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***Culicoides*, THE VECTOR OF EPIZOOTIC HEMORRHAGIC DISEASE IN WHITE-TAILED DEER IN KENTUCKY IN 1971**

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Abstract: The biting gnat, *Culicoides variipennis* (Coquillett), was shown to be a vector of epizootic hemorrhagic disease (EHD) in white-tailed deer, *Odocoileus virginianus*, in Kentucky because of virus isolations from parous females. Epidemiological evidence showed a close relationship of this vector to the animal host during an outbreak of EHD in penned deer. Larval breeding sites of *C. variipennis* were found and *C. variipennis* was the most abundant biting fly present during the outbreak. Females of *C. variipennis* were commonly observed biting deer, swine, cattle and, occasionally, man.

INTRODUCTION

During September and October, 1971, a severe outbreak of hemorrhagic disease appeared in white-tailed deer (*Odocoileus virginianus*) maintained in pens at the Mammoth Cave National Park in Kentucky, a field station of the Denver Wildlife Research Center, Fish and Wildlife Service, U.S. Department of the Interior.¹⁵ Deer usually died soon after the appearance of clinical signs, and mortality reached over 60%.¹⁵

Investigators at the time of the outbreak believed that the disease was probably epizootic hemorrhagic disease (EHD) of deer. However, bluetongue (BT), a similar disease that affects domestic and wild ruminants, was prevalent throughout the southeastern states and was suspected in cattle on farms in the Mammoth Cave area (unpublished data, this laboratory). Necropsy of deer immediately after death showed pathologic changes¹⁵ compatible with a diagnosis of either BT or EHD.^{20,21} The viruses of BT and EHD (BTV and EHDV) are morphologically indistinguishable in electron micrographs;^{11,18} they are related antigenically but can be separated by

serological tests. Because of their similarities, Borden *et al.*² and Murphy *et al.*¹¹ included BTV and EHDV in the same serologic subgroup within the orbiviruses—a new proposed taxonomic name for the BT-like virus group.

The suspect vector during the outbreak was *Culicoides variipennis* (Coquillett) (Diptera: Ceratopogonidae), a small bloodsucking gnat belonging to a group of flies known as biting midges. This fly was the suspect vector because 1) it was a known biological vector of BTV in laboratory studies,^{6,7} 2) “. . . similar clinical, pathological, and epizootiological patterns . . .”²⁰ for BT and EHD indicated that these two similar diseases probably had identical vectors, and 3) the known distribution for this species indicated that it should occur commonly in Kentucky.²² Subsequently, other published data indicated that *C. variipennis* was probably a vector of EHDV. Moore and Lee¹⁰ and Lee *et al.*⁹ reported the isolation of EHD group viruses from *Culicoides* collected in Nigeria. Moore and Lee¹⁰ concluded because of these isolations that “. . . *Culicoides* should also be considered in the search for the

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vector of EHD virus." Lee *et al.*⁹ concluded that circumstantial evidence points to the possibility that *Culicoides* is the vector of EHDV as well as of BTV. Boorman and Gibbs¹ in England demonstrated that EHDV (New Jersey isolate) multiplied in colony *C. variipennis* (subcolony of colony maintained at Denver) after flies ingested the virus. They concluded from their data that "*C. variipennis* may be capable of transmitting EHD virus, and that all members of the *nubeculosus* group of *Culicoides* warrant consideration as possible vectors of viruses belonging to the BT group."

MATERIALS AND METHODS

Epidemiological Criteria

Biting fly collections were made toward the end of the outbreak in penned deer at the Mammoth Cave area¹⁵ to determine whether EHDV or BTV were present in native biting-fly populations. Four additional criteria, which were used by Australian workers to assess insect species as potential vectors of arboviruses,¹⁶ were also used by us to determine the probable vector of the outbreak at the deer pens: 1) *Abundance*—Common species are more apt to be important vectors. 2) *Distribution*—A vector species should have a distribution the same as or greater than that of the disease. 3) *Host preference*—A vector species should have a host preference that includes the susceptible host, in this case deer. 4) *Susceptibility to infection*—Laboratory studies, particularly those that evaluate the susceptibility of vector populations to infection with the viruses being studied, should eliminate species that are not apt to be vectors.

