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Detection of Bluetongue Virus Serotype 17 in *Culicoides variipennis* by Nucleic Acid Blot and Sandwich Hybridization Techniques

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Molecular hybridization techniques were developed for the detection and surveillance of bluetongue virus (BTV) serotype 17 in the insect vector *Culicoides variipennis*, a biting midge. Radiolabeled RNA and cDNA probes were generated from sequences of the L3 segment of BTV serotype 17. These probes were used to detect BTV RNA in pools of infected *C. variipennis* by hybridizing the probes directly to analyte immobilized on nylon membranes or by using a nucleic acid sandwich hybridization test. Hybridization procedures were able to detect 1 infected *C. variipennis* in a pool of 50 and as little as 3.55 $\log_{10}$ 50% tissue culture infective doses per ml of virus. These hybridization techniques provide an alternative to virus isolation for the surveillance of BTV in vector populations.

Bluetongue virus (BTV) is an arthropod-borne pathogen that infects wild and domestic ruminants. BTV is the prototype virus of the genus *Orbivirus* in the family *Reoviridae*. There are 24 serotypes of BTV recognized worldwide, and 5 of these (serotypes 2, 10, 11, 13, and 17) are indigenous to the United States (1, 10, 27). BTV serotypes 10, 11, 13, and 17 occur throughout the United States; BTV serotype 2 is apparently restricted to Florida (10).

BTV infects domestic cattle and sheep and is an important cause of abortions and congenital malformations (4, 11, 20). The virus is also economically important in the United States, because many countries restrict imports of live animals, embryos, and semen from BTV-enzootic areas (9).

The principal vector of BTV in the United States is *Culicoides variipennis* (Diptera: Ceratopogonidae), a biting midge commonly referred to as a "punky" or a "no-see-um." The conventional method for BTV surveillance in vectors requires the isolation of virus in cell culture or embryonated chicken eggs with subsequent serological identification (18). Conventional methods for virus surveillance are labor-intensive, expensive, and time-consuming and may require elaborate laboratory and containment facilities. Typically, virus isolation and identification can take more than 2 weeks. Thus, test results are not available in time to permit the institution of effective control measures.

Nucleic acid hybridization offers a novel approach for detecting viruses in biological specimens. Nucleic acid hybridization techniques are not affected by many of the limitations of virus isolation, such as latent or integrated viruses, or the limitations of serological diagnosis, such as antigen-antibody equivalence or immunological tolerance (3). Such techniques have been used to detect BTV analyte in cells and tissues (6–8, 28, 30, 33).

However, blot hybridization techniques require laborious extraction procedures and, when RNA is the target, immobilization of the analyte on the solid support is inefficient (26, 34). Sandwich hybridization techniques minimize these problems. Sandwich hybridization requires two contiguous nucleic acid sequences from the genome of the pathogen to generate the capture and detector probes. The capture probe is DNA, thereby permitting more efficient immobilization on the solid support. The detector probe, which can be RNA or DNA, is typically radiolabeled. When a sample contains a nucleic acid sequence complementary to the capture and detector, the detector probe is indirectly bound to the solid support and a signal results. Sandwich hybridization techniques have been used to detect viruses (16, 31, 32) and bacteria (22, 23) in clinical samples.

We report the detection of RNA from BTV serotype 17 in *C. variipennis* pools by molecular hybridization with direct blot and sandwich hybridization techniques.

MATERIALS AND METHODS

Virus. BTV serotype 17 (strain 62-45S) was obtained from the Arthropod-Borne Animal Disease Research Laboratory, Laramie, Wyo. This virus strain is a cell culture-adapted strain that was originally isolated from sheep. BTV serotype 17 was propagated by inoculating BHK-21 cells at a multiplicity of infection of approximately 0.2.

