caused by BTV and EHDV depend on the multiple interactions that exist between these viruses, their vectors, the environment and the susceptibility of ruminants (Mellor, 2000) (Savini et al., 2011) (Legisa et al., 2014). In Mexico, serologic and virologic evidence has been reported for BTV in domestic ruminants (Hernandez et al., 1994), (Stott et al., 1989) (Suzan et al., 1983), (Mooehead-Jackson, 1981), (Teclaw et al., 1985). Particularly, in the northeastern region of Mexico, high rates of seropositivity against BTV and EHDV in populations of white-tailed deer (*Odocoileus virginianus texanus*) has been reported (Martínez et al., 1999). According to these data, favorable environment conditions for the presence and movement of the *Culicoides* vector in this area of the country are apparently occurring. The present study was performed to determine the presence of genetic sequences of the orbivirus BTV and EHDV in their potential arthropod vector (*Culicoides* midges), caught in selected regions in the northeast of Mexico.

MATERIALS AND METHODS

Study area and sample collection

Capture sites for biting midges include four distinct micro-regions located in the state of Nuevo Leon, in the northeast of Mexico (Figure 1). The sites show differences in altitude above sea level, temperature, average annual precipitation and predominant vegetation. Each site was located near pens of ranches with domestic ruminants (cattle, goats, sheep), not more than 20 maway and with the concurrence of wild ruminants (white-tailed deer) within an area of 5 km. The capture of midges was carried out with Mosquito Magnet® traps (Liberty Plus, American Biophysics Co., USA). The traps were set at 1.5 m above ground and operated from 1 h before sunset until 1 h after sunrise. Captured midges were deposited in a plastic bag and transported under refrigeration to the laboratory for further analysis. The capture lasted from February to November 2013. The four capture sites were visited once in a month.

Identification of specimens

Previous separation of other captured insects, Culicoides (*Culicoides*, Diptera: Ceratopogonidae) were identified with the aid of a binocular stereoscopic microscope (Carl Zeiss) according to the characteristics observed in the wings as reported by Rawlings (1996) and Borror et al. (1989). The identification is based on the characteristic pattern of dark and light areas of the wings, the dorsal side of the thorax, size and shape of the segment of the antennas, the relative position of the eyes, the shape of the spermathecal in females and the shape of the genitals in males (Meiswinkel et al., 2004; Werner and Kampen, 2007). The data of the specimens were recorded with the program of Microsoft Excel version 2007 and the samples of some specimens by a digital photography. After the identification, the specimens were separated according to sex and depending on the quantity, a sample 3 to 5 male and female midges were preserved in 70% ethanol while the remaining specimens were kept frozen at -80°C until use in RT-PCR.

Molecular detection of orbivirus (BTV and EHDV)

Pools of 7 to 15 female specimens were analyzed separately to determine the presence of genetic sequences of BTV and EHDV by RT-PCR. Total RNA from each batch was obtained using the commercial kit "FastRNA® Soil Pro - Direct Kit" and the Fast Prep-24 (www.mpbio.com), following the manufacturer's recommendations. RNA was recovered in 30 µl of DEPC-treated water and stored at -70°C until use. The quantity and quality of total RNA harvested was analyzed by spectrophotometry at 260 nm using the Epoch™ device and Gen5™ microplate data analysis software (www.biotek.com). For the RT-PCR analysis, total RNA concentration was adjusted to 1 µg/µl with DEPC-treated water. The RT-PCR reactions were performed with the help of the

