Detection of Bluetongue Virus RNA in Field-Collected *Culicoides* spp. (Diptera: Ceratopogonidae) Following the Discovery of Bluetongue Virus Serotype 1 in White-Tailed Deer and Cattle in Louisiana

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ABSTRACT   In November 2004, bluetongue virus (family *Reoviridae*, genus *Orbivirus*, BTV) serotype 1 (BTV-1) was detected for the first time in the United States from a hunter-killed deer in St. Mary Parish, LA. In 2005, sera surveys were conducted on three cattle farms near the area where the deer was found, and BTV-1–seropositive cattle were found on two of the three farms; in 2006, sera surveys from the cattle on the three farms did not detect any BTV-1–positive animals. The purpose of this study was to survey ceratopogonid populations at the three farms and test field-collected specimens for the presence of BTV and epizootic hemorrhagic disease virus (family *Reoviridae*, genus *Orbivirus*, EHDV). Miniature CDC light traps and New Jersey traps were used to capture ceratopogonids on the three farms from January 2006 through November 2007. In total, 3,319 ceratopogonids were captured, including 1,790 specimens of 10 different species of *Culicoides*. IR-RT-polymerase chain reaction (PCR) was performed to screen for BTV and EHDV in 264 pools representing 2,309 specimens collected at the farms. All positive samples were sequenced for serotype determination. Five pools of 275 (1.8%) were positive for BTV. Pools of four species of *Culicoides* were found to be positive: *Culicoides crepuscularis* (Malloch), *Culicoides debilipalpis* Lutz (two pools), *Culicoides haematopotus* Malloch, and *Culicoides furens* (Poey). The amplicons of the positive specimens were sequenced and found to be identical to both BTV-17 and BTV-13. During our study, no BTV-1 transmission was detected in cattle, and no BTV-1 was detected in specimens of ceratopogonids.

KEY WORDS   Ceratopogonidae, *Culicoides*, bluetongue virus

Infection with bluetongue virus (family *Reoviridae*, genus *Orbivirus*, BTV) can cause serious hemorrhagic disease with high mortality rates in sheep and deer (Osburn 1994, Howerth et al. 1988). Certain domestic ruminants, such as cattle and goats, rarely show clinical symptoms. Following infection of BTV, cattle can have a prolonged viremia and serve as reservoir hosts for BTV (MacLachlan et al. 1994).

There are 24 described serotypes of BTV worldwide; BTV has been found where suitable vectors are present (Tabachnick 1996). Until recently, five serotypes (2, 10, 11, 13, and 17) of bluetongue virus were known to naturally occur in the United States (Mullen et al. 1999). In November 2004, bluetongue virus serotype 1 was isolated for the first time in the United States from a hunter-killed deer in the marsh area of the Atchafalaya Delta in St. Mary Parish, LA (Johnson et al. 2006). Previously, the known range of BTV serotype 1 was confined to Africa, southern Europe, and Central and South America (Tabachnick 1996).

Shortly after the first report of BTV-1 in St. Mary Parish, serum surveys were conducted on three nearby cattle farms which were located within 30 km of the area where the deer was shot. Two of these three farms had BTV-1–seropositive cattle. The purpose of this study was to identify potential BTV vectors in the area of apparent BTV-1 transmission.

Materials and Methods

Animal Blood Samples. Blood samples were collected from March to July in both 2005 and 2006 from cattle on three farms in St. Mary Parish, LA. The three farms were within 30 km of the location where the BTV-1–positive deer was shot in 2004 (Johnson et al. 2006). All farms had some type of standing water in ditches or canals surrounding the pastures, which oc-

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casionally filled with floodwater from heavy rain events. Farm A (29.67237° N−91.27583° W) had ≈820 km^2 of pasture containing 127 head of cattle. Farm B (29.654885° N−91.284511 °W) was located 2.19 km southwest of farm A and had 215 km^2 of pasture with 72 head of cattle. Farm C (29.70298° N−91.38673°W) was located 11.49 km northwest of farm B and contained 134 km^2 of pasture with 21 head of cattle. Sera from cattle were screened for the presence of antibodies to bluetongue by Competitive Enzyme-Linked Immunoabsorbent Assay (cELISA), following the manufacturer's testing procedures. Samples which screened positive on cELISA were then tested by virus neutralization (VN) for antibodies against both viruses BTV-1 and -2. A standard VN microplate assay was conducted with 1:10 as the initial serum dilution. Animals with a four-fold or greater titer to BTV-1 than to BTV-2 were deemed BTV-1 antibody positive.