Biting Fly Collection

Adult biting flies were collected alive with 4 modified CDC (Center for Disease Control) light traps.¹⁷ Light trap collections were made at several locations in the deer pens on 11 evenings from 14-25 October. Light trap catches

were chilled, sealed in ampules, frozen in liquid nitrogen, and shipped to Denver for identification of the biting flies. Mosquitoes and parous *C. variipennis* females without a recent blood meal were pooled for virus assay according to species and the time and locality of collection. Nulliparous females (without a previous blood meal) of *C. variipennis* were determined by the pigmentation method of Dyce³ for Australian *Culicoides* (used successfully by Nelson and Scrivani¹² for *C. variipennis* in the United States), recorded, and discarded.

Two other groups of flies were examined besides those collected for virus assay. Station personnel had netted swarms of flies in the aisles between deer pens on 3 October. Several electric-screen traps had been operated since mid-June in open deer stalls; the dead flies below these traps were combined to determine the earlier abundance of possible vectors.

Virus Isolation and Identification

Pools of flies were handled by the diagnostic procedures normally used at the Denver laboratory to isolate BTV. Primary virus isolation was by intravenous inoculation of insect suspensions into 10-day embryonating chicken eggs.

Subsequently, after over 2 years of storage of the egg isolates at 4 C and with the recent development of improved EHDV identification procedures at the Denver laboratory,⁸ all isolates from insects were repassed by yolk-sac inoculation of 7-8 day eggs. The few isolates recovered were inoculated onto African green monkey kidney (VERO) cell cultures²¹ for BTV and EHDV identification by the indirect fluorescent antibody (FA) test.⁸

Larval Breeding Sites

Possible larval breeding sites were examined throughout the Park for the presence of larval populations of mosquitoes and *C. variipennis*. Several farms,

²¹ Gorgas Memorial Institute, Middle America Research Unit, Ancon, Canal Zone.

where a problem with a BT-like disease in cattle had occurred (unpublished data, this laboratory), were examined to determine the prevalence of *C. variipennis* larval breeding sites in the Mammoth Cave National Park region of Kentucky.

Vector Competence

Live larvae and pupae of *C. variipennis* were collected from two larval breeding sites from 17 October to 12 December and shipped to Denver for rearing. The reared adults were used in vector-competence studies to determine whether pertinent Kentucky field populations were susceptible to oral infection with a standard source of BTV. Adults were given a single infective blood meal of an egg-adapted strain of BTV (BT-262, accession no. 62-45S) to determine the infection rates of these field populations for a single infective blood meal. These infection rates were compared with those obtained for other field populations in 1971 (Jones and Foster, in manuscript) and with those obtained for the standard colony of *C. variipennis* (SONORA strain, 000 line)⁸ maintained at our laboratory. The susceptibility rate of this laboratory colony is approximately 30% for the egg-adapted BT-262 strain of BTV⁷ and the infection rate in response to a single infective blood meal has normally ranged from 17 to 38%. Infective blood meals, given through a membrane, consisted of a 9:1 mixture of defibrinated normal sheep blood and virus suspension. The virus concentration of an infective blood meal was $\cong 3 \times 10^6$ chicken-embryo median lethal doses (ELD₅₀) per ml.⁷

RESULTS

Biting Fly Collection for Virus Assay

The most common biting fly collected in light traps operated at the deer pens was *C. variipennis*. At least 30% of the females collected were parous (records for nulliparous females lost); the 22 pools of parous females without a recent blood meal contained 133 flies with pool size ranging from 1-18. The only other biting fly collected was the mosquito *Culiseta inornata* (Williston); almost all

of these were used for virus-isolation attempts with 3 pools containing a total of 27 flies.