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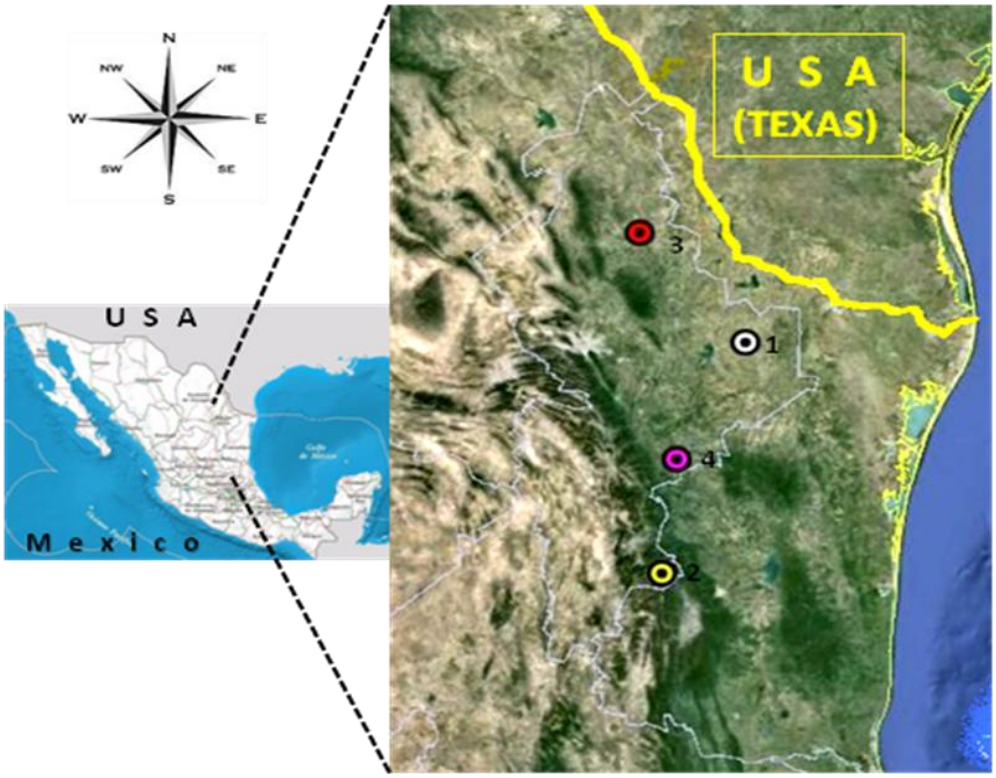

Figure 1. Relative position of the capture sites of the *Culicoides* spp in the regions at the Northeast of Mexico. 1,General Bravo; 2, Zaragoza, 3, Villaldama; 4, Linares.

commercial kits "SuperScript III One Step RT-PCR System with Platinum ® Taq High Fidelity" and "AccuPrimeTMSuperMix I" (www.invitrogen.com), following the manufacturer's recommendations. For the amplification of the gene segments from each virus, specific oligonucleotides were used as previously reported for BTV (Leblanc et al., 2010) and EHDV (Aradaib et al., 2003). In both cases, the oligonucleotides were designed based on highly conserved regions within the NS3 / NS3A of the segment 10 of BTV (BTV serotype 9) and of the NS1 gene segment 6 of the EHDV (EHDV serotype 1), respectively.

The names and sequences of the oligonucleotides for the amplification of genomic fragment of BTV are: BTV-20F 5´-ATGCTATCCGGGCTGATYC-3', BTV-285R 5´-ACRTCATCACGAAACGCTTC-3´. Both oligonucleotides amplify a fragment of 266 bp (Leblanc et al., 2010). The genetic sequence of EHDV was amplified in two steps, first by RT-PCR with the following oligonucleotides: 5'-TCGAAGAGGTGATGAATCGC-3' and 5'-TCATCTACTGCATCTGGCTG-3' and subsequently with nested PCR with the following oligonucleotides: 5'-CATGCGGCATATAGATTGGC-3' and 5'-GTCATCTAGCACGATGCGTG-3'. These latter oligonucleotides amplify a specific fragment of EHDV of 225 bp (Aradaib et al., 2003). The amplification of gene segments from both viruses was performed in a first instance with gradient RT-PCR to determine the optimum bonding temperature of the primers. All the PCR reactions were performed in a thermocycler (Maxygen, Union City, Calif. USA) to a final volume of 50 µl per tube. The reaction of the RT-PCR step was one initial denaturation at 95°C for 1 min followed by 30 min at 48°C (cDNA synthesis) and 95°C for 1 min. The thermal cycles of PCR amplification were: 1 min at 95°C followed by 40 cycles of 95°C for 30 s (denaturation of DNA), 50°C for 30 s (oligonucleotide binding) and 72°C for 40 s (DNA extension) and a final incubation at 72°C for 10 min. 5 µl of the amplification product were analyzed by electrophoresis in 2.5% agarose gels stained with commercial dye GelRed^{TM} according to the standard protocol. The size of the amplified product was compared against a molecular weight marker of 1 Kb (Axygen^{TM}). The pools were considered positive if the band size for each respective genetic sequence of the virus was observed. The image obtained of the gel was documented electronically with the help of the equipment and the program of MultiDoc-It Imaging System (www.uvp.com). In each run, negative and positive controls of the reaction were included. The positive control reaction of RT-PCR for detection of BTV was the RNA obtained from a commercial vaccine against the virus containing serotype 10 (Colorado Serum Company, Denver, USA.); for EHDV it was not possible to include a positive control. Negative controls consisted of sterile double distilled water free of RNase and DNase, containing the reaction mix except nucleic acid.