Farm Trap Study. From 1 January 2006, through 15 November 2007, trap studies were conducted at the three cattle farms. Routinely, miniature CDC black-light traps (model 512; John W. Hock Co., Gainesville, FL) were hung from tree branches ≈1.5–2.0 m above ground at each farm. There were two trap sites at each farm that were >100 m apart; the traps were deployed twice per month. One trap at each farm was baited with 2 kg of dry ice in an igloo container. The dry ice was rotated between the two sites at each farm every trap-night. In addition to this regular trapping effort, intensive trapping was conducted at farms A and C from 22 May to 6 June 2007, and at farms A and B from 21 August to 3 September 2007 (Becker 2008). Three trap types were used: 1) New Jersey Stainless Steel Light Trap with a 40-W incandescent light bulb (model 1112; John W. Hock Co., Gainesville, FL), 2) New Jersey Stainless Steel Light Trap modified with two F8T5 8-W blacklights (Becker 2008), and 3) miniature CDC blacklight trap baited with 2 kg of dry ice. The traps were rotated three times a week for three consecutive weeks resulting in 27 trap nights per farm and 54 trap nights for each of the two trials. There was a total of 147 trap nights during the entire study, including both the routine trapping and the intensive trap study. The traps were set out before dusk and retrieved after sunrise. Captured midges were immediately placed in a container with ≈15 kg of dry ice, transported to Louisiana State University, and stored at −80°C.

All ceratopogonids were identified to genus and specimens of *Culicoides* identified to species (Blanton and Wirth 1979; Downes and Wirth 1981). Pools of midges were created by separating ceratopogonids by species, farm, and month captured and placing them into chilled 1.5-ml microfuge tubes; pools contained a minimum of one specimen and a maximum of 50 specimens. Pooled midges were stored at −80°C until shipment on dry ice to the Arthropod-Borne Animal Diseases Research Laboratory, Laramie, WY, where they were again stored at −80°C until screened for BTV and epizootic hemorrhagic disease virus (family *Reoviridae*, genus *Orbivirus*, EHDV).

Infrared Reverse Transcriptase Polymerase Chain Reaction. The pools of female flies were macerated separately in gnat homogenization buffer with gold-plated tungsten beads (Spirit River, Roseburg, OR) by using a Tissue Lyser (QIAGEN, Valencia, CA) as described by Kato and Mayer (2007). Total RNA was extracted from the homogenate using an RNeasy kit (QIAGEN) following the manufacturers recommendations. Gnat homogenization buffer consisted of 400 μg/ml penicillin, 400 μg/ml streptomycin, 200 μg/ml gentamicin, 25 μg/ml ciprofloxacin, and 5 μg/ml amphotericin B prepared in Medium 199 with Earle's salts in 10% fetal bovine serum. The extracted RNA was screened by infrared reverse-transcription-polymerase chain reaction (IR-RT-PCR) for BTV and EHDV by using the protocols described by Kato and Mayer (2007). Infrared labeled primers EHDV 63-F1 (5′-AACAGTTACTACGCAAATCA-3′) and EHDV 245-R1 (5′-AGCCA TTTCAGCCAATCT-3′) were used to amplify a portion of the NS1 gene of EHDV. BTV-specific primers BTV 12 F (5′-TCGCTGCCAT-GCTATCCG-3′) and BTV 246R (5′-CGTACGATGC-GAATGCAG-3′) were used to amplify the highly conserved regions of the S10 gene of BTV. Nontemplate controls were used for each replicate.

The amplicons (PCR products) of positive pools were purified with a QIAquick PCR purification kit (QIAGEN) and sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were determined using an ABI 3700 capillary sequencer (Applied Biosystems), aligned and assembled with Chromas Lite 2.01 (Technelysium, Tewantin, Australia) and ClustalW (Kyoto University Bioinformatics Center, Kyoto, Japan), and compared with all BTV sequences in Gen-Bank by using the BLAST 2.0 program (National Center for Biotechnology Information, Bethesda, MD).

