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Detection of Dengue Virus Type 2 in *Aedes albopictus* by Nucleic Acid Hybridization with Strand-Specific RNA Probes

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A molecular hybridization technique with radiolabeled, strand-specific RNA probes was developed to detect dengue virus type 2 RNA in pools of infected *Aedes albopictus* mosquitoes. One infected mosquito in a pool of 25 could be detected, corresponding to a dengue virus type 2 titer of 2.75 $\log_{10}$ 50% tissue culture infectious doses.

The dengue virus complex (*Flaviviridae*) consists of four serotypes (DEN-1, DEN-2, DEN-3, and DEN-4; 12). Dengue virions possess a single-stranded RNA genome of positive polarity approximately 11 kilobases in length (13).

Dengue viruses are important mosquito-borne disease agents of humans. Dengue virus infections typically result in an acute, self-limited disease characterized by fever, myalgia, and rash. Less frequently, dengue virus infection causes a more severe disease form, dengue hemorrhagic fever-shock syndrome (4). *Aedes albopictus* and *Aedes aegypti* are the primary mosquito vectors of dengue virus.

The increasing incidence of dengue virus infections throughout the tropics has prompted increased efforts to develop rapid and reliable diagnostic techniques. Conventional dengue virus surveillance involves isolation of virus from mosquitoes or mosquito pools, amplification in a bioassay such as suckling mice or cell culture, and serological identification. Nucleic acid hybridization offers a novel approach to detect viruses in biological specimens (3, 5, 10). Nucleic acid hybridization has been used successfully to detect purified dengue virus RNA and viral RNA in dengue virus-infected Vero cells and C6/36 cells by using cDNA probes or synthetic oligonucleotide probes (5, 9, 10). We report the detection of dengue virus RNA in mosquito pools by molecular hybridization.

A low-passage DEN-2 human isolate (PR152) was propagated in an *A. albopictus* C6/36 cell culture in Leibovitz's L-15 medium and 4% fetal calf serum at 27°C. Virus was concentrated from the culture medium by precipitation with 7% polyethylene glycol at 4°C for 12 to 14 h and centrifugation at 30,000 rpm for 4 h. Virus from 200 ml of medium was suspended in 1 ml of TNE buffer (10 mM Tris hydrochloride [pH 8.0], 0.1 M NaCl, 1 mM EDTA) and titrated at 4.0 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$)/ml in C6/36 (*A. albopictus*) cells (see below). The low titer of the virus stock may reflect some virus inactivation during concentration and storage.

A. albopictus (Oahu) females were inoculated intrathoracically with 1.0 μl of a 1:10 dilution of DEN-2 concentrate and maintained for 10 to 14 days at 26°C (14). Mosquito infection was determined by direct immunofluorescence assay of head squashes (1). Pools of 25 mosquitoes were constructed with uninfected mosquitoes and mosquitoes which were virus positive by immunofluorescence assay (Table 1).

The mosquito pools were triturated in 10 ml of diluent (L-15 medium, 500 U of penicillin per ml, 50 μg of streptomycin per ml, 5 μg of amphotericin B [Fungizone] per ml). Serial 10-fold dilutions of each pool were combined with C6/36 (*A. albopictus*) cells and plated in quadruplicate in chambered slides (Miles Scientific, Div. Miles Laboratories, Inc., Napierville, Ill.). After incubation at 27°C for 7 days, slides were fixed in cold acetone and examined for virus antigen by a direct immunofluorescence technique (1).

Total RNA was extracted from pooled mosquitoes (8). After trituration in 300 μl of a 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, the lysis mixture was extracted twice with a phenol-chloroform mixture and twice with chloroform and then ethanol precipitated. The pellet was suspended in 100 μl of 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA and stored at −70°C until assayed.

To make the RNA probes, a 1.95-kilobase DEN-2 cDNA representing sequences from the NS5 gene region of the viral genome (5, 17) was digested with *Eco*RI restriction endonuclease. The resulting fragments of approximately 750 and 1,200 base pairs were ligated into in vitro transcription plasmids pSP64 and pSP65 (11). The 1.95-kilobase cDNA was previously demonstrated to be specific for DEN-2 RNA in cross-hybridization experiments under stringent conditions with viral RNA from all serotypes (unpublished data). RNA probes were transcribed from linearized DNA templates in the presence of [^{32}P]CTP and SP6 polymerase. Specific activities of labeled RNA were greater than 10^8 cpm/μg.