C. variipennis. *C. variipennis* midges used in this study were from the AK colony (Bruneau strain), which originated from field material collected in Idaho in 1973 (13). Rearing, infection, and maintenance of the midges were described previously (12). Briefly, the midges were anesthetized with carbon dioxide and intrathoracically inoculated with the virus suspension by using a microcapillary tube drawn to a fine point. This procedure ensured that all midges became infected. Midges were extrinsically incubated for 14 days postinfection at 26°C and 40% relative humidity and then frozen at −70°C until processed. The prevalence of infection in the midges was determined by examining head tissues for viral antigen by an indirect immunofluorescence assay (2). Pools of 50 *C. variipennis* containing various numbers of infected and uninfected midges were constructed (see Table 1). Duplicate pools were constructed; one was used for virus isolation and one was used for hybridization.

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Virus isolation. The *C. variipennis* pools were triturated in 1 ml of diluent (L-15 [Leibovitz] medium containing 100 U of penicillin per ml, 100 μg of streptomycin per ml, 100 μg of gentamicin per ml, and 8% heat-inactivated fetal bovine serum). Serial 10-fold dilutions of each pool were plated in 96-well, flat-bottomed microtiter plates along with 7,500 BHK-21 cells per well. Plates were incubated at 34°C in a humid incubator without CO_2. Endpoints were determined after 5 to 7 days, and titers were calculated (15).

RNA extraction and blotting. Total RNA was extracted from pooled *C. variipennis* after trituration in 300 μl of lysis buffer (7 M urea, 0.35 M NaCl, 0.1 M Tris hydrochloride [pH 8.0], 0.01 M EDTA, 2% sodium dodecyl sulfate) at 4°C (14). The lysis mixture was extracted once with phenol-chloroform (1:1) and twice with chloroform and ethanol precipitated at −70°C for at least 1 h. The RNA was pelleted in a microcentrifuge for 15 min at 4°C and suspended in 100 μl of 0.5% sodium dodecyl sulfate.

RNA from *C. variipennis* pools was denatured, blotted, and hybridized by a modification of the procedure previously described (21). One-fifth (20 μl) of the RNA extracted from each pool was suspended in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA, denatured at 100°C for 5 min, and quenched on ice. An equal volume of 2× denaturation buffer (14% formaldehyde, 12× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) was added to each denatured sample. Serial 10-fold dilutions of each sample were made in 1× denaturation buffer and applied to nylon filters (0.45-μm-pore Nytran; Schleicher & Schuell, Inc., Keene, N.H.) with a Minifold II slot blot apparatus (Schleicher & Schuell) under low vacuum pressure. The filters were rinsed in 6× SSC, and the RNA was cross-linked to the filters by exposure to UV light (160 J/m^2) for 3 min (5).

Probe constructs. The construct pBTV17-3 was produced by cloning a 2,772-base-pair (bp) cDNA fragment of the L3 RNA genome segment of BTV serotype 17 (24). The L3 genome segment codes for the viral polypeptide P3. The cDNA is group reactive and hybridizes with L3 RNA segments from all 5 indigenous and 14 exotic BTV serotypes (28).

Sequences from pBTV17-3 were subcloned into an in vitro transcription plasmid and into M13 replicative-form DNA to produce RNA transcript and single-stranded DNA probes for use in direct and sandwich hybridizations (R. Schoepp, Ph.D. dissertation, Colorado State University, Fort Collins, 1989). Construct pBTV17-3 was digested with the enzymes *Bam*HI and *Hin*dIII, resulting in fragments of 438, 674, and 1,660 bp. The 674-bp fragment was subcloned into the *Bam*HI site of the transcription plasmid pGEM-1 (Promega Corp., Madison, Wis.) to produce the construct pRSG1.674. The 1,660-bp fragment contiguous to the 674-bp fragment had *Hin*dIII and *Bam*HI ends and was ligated into the double-stranded replicative-form DNA of M13mp18 and M13mp19 to produce the constructs RSM38.1660 and RSM39.1660. The polarities of the RNA transcripts and the recombinant M13 single-stranded DNA capture sequences were determined. The probes transcribed from the SP6 promoter (RSG1.674/SP6) and the T7 promoter (RSG1.674/T7) were of positive and negative senses, respectively (Schoepp, Ph.D. dissertation; data not shown). The constructs RSM38.1660 and RSM39.1660 yielded single-stranded recombinant DNA of positive or negative sense, respectively (Schoepp, Ph.D. dissertation; data not shown).