Results

Animal Blood Samples. In 2005, 49 of 220 cattle from the three farms tested positive for BTV using cELISA test. Of these, four cattle had at least a four-fold greater titer to BTV-1 than BTV-2 by using VN test and were considered to be BTV-1–positive animals. Farm B had three BTV-1–seropositive cattle and Farm C had one BTV-1–positive cow. The same cattle at the three farms were retested in 2006 and 50 cattle were BTV positive for cELISA. However, using VN test none of these animals were at least four-fold greater in titer to BTV-1 than BTV-2.

Farm Trap Study. A total of 2,309 specimens (264 pools) of ceratopogonids representing 10 species of *Culicoides* and two other genera (*Atrichopogon* spp. and *Forcipomyia* spp.) was screened for BTV by using IR-RT-PCR (Table 1). More than 88% of specimens of *Culicoides* captured at all farms were from three species: *Culicoides debilipalpis* Lutz, *Culicoides arboricola* Root & Hoffman, and *Culicoides crepuscularis* (Malloch). The most specimens were captured in the month of August in both 2006 and 2007. The predominant species at farm A was *C. arboricola*, whereas *C.*

Table 1. Number of pools and specimens of ceratopogonids captured at farms in 2006 and 2007 in St. Mary Parish, LA, and tested for BT virus and EHD virus by IR-RT-PCR

Location	Species/genus	No. specimens	No. pools
Farm A	*Atrichopogon* spp.	10	2
	Forcipomyia spp.	217	16
	C. arboricola	229	23
	C. biguttatus	2	1
	C. crepuscularis	56	16
	C. debilipalpis[a]	201	19
	C. furens	1	1
	C. haematopotus[a]	36	14
	C. hinmani	16	8
	C. paraensis	32	11
	C. stellifer	8	6
Farm B	*Atrichopogon* spp.	13	5
	Forcipomyia spp.	241	18
	C. arboricola	204	11
	C. biguttatus	1	1
	C. crepuscularis[a]	89	11
	C. debilipalpis[a]	245	13
	C. haematopotus	1	1
	C. hinmani	13	1
	C. paraensis	8	4
	C. stellifer	5	2
Farm C	*Atrichopogon* spp.	79	9
	Forcipomyia spp.	129	10
	C. arboricola	120	13
	C. biguttatus	3	2
	C. crepuscularis	105	13
	C. debilipalpis	180	13
	C. furens[a]	17	3
	C. haematopotus	32	11
	C. hinmani	1	1
	C. paraensis	12	3
	C. stellifer	3	2
TOTAL		2309	264

[a] Denotes one BTV-positive pool.

debilipalpis was the most frequently captured species at farms B and C.

Infrared Reverse Transcriptase Polymerase Chain Reaction. Of the 264 pools, five tested positive for the presence of BTV. All positive midges were captured using miniature CDC blacklight traps. There was at least one positive pool from each of the three locations. There were 80 pools of 681 specimens from farm A. Of 19 pools of *C. debilipalpis*, one was positive for BTV, and these specimens were captured in October 2006. One pool comprised of a single specimen of *Culicoides haematopotus* Malloch was positive for BTV; the specimen was captured in August 2007. In total, 820 specimens in 67 pools from Farm B were screened for BTV. One out of 11 pools of *C. crepuscularis*, captured in June 2006, and one out of 13 pools of *C. debilipalpis*, captured in September 2006, were positive for BTV. The specimens in positive pools of both *C. crepuscularis* and *C. debilipalpis* were collected during the seasonal peaks of both species populations (Fig. 1). In total, 117 pools of 808 specimens from Farm C were screened for BTV, and one of three pools of *C. furens* was positive for BTV. The positive pool was a single specimen of *C. furens* that was captured in May 2006. There were no positive pools for EHDV.

The amplicons of the positive pools were sequenced for serotype. We sequenced NS3 gene from RNA extracts, which were 100% identical to that of BTV-13 or BTV-17 by using GenBank (AY426597.1, AF044713.1, AF044712.1, AF044375.1, AF044374.1, and L08630.1). None of the amplicons were matches for BTV serotype 1.
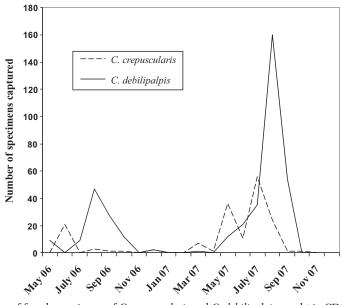

Fig. 1. Total numbers of female specimens of *C. crepuscularis* and *C. debilipalpis* caught in CDC light-traps baited with dry ice on three farms in St. Mary Parish, LA.