Virus Isolation and Identification

Data from the initial intravenous inoculation of eggs indicated that 12 of 22 pools of parous females of *C. variipennis* were positive for the isolation of an agent. Three of these isolates were identified as EHDV from pools that contained 4, 8, and 8 *C. variipennis* females. Two of the three isolates were identified elsewhere⁴ as EHDV by passage of egg-isolate material into susceptible deer and by identification of the agent as EHDV by an indirect FA test. Subsequent re-passage of all egg isolates yielded an additional agent (from only 4 parous females) that we identified as EHDV by the indirect FA test. Virus from the blood from one deer (No. 6)⁴ was re-confirmed as EHDV by the indirect FA test. These two isolates were the only two that we could reisolate and identify after over 2 years of storage at 4 C. None of the isolates were identified as BTV.

Abundance of the Vector

Light trap catches at the deer pens in the park were small because of cool nights. Nevertheless, *C. variipennis* was shown to be the most common biting fly present in late fall of 1971; a few mosquitoes were collected—all *C. inornata*. Our investigations gave four indications that *C. variipennis* was abundant in October and probably had been abundant at the deer pens during much of the summer: 1) swarms of flies (almost all males) collected in the aisles between pens on 3 October proved to be this species; 2) examination of the combined catch from electric-screen traps that had been operated since mid-June in open deer stalls yielded large numbers of *C. variipennis* to the virtual exclusion of other biting flies; 3) *C. variipennis* females were commonly seen attacking deer in the pens in October during daylight hours when it was warm; and finally, 4) larval breeding sites of this species were found in the pens.

Larval Breeding Sites

A moderate larval population (coded Maple Springs Ranger Station, Cuva 71 Ky 1) of *C. variipennis* was found in a breeding site in a deer pen in October, 1971. This site was a 1 m² permanent-type breeding area of soft mud that was exposed to direct sunlight and was produced by a slight flow of water from a leaking water pipe. Other likely breeding sites of possible vectors close to the deer pens were inactive. However, the weather had been dry and some deer pens had had standing water and wet soft-mud sites in low areas during the summer; these wet areas were possible breeding sites for earlier larval populations of mosquitoes and *Culicoides*. An examination in April, 1972, of one such low shady area with standing water and a considerable amount of exposed soft mud disclosed a moderate larval population of *C. variipennis* (coded Maple Springs Ranger Station, Cuva 72 Ky 4); no mosquito larvae were found at this site.

Except for very sparse larval populations of mosquitoes over 2 km from the deer pens, no other larval breeding sites of biting flies were located within the Park in October, 1971. Four farms in Edmonton County, which were within 20 km of the Park, were examined. Two were positive for good larval breeding sites of *C. variipennis*; the other two farms had possible breeding sites, but no larval populations were found. One farm had a moderate larval population in a small nonpermanent-type breeding site where water collected in a muddy depression in a cow yard. The other farm, which bordered the Park and was about 6 km from the deer pens, had an extensive larval breeding site of *C. variipennis* (coded Arthur, Cuva 71 Ky 2); a dense larval population was evident throughout the wide soft-mud margins of a shallow 1000 m² pond that was exposed to direct sunlight and was highly polluted by drainage from hog pens. An examination in 1972 of a farm near Adairville, Kentucky, disclosed an extensive breeding area of *C. variipennis* along the mud margins of a dirt stock tank used by cattle. The density of the

larval population was moderate when the water level was low enough to expose mud margins; density was slight when the tank was full with little exposed mud. Our data indicate that *C. variipennis* occurs commonly throughout Kentucky and that larval breeding sites are common when ecologic conditions provide suitable sites for larval breeding.

Biting Records for the Vector

Females of *C. variipennis* were commonly seen attacking deer in the deer pens in the fall of 1971, usually by flying up to the belly area. One natural biting record was obtained when a female landed on the hock of a fawn, disappeared into the short hairs, and reappeared engorged after about 5 min. This species was also noted commonly attacking sentinel cattle established in the pens [South-eastern Cooperative Wildlife Disease Study (SCWDS), University of Georgia, Athens, Georgia], again by flying up to the belly area. Flies were not noted attacking sentinel sheep in the same deer pen as the cattle. A few females of *C. variipennis* bit the investigators. *C. variipennis* females were commonly seen biting swine at the hog farm.