Labeling of probes. RNA was labeled by incorporation of [α-^{32}P]CTP during transcription from templates of linearized pRSG1.674. The protocol used was a modification of one described by Melton and associates (19) and recommended by the kit manufacturer (Promega). For production of the cDNA probe, pBTV17-3 was nick translated to incorporate [α-^{32}P]dCTP. Nick translation kits were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), and the reaction was carried out as suggested by the manufacturer.

Direct hybridization. Direct blot hybridizations with either the cDNA probe (pBTV17-3) or the RNA transcript probe (RSG1.674/T7) were performed in essentially the same manner. Filters were prehybridized at 42°C for 2 h in hybridization solution containing 50% deionized formamide, 6× SSC, 5× Denhardt solution, 0.1% sodium dodecyl sulfate, 200 μg of salmon sperm DNA per ml, and 200 μg of yeast tRNA per ml. Hybridization with fresh solution containing probe (10^6 cpm/ml) was done at 42°C overnight (16 h). Posthybridization washes consisted of four 5-min washes in 2× SSC–0.1% sodium dodecyl sulfate at room temperature, two 25-min washes in 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C, one 10-min wash in 2× SSC containing 10 μg of RNase A per ml at room temperature (omitted when the cDNA probe was used), and a final rinse in 2× SSC. The filters were dried and exposed to Fuji RX X-ray film for 72 h at −70°C.

Sandwich hybridization. Hybridizations were performed on squares (10 by 10 mm) of Nytran membrane. Membranes of this size were easily assembled in the Minifold I dot blot apparatus (Schleicher & Schuell) for application of the capture probe and were ideal for use in 24-well tissue culture plates in which hybridizations and posthybridization washes were done. The capture probe (0.5 μg of single-stranded RSM39.1660 DNA) was applied in 100 μl of 10× SSC under low vacuum pressure (approximately 200 mm Hg [ca. 26.7 kPa]). After blotting, the filters were UV cross-linked for 3 min to bind the DNA and then stored at 4°C until used.

Five hundred microliters of the hybridization solution described above but without the salmon sperm DNA was added to each filter, and the plates were incubated at 60°C for 2 h. The hybridization solution was removed, and denatured analyte was added to each well with 0.5 ml of fresh hybridization solution containing 10^6 cpm of radiolabeled RNA detector probe per ml. The plate was covered, sealed with tape, and hybridized for 16 h in a 60°C water bath. Sandwich hybridization containing capture and detector probes but no analyte was included to ensure that the two components did not self-hybridize. Posthybridization washes were identical to those described for direct hybridization, except that the RNase A wash was omitted. The filters were dried and exposed to film for 72 h at −70°C.

RESULTS

Sensitivity and specificity of direct hybridizations with the RNA probe to detect purified BTV RNA. BTV serotypes 2, 10, 11, 13, and 17 as well as epizootic hemorrhagic disease virus (EHDV) serotypes 1 and 2 were each purified on sucrose gradients, and genomic RNA was extracted from each virus preparation. One hundred nanograms of each genomic RNA was serially diluted 10-fold and immobilized on nylon membranes. Sensitivity and specificity were determined by hybridizing each viral RNA with RNA probe RSG1.647/T7. Under conditions of low-stringency hybridization, as little as 1.0 ng of genomic L3 RNA from all five BTV serotypes was detected (Fig. 1). As little as 100 pg of genomic RNA from BTV serotype 11 was detected. There

