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Detection of Dengue Virus NS1 Antigen in Infected *Aedes aegypti* Using a Commercially Available Kit

Natalia V. Voge, Irma Sánchez-Vargas, Carol D. Blair, Lars Eisen, and Barry J. Beaty*
Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado

Abstract. Epidemic dengue has emerged throughout the tropical world. In the continued absence of a vaccine against dengue virus (DENV), mosquito vector surveillance and control programs are essential to reduce human infections. An effective test to detect DENV in infected mosquitoes would be a valuable addition to the surveillance effort. We investigated DENV detection in infected *Aedes aegypti* using a commercially available DENV non-structural protein 1 (NS1) ELISA kit (Platelia Dengue NS1 Ag), and by reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation assays. The DENV-infected mosquitoes were subjected to field-relevant conditions and assayed individually and pooled with uninfected mosquitoes. Overall, DENV NS1 antigen was detected in 98% of infected mosquitoes/pools versus 79% for RT-PCR and 29% for virus isolation. Our results indicate that NS1 is an excellent analyte for detection of DENV in *Ae. aegypti* and that the tested NS1 antigen kit provides a sensitive, rapid, and convenient test for DENV surveillance in mosquitoes.

INTRODUCTION

Dengue is the most important mosquito-borne arboviral disease. The four dengue virus (DENV) serotypes (genus *Flavivirus,* family *Flaviviridae*) now circulate pan-tropically,[1–4] and new genotypes associated with increased virulence have expanded from endemic areas of Asia into the Americas.[5–8] Dramatic increases in epidemic dengue fever and dengue hemorrhagic fever have occurred in recent decades, resulting in an estimated 100 million cases of dengue fever and 500,000 of dengue hemorrhagic fever per year.[9] Although most cases occur in the endemic areas of the Americas, Southeast Asia, and the Pacific, dengue is a threat to the continental United States; dengue cases are diagnosed each year in travelers returning from endemic areas and autochthonous cases have been documented in Texas and recently also in Florida.[10–13] New and more effective tools and approaches for surveillance and control of dengue are sorely needed.[14]

In the absence of a vaccine, dengue prevention is focused upon controlling mosquito vectors. Development of improved surveillance methods for DENV in mosquito populations would be of great value for public health and vector control programs.[14,15] For example, monitoring of mosquito populations for DENV infection could provide improved risk assessment for DENV infections in humans, and would allow vector control programs to better target their interventions to areas at greatest risk for ongoing or impending epidemics and to respond rapidly and more effectively to the emergence of DENV in new areas with susceptible human populations.[15] Although not typically used by DENV control programs, pathogen surveillance in vector populations is widely used for other arboviruses, for example West Nile virus (WNV).[16,17] The WNV surveillance in mosquitoes provides important spatial and temporal information about virus circulation and areas needing to be targeted for control and prevention efforts. In mosquito-based arbovirus surveillance, adult mosquitoes are typically collected using bait or oviposition traps, identified to sex and species, separated into pools that are assayed using one or a combination of tests, and field infection rates are then determined.[16,18–21] Because thousands of mosquitoes commonly are collected in surveillance programs, it is cost prohibitive to process individual mosquitoes, and pools of 100 or more mosquitoes are often tested to reduce costs.

The DENV is maintained in mosquito-human transmission cycles; *Aedes aegypti* and *Aedes albopictus* are the most important vectors.[1] *Aedes aegypti*, the principal urban vector, lives in close contact with humans and commonly transmits the virus to humans in and around homes or other indoor environments.[15,22] Mosquitoes become infected after feeding on a viremic human and after a 4–14 days extrinsic incubation period, the virus can be transmitted to susceptible humans.[23] The mosquito is infected for life and can transmit the virus each time she bites a susceptible human.