Vector Competence

The tests conducted over a two month period with adults reared from larvae and pupae collected in 1971 from the deer-pen and hog-pond breeding sites indicated that these two populations were completely resistant to infection with the BT-262 strain of BTV in the fall of 1971. The infection rates were 0% for a single infective blood meal (numbers of flies assayed: deer pen—50, hog pond—37) compared with 37% (11/30) and 23% (7/30) for control colony flies tested at the same time. Two field populations (Texas and Colorado) tested in 1971 had infection rates of 68 and 21%. However, adults reared from larvae collected at the hog-pond site in April, 1972 (Arthur, Cuva 72 Ky 2) were relatively susceptible to the same virus strain: the infection rate for a single infective blood meal was 27% for the 30

flies assayed compared with 31% (range 21-38%) for 107 flies assayed for the control colony. Flies reared from the low-area breeding site in the deer pens in 1972 (Maple Springs R.S., Cuva 72 Ky 4), presumably the same population as flies from the nearby 1971 breeding site at a leaking water pipe, remained resistant to oral infection with the BT-262 virus strain; the infection rate was 0% for the 11 flies assayed.

DISCUSSION

Two related viruses were apparently involved in the 1971 disease epizootic in deer at Mammoth Cave National Park. Thomas *et al.*¹⁰ found both serologic and virologic evidence that both BTV and EHDV were present in the overall epizootic, which involved several states, but that EHD was the prevalent virus at Mammoth Cave National Park. Substantively, their serologic results (their Table 2) showed that antibody developed primarily to EHDV and not to BTV in both resident and sentinel (SCWDS) deer at the Park. The viruses of both EHD and BT were also recovered from a resident penned deer at Mammoth Cave (SCWDS case history, personal communication with F. C. Thomas) as shown by the data for specimen 3 of their Table 1. The data for this deer further explains the statement by Prestwood *et al.*¹³ that "Both BTV and EHDV were isolated from a single animal in Kentucky."

The recovery of EHDV from three pools of *C. variipennis* collected at the deer pens, together with the lack of recovery of an isolate of BTV, suggested that the outbreak in penned deer at Mammoth Cave was primarily EHD. In addition, one of these isolates was highly pathogenic to deer.⁴ Our vector-competence studies with field populations (Jones and Foster, in manuscript) further indicated that the epizootic at Mammoth Cave National Park was EHD, not BT. The data obtained from testing different BTV serotypes suggested that a population of *C. variipennis* collected during

an epizootic would be most susceptible to oral infection with a BTV strain belonging to the same serotype as the BTV strain collected during the epizootic. The data indicated that the possession of a high degree of oral susceptibility to one BTV strain would convey at least moderate susceptibility to other BTV strains belonging to different serotypes. Thus, the complete resistance of two populations of *C. variipennis* collected during the disease outbreak at Mammoth Cave National Park (0/50 and 0/37), indicated that the populations at the Park were resistant to oral infection with BTV in 1971. The recovery of several isolates of EHDV from small numbers of parous females collected at the same time, indicated that the population at the deer pens was highly susceptible to oral infection with EHDV.

The isolation of a BT-like virus, identified as EHDV here and elsewhere,⁴ from field-collected parous *C. variipennis* females is the first time that this virus group has been recovered from the naturally-infected vector in the United States. The only earlier records of arboviruses recovered from field-collected *C. variipennis* in the United States are for But-tonwillow, Lokern and Main Drain viruses in California.^{12,14}

The abundance of *C. variipennis* in comparison with other biting flies during the outbreak, the common distribution of this species in an area exceeding that of the outbreak at the deer pens, a host preference for the species that included deer, and the susceptibility of *C. variipennis* females to infection with a strain of EHDV from the outbreak,⁴ indicated that *C. variipennis* was the vector responsible for the outbreak of hemorrhagic disease in penned white-tailed deer at Mammoth Cave National Park. This diagnosis was confirmed and the primary disease agent identified as EHDV by the isolation of EHDV from parous females collected during the outbreak and by the complete resistance of the Park population to infection with a strain of BTV.

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