Statistical analysis

The association between the presence of *Culicoides* spp. with the micro-region, altitude above the sea level and the monthly average of local temperature was analyzed by Chi-square using the WinEpi program, considering $p < 0.05$ as significant. The data were processed in the program Working in Epidemiology (Blas 2006, http: //www.winepi.net/), with a confidence level of 95%.

RESULTS

The results of the study are shown in the Table 1,

Table 1. Relationship between the molecular detection of Bluetongue Virus (BTV) and Epizootic Hemorrhagic Disease Virus (EHDV) in pools of *Culicoides* spp. and the capture site in northeastern regions of Mexico.

Town	Geographical Position[1]	Altitude[2]	Season of the Year[3]					
			Spring [272][4]		Summer [274]		Autumn [22]	
			BTV	EHDV	BTV	EHDV	BTV	EHDV
General Bravo[368][4]	25°49´2.24´´N, 99°15´31.47´´O.	137	5/11[5]	3/11	7/15	3/15	0/2	0/2
Linares [101]	24°51´13.69´´N, 99°32´40.76´´O.	345	2/8	0/8	1/3	0/3	0/2	0/2
Villaldama [94]	26°29´04.06´´N, 100°26´10.32´´O.	434	2/5	1/5	1/3	2/3	0/1	0/1
Zaragoza [1]	23°57´10.24´´N, 99°46´12.24´´O.	1454	0/0	0/0	0/1	0/1	0/0	0/0
Total .			9/24	4/24	9/22	5/22	0/5	0/5

[1]Capture site. [2]Altitude above the sea level. [3]Spring = March to May, Summer June to August, Autumn= September-November. [4][ ] Analyzed female midges captured by season or town. [5]Lots + / N° total of batch analyzed.

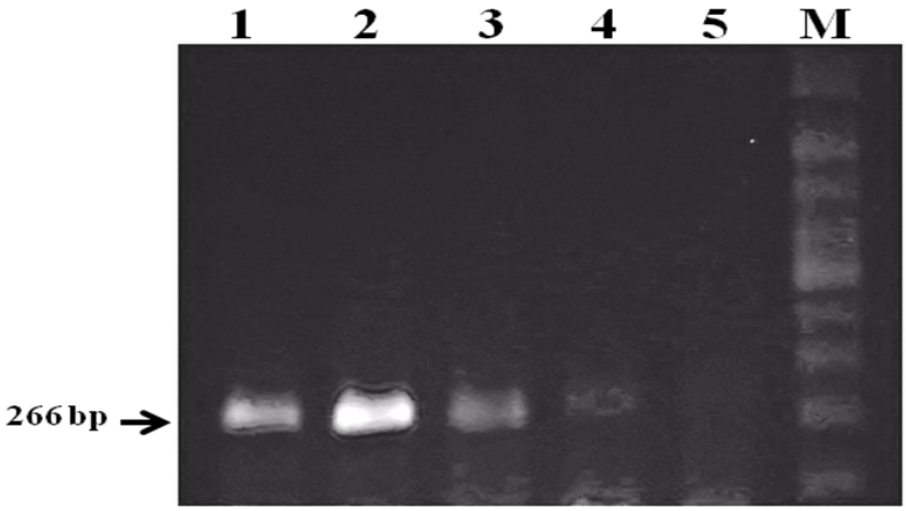

Figure 2. Agarose gel electrophoresis of the amplification product of 266 bp (arrow) fragment of the gene NS3/NS3A of BTV in *Culicoides* pools. Lanes: 1, Positive Control (commercial vaccine); 2-4, representative samples of the sites in General Bravo (2), Villaldama (3) and Linares (4); 5, negative control (distilled water); M, molecular weight marker.