The DENV can be detected in individual or pooled mosquitoes by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) for viral antigens, by reverse transcription-polymerase chain reaction (RT-PCR) for viral RNA, and by isolation of infectious virus.[20] However, DENV surveillance in mosquito vectors using these diagnostic techniques can be prohibitively expensive, may require special reagents, equipment or laboratory facilities or extensive training of personnel, and may be laborious and time-consuming. An ideal test method for DENV surveillance in vectors would be simple to perform, rapid, inexpensive, cost-efficient, sensitive and specific, and capable of detecting the pathogen under field-relevant conditions. For example, the triturated suspensions of large pools of mosquitoes, which are viscous and contain particulates and environmental contaminants, can complicate pathogen detection, especially by virus isolation and RT-PCR. In some circumstances, e.g., remote locations, mosquito traps may not be visited for extended periods of time, resulting in mosquito desiccation. In addition, mosquitoes may be subjected to cycles of freezing and thawing during identification, pooling, processing, and assaying the samples. All of these field-relevant conditions can result in infectious virus inactivation and/or destruction of viral analytes.[21]

The RT-PCR is widely used for detection of arboviruses, including DENV,[22] in field-collected or simulated field-collected mosquito pools. The RT-PCR has been shown to

*Address correspondence to Barry J. Beaty, Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523. E-mail: Barry.Beaty@colostate.edu

detect one mosquito infected with Japanese encephalitis virus in a pool of 1,000 mosquitoes after 14 days of simulated tropical conditions.[24] Dengue virus RNA can be detected in mosquitoes captured over a period of 28 days on sticky lure traps using RT-PCR,[25] and nested PCR has been shown to detect DENV in one infected mosquito head in pools of up to 59 negative mosquito heads.[26] Chikungunya virus RNA can be detected in *Ae. aegypti* mosquitoes stored at 28°C for 12 weeks.[27] Although an excellent test, RT-PCR is expensive and requires trained personnel, specialized equipment, and laboratory facilities.

Antigen detection systems using in-house ELISAs can be used for arbovirus surveillance in mosquitoes.[18,19] Antigen detection kits are commercially available to detect WNV and Saint Louis encephalitis virus in mosquitoes.[17] Recently, Tan and others[28] showed that a commercially available ELISA kit designed to detect DENV nonstructural protein 1 (NS1) in human serum (Dengue NS1 Ag Strip; Bio-Rad Laboratories, Marnes-la-Coquette, France; Catalogue no. 70700) also could be used to detect DENV NS1 in infected *Ae. aegypti*. Dengue virus NS1 antigen was detected in mosquitoes at 10 days after infection in the laboratory with DENV serotypes 1, 2, 3, or 4, as well as in field-collected DENV-infected mosquitoes. The test was as sensitive as real-time RT-PCR in detecting DENV-infected mosquitoes.[28] Another NS1 test (Panbio Dengue Early ELISA; Panbio, Brisbane, Australia; Catalogue no. E-DEN02P) also proved to be sensitive for detection of DENV in experimentally infected *Ae. aegypti* mosquitoes; NS1 was detected in pools of up to 50 mosquitoes at Days 0, 5, and 15 post infection (PI). This portable test could be performed in 30 min, allowing for rapid monitoring of DENV in mosquitoes in the field.[29]

We expanded upon these studies and evaluated the use of a third test—the Platelia Dengue NS1 Ag kit (Bio-Rad Laboratories; Catalogue no. 72830)—to detect DENV NS1 antigen in mosquitoes subjected to conditions and processing steps commonly encountered in mosquito-based surveillance systems.[16,18] We constructed pools of *Ae. aegypti* containing up to 999 uninfected mosquitoes and one infected mosquito subjected to simulated field conditions including drying (desiccation) and/or freeze-thaw cycles. The pools were then assayed for infectious DENV by virus isolation, for DENV RNA by RT-PCR and nested PCR, and for DENV NS1 antigen using the Platelia Dengue NS1 Ag kit (Bio-Rad Laboratories).

METHODS

DENV preparation. To prepare virus for mosquito infections, C6/36 cells were cultured to confluent monolayers at 28°C using modified Eagle's medium supplemented with 7% fetal bovine serum, L-glutamine, non-essential amino acids, and penicillin/streptomycin. High passage DENV-2 (Jamaica 1409) was used to infect confluent monolayers at MOI 0.01. After 7 days medium was replaced, and at 12–14 days medium was harvested and frozen at −70°C. Viral titer was determined by plaque assay.[30]

DENV titration by plaque assay. To titrate DENV, LLC-MK2 monkey kidney cells were grown to confluent monolayers in 12-well plates, infected with 10-fold serial dilutions of virus for 1 hour, and then overlaid with an agar-nutrient mixture.[31] After 7 days incubation at 37°C cells were stained with 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution and incubated for 4 more hours.[30] Viral titers were determined by counting plaques.