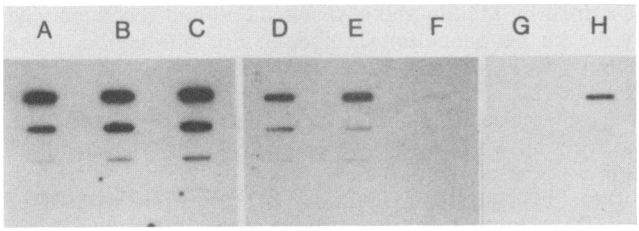

FIG. 1. Sensitivity and specificity of the RNA probe RSG1.647/T7. Viral RNA was extracted from purified BTV serotypes 2 (A), 10 (B), 11 (C), 13 (D), and 17 (E) and EHDV serotypes 1 (F) and 2 (G). The preparations were enriched for double-stranded RNA by LiCl precipitations. One hundred nanograms of each preparation was serially diluted from undiluted to 1:1,000 before application to nylon membranes. One hundred picograms of BTV serotype 17 L3 cDNA was serially diluted and used as a hybridization probe control (H).

was also weak hybridization to the cognate genes of both EHDV serotypes (Fig. 1). The RNA probe also detected 10 pg of homologous L3 cDNA (Fig. 1).

Detection of BTV analyte in pools of *C. variipennis* with the RNA probe. *C. variipennis* midges were experimentally infected with BTV serotype 17, and various ratios of infected and uninfected midges were formed into pools of 50. The composition and virus titers of the pools are shown in Table 1. BTV serotype 17 titers ranged from 4.93 to 6.43 $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$)/ml. RNA was extracted from similarly constructed pools, and the analyte was serially diluted 10-fold before being immobilized on nylon membranes. Hybridizations were performed with the RNA probe RSG1.674/T7. One infected *C. variipennis* in a pool of 50 could be detected by this technique (Fig. 2).

Detection of BTV analyte in pools of *C. variipennis* with the cDNA probe. With the cDNA probe (pBTV17-3), *C. variipennis* pools containing 25, 10, 5, and 1 infected midge(s) were easily detected (Fig. 3). BTV analyte could be detected in the serial dilutions of the pools. The calculated sensitivity ranged from 3.55 to 3.93 $\log_{10}$ $TCID_{50}$/ml. The pool (D) containing a single infected *C. variipennis* produced a signal that was similar in intensity to that produced by the pools (A, B, and C) containing greater numbers of infected *C. variipennis*. The negative control pool (E) containing 50 uninfected midges yielded a weak signal; this nonspecific signal was attributed to hybridization under conditions of low stringency.

Detection of BTV analyte in pools of *C. variipennis* by sandwich hybridization. The analyte used for hybridization with the cDNA probe was also used in the sandwich hybridization assay. BTV RNA could be detected in all the infected *C. variipennis* pools (Fig. 3). Since sandwich hybridization could detect a single infected midge in a pool of 50, the level of sensitivity was the same as with direct hybridization with RNA and cDNA probes. There was little or no signal from the uninfected *C. variipennis* pool.

TABLE 1. Composition and virus titers of *C. variipennis* pools

Pool	No. of *C. variipennis*		Titer ($\log_{10}$ $TCID_{50}$/ml)
	Infected	Uninfected	
A	25	25	6.43
B	10	40	5.93
C	5	45	5.55
D	1	49	4.93
E	0	50	0.00
Control	5	0	5.55

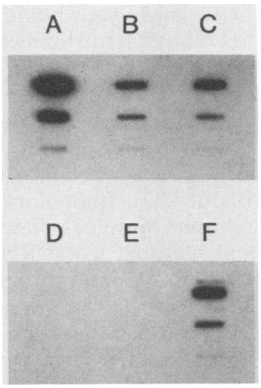

FIG. 2. Detection of BTV serotype 17 RNA in infected *C. variipennis* with the RNA transcript probe RSG1.674/T7. RNA was extracted from pools of 50 *C. variipennis* consisting of 25 infected and 25 uninfected midges (A), 10 infected and 40 uninfected midges (B), 5 infected and 45 uninfected midges (C), 1 infected and 49 uninfected midges (D), 0 infected and 50 uninfected midges (E), and 5 infected and 0 uninfected midges (F). Each sample was serially diluted 10-fold from undiluted to 1:1,000 before application. BTV serotype 17 RNA was detected by hybridization with the negative-polarity ^{32}P-labeled RNA probe transcribed from pRSG1.674/T7.