according to the geographical position (town) and season of year. A total of 564 female midges distributed on 51 pools were analyzed by RT-PCR. From the total of pools, 18 and 9 were positive for BTV and EHDV, respectively. When the season is taken into account, spring and summer showed the highest occurrence of both Orbiviruses, with a frequency of 9/24 in spring and 9/22 in summer for BTV and 4/24 in spring and 5/22 in summer for EHDV. In the autumn months (September to November) it was not possible to detect positive pools. By geographical position (Figure 2) the town of Bravo showed the highest occurrence of positive pools with a frequency of 12/28 for BTV and 6/28 for EHDV. Through the study, the capture site located in the town of Zaragoza (Figure 1), with an altitude above sea level of 1454 m, is the only one place where practically no midges were captured. In this site it was possible to catch a single specimen in summer. However, and taking into account this specimen, in virtually all analyzed micro-regions (Figure 1), at least one midge was captured. With these data, it appears that the amount of captured midges was dependent on the micro-region and season of year ($p<0.1$) (Table 1). Though, in the study the quantity of harvested midges could have been influenced by the device type (Mosquito Magnet®) used to trap the Culicoides midges as well as by their monthly use at each site, the usage frequency (from sunset to sunrise, ~12 h) and local wind (climate) conditions at time of trapping. In our study, the primary objective was the detection of genetic sequences Orbiviruses BTV and EHDV in *Culicoides* circulating in northeastern Mexico. So biting midges trapped were identified only at basic phenotypic level to determine if they belong to the genus *Culicoides*. In addition, the basic phenotypic identification

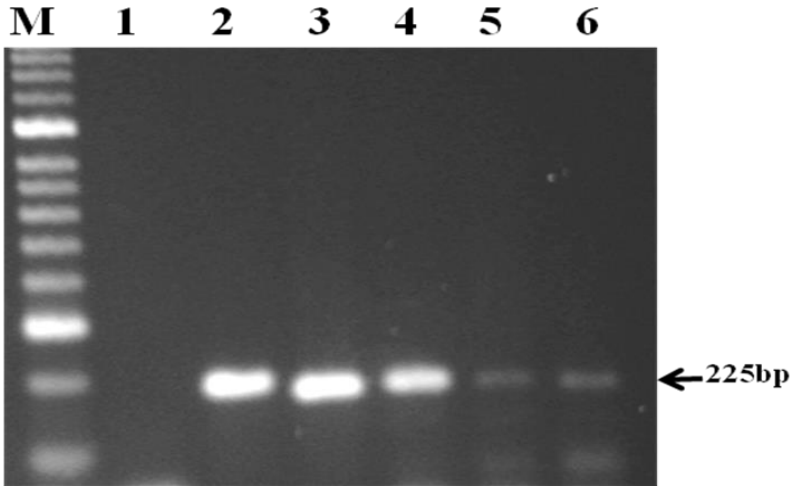

Figure 3. Agarose gel electrophoresis (stained with Gel Red™) of RT-PCR and Nested PCR for the detection of EHDV, in which a specific PCR product of ~ 225 bp (arrow) of the NS1 gene segment 6 of the EHDV is shown. M, molecular weight marker; lanes: 1, negative control (double distilled water); 2-6, representative samples of pools of midges collected from different parts of the Northeastern regions of Mexico.

of all midges captured showed that all specimens corresponded to the *Culicoides variipennis* complex (Coquillett) (Wirth, 1957; Borror et al., 1989; Rawlings, 1996). Midges of *C. variipennis* complex comprise the species *C. variipennis*, *C. sonorensis* and *C. occidentalis* (Schmidtmann et al., 2011; Tabachnick, 1996). In the present study, no further analyses were performed in Culicoides midges to determine the species. For molecular detection of BTV on midges, preliminary gradient-PCR experiments were conducted using a BTV vaccine strain serotype 10 (data not shown). On the other hand, unfortunately we were unable to include a positive control in our experiments to detect EHDV, so the findings are based on the band size as previously described (Aradaib et al., 2003). Examples representing the PCR amplification products of 266 bp for BTV and 225 bp for EHDV from each studied region are shown in Figures 1 and 2, respectively. Amplicones were of the same molecular weights as those expected from amplification of BTV (LeBlanc et al., 2010) and EHDV (Aradaib et al., 2003) viral nucleic acids. PCR assays performed separately for both viruses in each of the 51 pools of Culicoides midges generally resulted in bands of the expected size of variable intensity (Figures 2 and 3).