DENV infection of *Aedes aegypti* mosquitoes. *Aedes aegypti* (Puerto Rico Rex-D strain) eggs were hatched and reared to adults at 28°C/75–80% relative humidity with a photo-cycle of 16:8 L:D. To infect mosquitoes, adult females were placed in cartons and deprived of sugar and water 4 hours before injection. Mosquitoes were intrathoracically injected with 1×10^3 to 1.5×10^3 plaque-forming units (PFU) DENV-2 (Jamaica 1409) in a volume of ~0.5 μL. Mosquitoes were frozen at −70°C on Days 1, 3, 7, 14, or 21 PI. To assay for DENV infection, individual mosquito heads were severed, squashed on a glass slide, fixed in acetone, and assayed for DENV envelope protein antigen by indirect IFA.[23] The corresponding mosquito bodies were kept at −70°C.

Construction and processing of mosquito pools. The bodies of mosquitoes injected with DENV were used to construct mosquito pools along with age-matched uninfected mosquitoes. Mosquitoes processed at 1 day PI did not contain detectable antigen in head tissues but were shown by other assays to be DENV-infected (see Results section). Head-squash preparations of all mosquitoes processed at 3–21 days PI exhibited 3 + or 4 + IFA scores.

We constructed pools containing one DENV-infected and varying numbers of uninfected mosquitoes (0, 9, 99, 499, or 999) for total pool sizes of 1, 10, 100, 500, or 1,000 mosquitoes. To address heterogeneity in virus loads likely to be encountered in field-collected mosquitoes caused by differences in vector competence, times of extrinsic incubation, and environmental factors,[23,32,33] mosquitoes harvested 1, 3, 7, 14, or 21 days PI, respectively, were added to the pools of uninfected mosquitoes. Mosquito pools were triturated using cold pestles in 1.5 mL of L-15 medium for pools of 1 and 10 mosquitoes, 2 mL for pools of 100 and 500 mosquitoes, and 3 mL for pools of 1,000 mosquitoes. Medium (pH 7.2 ± 0.2) contained HEPES, FBS, L-glutamine, essential amino acids, and penicillin-streptomycin. After trituration pools were centrifuged at 1,000 rpm for 10 min and supernatants were tested for NS1 Ag without further processing.

For experiments to simulate field conditions and processing, additional pools containing one infected mosquito and 0, 9, 99, or 999 uninfected mosquitoes were constructed. To address effects of freezing and thawing during processing, the constructed pools containing the bodies of uninfected mosquitoes to which one infected mosquito body was added were subjected to either 1 hour of freezing at −20°C and 1 hour of thawing at room temperature or five identical freeze-thaw cycles. To address effects of drying (desiccation) of mosquitoes, an additional set of pools was constructed with infected mosquitoes that had been maintained for 1, 3, 14, or 30 days at room temperature in a biosafety cabinet.

DENV NS1 antigen detection using the Platelia Dengue NS1 Ag kit. The Platelia Dengue NS1 Ag kit (Bio-Rad Laboratories) was used to detect DENV NS1 antigen in mosquitoes. The test is a one-step sandwich format enzyme immunoassay for qualitative or semi-quantitative detection of DENV NS1 antigen from all four dengue virus serotypes.[34] Fifty microliters (50 μL) of the supernatant of centrifuged mosquito homogenate suspensions were placed into each well of a 96-well plate and incubated with 150 μL anti-NS1 monoclonal antibody (MAb) conjugated with horseradish peroxidase in phosphate buffer, Tween 20, and fetal calf serum for 90 min at 37°C. When NS1

was present, an immune-complex MAb-NS1-MAb-peroxidase formed and was revealed by adding a chromogenic substrate (tetramethylbenzidine) and H_2O_2 to initiate color development. The reaction was stopped by the addition of 100 μL of 1 N sulfuric acid. Absorbance was determined at 450 nm (A_{450}). Sample A_{450} values were compared with those of positive standards included in the kit[34]; each sample was assayed in triplicate.