DISCUSSION

Surveillance of vectors for arthropod-borne virus infections is difficult because of the large numbers of arthropods collected. Since the prevalence of infection in arthropod vectors is usually low, assays to monitor vector infection rates must be sensitive enough to detect one infected vector in a large pool. Techniques that permit direct detection of BTV in pools provide rapid diagnostic capability. Hybrid-

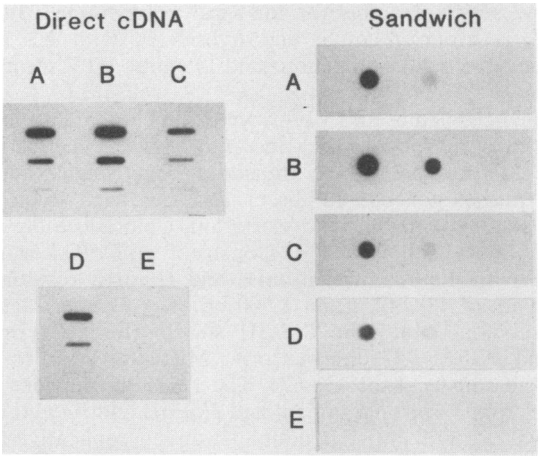

FIG. 3. Detection of BTV serotype 17 RNA in infected *C. variipennis* with a direct cDNA probe or sandwich hybridization. RNA was extracted from pools of 50 *C. variipennis* consisting of 25 infected and 25 uninfected midges (A), 10 infected and 40 uninfected midges (B), 5 infected and 45 uninfected midges (C), 1 infected and 49 uninfected midges (D), and 0 infected and 50 uninfected midges (E). For direct cDNA hybridization, each sample was serially diluted 10-fold (undiluted to 1:1,000) before application. BTV serotype 17 RNA was detected by hybridization with the nick-translated ^{32}P-labeled cDNA probe pBTV17-3. For sandwich hybridization, each sample was also serially diluted 10-fold before application. BTV serotype 17 RNA was detected by hybridization with the single-stranded DNA capture probe (RSM39.1660) and the single-stranded RNA detector probe (RSG1.674/T7).

ization techniques permit direct detection of the pathogen and eliminate concerns about antigen degradation.

The RNA probe was cross-reactive with L3 RNA from all five indigenous serotypes of BTV and detected as little as 100 pg of BTV genomic RNA or 15.4 pg of the L3 genome segment. All three hybridization techniques used (RNA probe, cDNA probe, and sandwich hybridization) readily detected 1 infected midge in a pool of 50. BTV isolation from a similarly constructed pool produced a titer of 4.93 $\log_{10}$ $TCID_{50}$/ml. It is important to note that the hybridization sensitivities resulting from direct and sandwich hybridizations cannot be directly compared, since the analyte used in each assay was prepared at different times and subjected to different periods of storage at −70°C. Storage may have adversely affected the analyte and lowered the sensitivity of some hybridization assays.

Studies are currently in progress to optimize and compare directly the hybridization techniques and to determine the diagnostic efficacy of developed techniques in prospective trials. Sandwich hybridization may be particularly promising, because theoretically the need for labor-intensive extraction of samples is reduced or eliminated (25) and because it could be used to purify analyte for amplification by the polymerase chain reaction (29) or for signal amplification by Q beta-replicase (17).

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