Discussion

Specimens of four ceratopogonid species [*C. crepuscularis*, *C. debilipalpis*, *C. haematopotus*, and *Culicoides furens* (Poey)] were found to be positive for BTV serotype 17 or 13. Three of these species (*C. crepuscularis*, *C. haematopotus*, and *C. furens*) have not been highly considered as vectors of BTV in the United States.

Previous studies have incriminated *C. debilipalpis* as a vector of BTV. This species is known to feed in large numbers on deer, especially in the southern United States. One study in Georgia reported capturing >20,000 specimens of *C. debilipalpis* from a caged deer in one morning (Smith and Stallknecht 1996). Moreover, Mullen et al. (1985) showed that laboratory-fed *C. debilipalpis* were able to harbor BTV through replication and transmit the virus 14 d after a bloodmeal.

Female specimens of *C. crepuscularis* are considered to be ornithophilic (Hair and Turner 1968), but specimens have been reported biting people and feeding on ewes and steers (Pickard and Snow 1955; Raich et al. 1997). This species was dominant in the marsh area where the BTV-1–positive deer was shot, making up >95% of the total specimens of *Culicoides* captured (Becker 2008). In northern Colorado, White et al. (2005) found genome segments 7 and 3 of BTV in larvae of *C. crepuscularis* collected in cattle pastures, but BTV was not isolated from the larvae.

Specimens of *C. furens* feed on humans and are very abundant in coastal areas. In Florida, this species is the most important human-biting midge (Linley 1983). Some specimens were also captured in the light-trap where the BTV-1–positive deer was shot (Becker 2008). This species has been suggested as a potential vector of BTV in Central America and the Caribbean region (Saenz et al. 1994).

Specimens of *C. haematopotus* are largely found associated with livestock and wooded areas throughout the United States (Blanton and Wirth 1979). Mullen et al. (1999) captured some specimens of *C. haematopotus* from cattle in Alabama and Smith et al. (1996) aspirated specimens from deer in an area where BTV and EHDV were enzootic. However, no previous studies in the United States have shown any BTV-positive field-collected specimens of *C. haematopotus.*

We did not catch any specimens of *C. sonorensis*, which is the main vector of BTV in the United States. Nor did we capture any specimens of *C. insignis*, which is the vector of BTV-1 in the Caribbean and Central and South America. During 2005, the Southeastern Cooperative Wildlife Disease Study group at the University of Georgia, Athens, GA, collected and tested serum samples from 399 hunter-killed deer in Louisiana and found six deer to be antibody positive for BTV-1 (D. Stallknecht, personal communication). Three of the six deer came from St. Mary Parish which indicates that BTV-1 was being transmitted in the area of our study. Therefore, it is probable that BTV-1 can be transmitted by native species of *Culicoides*, other than *C. sonorensis.*

In Louisiana, bluetongue disease in deer normally occurs in the fall (F. Enright, personal communication). Vertical transmission of BTV in insects has not been proven and BTV is not contagious. Thus, an insect vector must take a bloodmeal from an animal infected with BTV, and the virus has to replicate in the salivary glands of the insect before it can be transmitted ($\approx$14 d). Death in deer occurs $\approx$14 d after infection. Therefore, it takes $\approx$1 mo for an insect vector of BTV to transmit the virus to a healthy deer and for that animal to die, and species of *Culicoides* that are abundant right before and during the fall would be suspect vectors. In our study, BTV-positive specimens of *C. debilipalpis* were captured in September and October (Fig. 1); there was a large population peak of this species in August and September, and some specimens were caught in October. Because we collected BTV-positive specimens of *C. debilipalpis* during the time when BTV outbreaks in deer occur, and Mullen et al. (1985) showed that *C. debilipalpis* was a competent BTV vector, this species should be considered as a potential BTV vector in southern Louisiana.

We did not detect any BTV-1– or EHDV-positive ceratopogonids. Potentially capturing and testing more ceratopogonids in the area would be adequate for finding BTV-1–positive specimens. Our trap study started after the BTV-1–positive cattle were detected, and no new seropositive cattle were identified after we started. No BTV-1 transmission was detected in cattle and no BTV-1 was detected in specimens of ceratopogonids during our study. However, BTV-1 could be transmitted by insects that are not captured in light-traps or even insects that were captured but not examined for BTV.

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