DENV RNA detection using RT-PCR and nested PCR. Mosquito pools were homogenized in L-15 medium, suspensions were centrifuged at 1,000 rpm for 10 min, supernatants were filtered sequentially through 0.45 and 0.20 μm diameter pore Acrodisc HT Tuffryn membranes (Pall Corporation, Ann Arbor, MI) and total RNA was extracted from the filtrates using TRIzol LS (Invitrogen, Carlsbad, CA) following manufacturer recommendations. The RNA concentration and quality were determined for each sample by the 260/280 nm ratio using a Nanodrop Spectrophotometer (ND-100).

The RT-PCR was performed using the Qiagen One-step kit (Qiagen Inc., Santa Clarita, CA). The reaction mixture consisted of 100 ng (5 μL) RNA, 5 μL 5X RT buffer, 1 μL dNTPs (10 mM), 1 μL each, forward and reverse primers (10 μM), 1 μL enzymes (from the Qiagen mix containing Omniscript reverse transcriptase, Sensiscript reverse transcriptase, and HotStarTaq DNA polymerase), 0.25 μL RNase inhibitor, and RNase free water to a total volume of 25 μL. Primers were designed to amplify a 362 nt region of the DENV-2 NS3 gene: sense DV1 (GGRACKTCAGGWTCTCC) and antisense DSP2 (CCG GTGTGCTCRGCYCTGAT).[35] Reverse transcription was performed at 52°C for 45 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 60 sec, and 68°C for 60 sec, with a final extension at 72°C for 10 min. All samples were analyzed by agarose gel electrophoresis followed by staining with ethidium-bromide and visualization using a UV transilluminator.

To increase sensitivity, RT-PCR products were further amplified by nested PCR.[26,36] The reaction mixture consisted of 10 μL 2X PCR master mix (Promega, Madison, WI), 1 μL (10 μM) each forward primer (5′ AATTGTCGACAGAAA AGGAAA), and reverse primer (5′ GGCTGGGGTTTGGT ATC), 3 μL from the completed RT-PCR and H_2O to 20 μL. The protocol was 94°C for 2 min, and then 30 cycles of 94°C for 30 sec, and 55°C for 1 min, with an extension at 72°C for 10 min. Products were analyzed as previously described.

DENV detection in mosquito pools by virus isolation. Mosquito pools were homogenized, centrifuged, and filtered as described previously for DENV RNA preparation and filtrates were titrated by plaque assay as described previously for DENV titration.

RESULTS

Detection of DENV in mosquito pools under ideal laboratory conditions. Mosquito pools were constructed using DENV-infected mosquitoes that had been processed using standard laboratory protocols. The DENV-infected mosquitoes at 1 to 21 days PI were pooled with uninfected mosquitoes (0, 9, 99, 499, or 999), and the resulting pools of 1 to 1,000 mosquitoes were assayed for infectious DENV by plaque titration, for DENV RNA by RT-PCR/nested PCR, and for DENV NS1 antigen using the Platelia Dengue NS1 Ag kit (Tables 1 and 2).

Infectious DENV was isolated from pools containing 1 infected mosquito in a total of 1 and 10 mosquitoes; virus titers in the pools ranged from 1.72 $\log_{10}$ PFU/mL to 4.72 $\log_{10}$ PFU/mL (Table 2). The virus titers of given sized pools increased from Day 1 to 7 PI and were highest in pools constructed with mosquitoes harvested at 7 and 14 days PI. The DENV was below the level of detection by plaque assay in pools with ≥ 100 mosquitoes, regardless of the days PI of the infected mosquito in the pool (Table 2).