DISCUSSION

The detection of the BTV and EHDV and its biological vector *Culicoides* spp. in certain areas of livestock production is important for the design of management programs and for the health of domestic and wild animals susceptible to these viruses (Hoffmann et al., 2009; Charron et al., 2013; Coetzee et al., 2012; Savini et al., 2011). In endemic areas both viruses can potentially be transmitted by the same vector (Mellor et al., 2000; Cêtre-Sossah et al., 2014). The presence of *Culicoides midges* has been reported in Mexico since 1948 (Macfie, 1948). Apparently, these types of biting midges are widely dispersed in USA and Latin America (Mullens and Dada, 1992; Holbrook et al., 2000; Lager, 2004). It has been reported that *C. varipennis var. sonorensis* midges are competent to transmit the virus of the Orbivirus genuses (Family Reoviridae), including BTV and EHDV (Ruder et al., 2012; Tabachnick, 1996), as well as and other infectious agents (Drolet et al., 2005). In the US, *C. variipennis var. sonorensis* midges have been reported as the primary vector of EHDV and BTV (Foster et al., 1977; Tabachnick, 1996; Tabachnick, 2004). Apparently, very little information is available about the main vector (s) involved in the transmission of BTV and EHDV outside US in the American continent (Lager, 2004). Although several studies have implicated *Culicoides variipennis var. insignis* as the predominant vector in South America, and it has been captured in Central America and the Caribbean (Lager, 2004; Mo et al., 1994; Ronderos et al., 2003), there are no recently reports of its presence in Mexico. The detection and monitoring of these midges are important for the control of the pathogens that they can potentially transmit (Maclachlan and Mayo, 2013). In the northeastern region of Mexico, serological evidence has been reported in wild and domestic ruminants for both BTV and EHDV (Suzan et al., 1983), (Teclaw et al., 1985; Martinez et al., 1999). On the other side, BTV has been isolated from cattle and sheep in this and other regions of Mexico (Stott et al.,

1989). However, to date the detection of genetic sequences of BTV and or EHDV from *Culicoides* biting midges circulating in the northeastern of Mexico has not been reported. In this work, genetic sequences of both viruses were detected in pools containing only *Culicoides*, obtained in spring and summer of certain sites (Figure 1 and Table 1). The BTV genomic sequence was found in relatively high proportions in all sites of capture (Figure 2 and Table 1). The putative genomic sequence of EHDV (Figure 3) was found in pools of *Culicoides* captured in only two of the four sites analyzed (Figure 1 and Table 1). These observations are consistent with the high seropositivity against both viruses, recorded in ruminants from this geographical area of Mexico. Taking together, the data presented in this study strongly suggests that *Culicoides spp* do contain genetic sequences of the Bluetongue virus and the Epizootic Hemorrhagic Disease Virus in the northeastern regions of Mexico. The circulation of this potential vector is variable and depends on the season of the year and the conditions of the micro-regions under study. These observations are consistent with other studies in other parts of the world (Kedmi et al., 2011). The detection of genetic sequences of BTV and EHDV implies that it is very likely that both viruses are circulating in this area of Mexico.

This result is in accordance with the high seropositivity recorded in previous serological studies in this region (Suzan et al., 1983; Teclaw et al., 1985; Martinez et al., 1999). These observations suggest that eventually the population of domestic and wild ruminants could be affected with the infection of one or both viruses (Sailleau et al., 2012; Toye et al., 2013). Under this context, clinical alterations and the concerned pathologies caused by the infection of these Orbivirus may be going undetected or misdiagnosed in populations of wild and domestic ruminants, which make it difficult to control (Maclachlan and Mayo, 2013). The possible presence of both viruses in the areas and populations studied may result in disease outbreaks, which could compromise the health and management of the livestock in the production system. According to the above mentioned, further studies are needed to determine the potential impact of both viruses in the health of susceptible animals in the northeast of Mexico.

Conflict of interests

The author(s) have not declared any conflict of interest.

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