RNA from mosquito pools was denatured in 7% formaldehyde–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C for 15 min. Samples were diluted 10-fold and applied to a nylon filter (Nytran) with a slot-blot apparatus. The filter was briefly washed in 6× SSC and exposed to a UV source (160 J/m^2) for 3 min to cross-link the RNA to the filter (2). Filters were prehybridized at 42°C for 3 h with 50% formamide–6× SSC–5× Denhardt solution–0.1% sodium dodecyl sulfate containing 200 μg each of salmon sperm DNA and tRNA per ml. Hybridization with fresh prehybridization mixture containing probe (10^6 cpm/ml) was conducted at 42°C for 16 h. Posthybridization washes consisted of four 5-min washes at room temperature

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TABLE 1. Composition and virus titer of mosquito pools

Pool	No. of mosquitoes		Log_{10} $TCID_{50}$ titer[a]
	Uninfected	Infected	
A	0	25	5.25
B	13	12	4.75
C	20	5	3.25
D	24	1	2.75
E	25	0	0.00

[a] Titrations were performed in C6/36 cells by a direct immunofluorescence technique (see text).

with 2× SSC–0.1% sodium dodecyl sulfate, two 15-min washes at 50°C with 0.1× SSC–0.1% sodium dodecyl sulfate, and one 10-min wash with 2× SSC containing 10 μg of RNase A per ml at room temperature. The filters were air dried and exposed to Du Pont Cronex 4 X-ray film.

After incubation for 10 days, mosquitoes infected with DEN-2 were used to construct pools A to E (Table 1). The titers of the pools ranged from 5.25 log_{10} $TCID_{50}$/ml (pool A) to 2.75 log_{10} $TCID_{50}$/ml (pool D), representing minimally from 1.17 to 0.0037 pg of genomic RNA.

The mosquito pools were analyzed for the presence of DEN-2 RNA with labeled RNA probe from in vitro transcription of clone KO64-750 (Fig. 1a) and clone KO65-1200 (Fig. 1b). In both cases, DEN-2 RNA from one infected mosquito in a pool of 25 could be easily detected after a 7-day exposure. This represented a minimum of 560 infectious virus particles or 0.0037 pg of virion RNA. The apparent difference in sensitivities between the two probes (Fig. 1a and b) may reflect RNA probe degradation. Alternatively, variations in sensitivity may be due to the efficiency of binding of the RNA samples to the nylon membrane. Lanes F (Fig. 1a and 1b) represent 1 ng to 10 pg of purified virus RNA and suggest a sensitivity of detection of 10 pg of dengue virus RNA. Thus, the hybridization technique probably detects large quantities of intracellular DEN-2 RNA and, perhaps, RNA of defective virus particles in mosquitoes. Alternatively, virus titers of the mosquito pools might have been greater had they been determined in mosquitoes instead of in C6/36 cells (15).

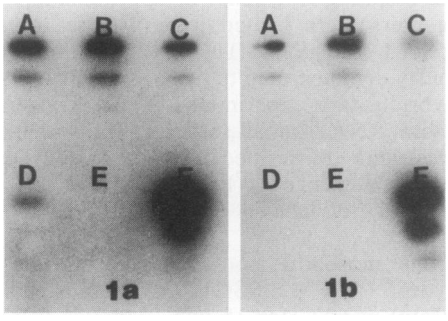

FIG. 1. Direct detection of DEN-2 RNA from infected *A. albopictus*. Mosquito RNA was extracted from pools consisting of 25 infected mosquitoes (A), 12 infected and 13 uninfected mosquitoes (B), 5 infected and 20 uninfected mosquitoes (C), 1 infected and 24 uninfected mosquitoes (D), and 25 uninfected mosquitoes (E); (F) purified DEN-2 RNA. Each sample was serially diluted 10-fold before application. Samples A to E range from undiluted RNA to 1:1,000 dilutions. Sample F represents from 1 ng to 10 pg of purified DEN-2 RNA. DEN-2 RNAs in samples A through F were detected by hybridization with transcription plasmids KO64-750 (a) and KO65-1200 (b).

Surveillance of vectors for arbovirus infections requires pooling of insects. The number of mosquitoes assayed is generally large and the rate of virus infection is low (16). Therefore, assays which monitor vector infection rates must be sensitive enough to detect one infected mosquito in a large pool. Enzyme immunoassay techniques can detect 1 infected mosquito in pools of 100 (6, 7). The La Crosse virus enzyme immunoassay detected 2 to 3 log_{10} $TCID_{50}$ of infectious virus, a sensitivity rate comparable to hybridization to DEN-2 RNA in infected mosquito pools. While antigen detection by enzyme immunoassay is a sensitive and specific technique, antigen degradation and the need for cold chains to preserve the samples is a concern for field use. Once the RNA is bound to the substrate, nucleic acid is stable. The durability of the membrane should allow rehybridization to samples with alternate probes. The convenience of RNA-RNA hybridization as a surveillance diagnostic tool can be further improved by developing nonradiolabeled probes.

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