Mosquito pools were assayed by RT-PCR/nested PCR for the presence of DENV RNA. Amplicons of 362 bp for RT-PCR and 332 bp for nested PCR were detected in pools containing 1–500 mosquitoes regardless of the days PI of the DENV-infected mosquitoes (Table 2). In contrast, DENV RNA was not detected by either RT-PCR or nested PCR in pools containing 1,000 mosquitoes.

The DENV NS1 antigen was detected both visually and spectrophotometrically using the Platelia Dengue NS1 Ag kit in all mosquito pools that had been prepared using ideal laboratory conditions (Table 2). The presence of NS1 was revealed by a yellow color, and the wells of the larger pools also contained a dark precipitate. The A_{450} values above 1 are considered to be positive and indicate the presence of NS1. The mean A_{450} values of pools containing a single infected mosquito, harvested from 7 to 21 days PI, were similar for pool sizes ranging from 1 to 500 mosquitoes. In contrast to detection by virus isolation or RT-PCR/nested PCR, DENV NS1 antigen also could be detected in pools of 1,000 mosquitoes containing a single DENV-infected mosquito harvested from 1 to 21 days PI (Table 2). In pools with 1,000 mosquitoes, compared with those with fewer mosquitoes, A_{450} values were lower but still above the cutoff threshold of 1. The mean A_{450} values of uninfected (negative control) pools ranged from 0.12 to 0.17. The range of A_{450} values in triplicate readings of both positive and negative pools was very small and consistent (Table 2).

Table 1

Summary of test sensitivities under the assayed conditions, including where DENV-infected mosquitoes were subjected to normal laboratory conditions versus drying (desiccation), freeze-thaw cycles, or a combination of drying and freeze-thaw cycles*

	Percentage of positive pools (no. positive/examined) in relation to the conditions to which DENV-infected mosquitoes contained in the pools were exposed				
Test method	Normal laboratory conditions	1–30 days of drying	1–5 freeze-thaw cycles	30 days of drying and 5 freeze-thaw cycles	Grand total for all conditions
Virus isolation	40% (8/20)	10% (5/48)	62% (15/24)	0% (0/3)	29% (28/95)
RT-PCR	75% (15/20)	75% (36/48)	92% (22/24)	75% (3/4)	79% (76/96)
Nested PCR	80% (16/20)	92% (44/48)	83% (20/24)	75% (3/4)	86% (83/96)
NS1 Ag kit	100% (20/20)	98% (47/48)	100% (24/24)	75% (3/4)	98% (94/96)

*These summary data include all tested pools from Tables 2–5.
DENV = dengue virus; RT-PCR = reverse transcription-polymerase chain reaction.

TABLE 2

Effects of mosquito pool size and time after intrathoracic injection (day PI) on detection of DENV by virus isolation, RNA detection, and NS1 antigen detection*

Pool size	Day PI	Titer (log_{10} PFU/mL)	RT-PCR	Nested PCR	Mean A_{450} (range)
1	1	1.94	positive	positive	5.18 (5.15–5.20)
1	7	4.34	positive	positive	6.35 (6.27–6.45)
1	14	4.72	positive	positive	6.17 (6.15–6.20)
1	21	3.54	positive	positive	6.08 (5.98–6.16)
1	Uninfected	negative	negative	negative	0.12 (0.12–0.12)
10	1	1.72	positive	positive	6.08 (5.92–6.08)
10	7	4.25	positive	positive	6.64 (6.27–7.35)
10	14	3.49	positive	positive	6.12 (6.07–6.20)
10	21	3.11	positive	positive	6.06 (6.06–6.06)
10	Uninfected	negative	negative	negative	0.15 (0.15–0.17)
100	1	ND	negative	positive	2.67 (2.24–2.98)
100	7	ND	positive	positive	6.25 (6.12–6.33)
100	14	ND	positive	positive	6.03 (6.02–6.06)
100	21	ND	positive	positive	6.17 (6.07–6.29)
100	Uninfected	negative	negative	negative	0.14 (0.14–0.14)
500	1	ND	positive	positive	3.61 (3.40–3.85)
500	7	ND	positive	positive	6.18 (6.11–6.23)
500	14	ND	positive	positive	6.01 (6.00–6.01)
500	21	ND	positive	positive	6.08 (5.97–6.17)
500	Uninfected	negative	negative	negative	0.17 (0.17–0.17)
1,000	1	ND	negative	negative	1.49 (1.24–1.49)
1,000	7	ND	negative	negative	3.07 (3.00–3.75)
1,000	14	ND	negative	negative	4.10 (4.09–4.10)
1,000	21	ND	negative	negative	6.65 (6.63–6.65)
1,000	Uninfected	negative	negative	negative	0.14 (0.14–0.14)

*Pools of 1 infected mosquito in the total pool size shown were assayed by virus titration, RT-PCR, nested PCR, and NS1 Ag detection for DENV infection.
DENV = dengue virus; PI = post-infection; PFU = plaque-forming units; RT-PCR = reverse transcription-polymerase chain reaction; ND = titer below level of detection; positive/negative = PCR product detected/not detected on gel.

All three tests (virus isolation, viral RNA detection by RT-PCR/nested PCR, and NS1 detection using the Platelia Dengue NS1 Ag kit) could be used to detect DENV in pools of 1 and 10 mosquitoes. False negative results were obtained in the virus isolation test for pools containing ≥ 100 mosquitoes and by RT-PCR/nested PCR for pools containing 1,000 mosquitoes. The Platelia Dengue NS1 Ag kit was the most sensitive test for detecting DENV in pools that contained infected specimens prepared under ideal laboratory protocols.

Detection of DENV in pools containing infected mosquitoes subjected to drying. To address the ability of the assays to detect DENV in mosquitoes in more field-relevant conditions, DENV-infected mosquitoes at 1, 7, 14, and 21 days PI were subjected to drying at room temperature for a further 1, 3, 14, or 30 days. The dried mosquitoes were used to construct pools of 1 infected in a total of 1, 10, and 100 mosquitoes, which were assayed for DENV by virus isolation, RT-PCR/nested PCR, and NS1 antigen (Tables 1 and 3).

Drying negatively affected DENV viability; only 10% (5 of 48) of the infected pools yielded virus isolates (Table 3). Virus isolation was most successful for small pool sizes and for a drying period not exceeding 3 days. However, the titers were low, ranging from 1.12 log_{10} PFU/mL to 1.94 log_{10} PFU/mL. Only a single virus isolate was made from a pool containing a mosquito at 14 days PI. Virus was not isolated from any pools containing DENV-infected mosquitoes at 1 or 21 days PI, or from any pools that contained DENV-infected mosquitoes that had dried for 14 or 30 days.

The DENV RNA could be detected in most of the infected pools using RT-PCR and nested PCR tests, with the nested PCR being more sensitive for detecting DENV RNA in pools constructed with DENV-infected mosquitoes at 1 day PI (Tables 1 and 3). Overall, the sensitivities were 92% (44 of 48) for nested PCR and 73% (35 of 48) for RT-PCR across drying conditions, due in part to false negative results in the RT-PCR test for pools constructed with mosquitoes at 1 day PI (Tables 1 and 3).

The DENV NS1 antigen was detected in almost every pool containing a DENV-infected mosquito, except for one pool with a total of 100 mosquitoes where the infected mosquito had been subjected to 30 days of drying (borderline value of 0.94; Table 3) and one other pool that was subjected to the most extreme circumstances (pool size of 1,000, with the infected mosquito dried for 30 days and subjected to 5 freeze-thaw cycles; Table 4). Only the mean A_{450} value is presented for each of the pools, because the range of values in triplicate samples varied minimally (see Table 2 for representative results). The A_{450} values for pools containing mosquitoes at 7–21 days PI were consistently high regardless of the number of days dried (Table 3). The A_{450} values for pools constructed with mosquitoes at 1 day PI declined with the time of drying, notably in pools with 100 mosquitoes. It is likely that minimal virus replication has occurred and thus only a small amount of NS1 antigen has been produced in infected mosquitoes by 1 day PI or that NS1 antigen detected at 1 day PI was injected with the virus inoculum. In either case, the amount of NS1 antigen may be near the threshold of detection.

TABLE 3

Effects of drying infected mosquitoes on detection of DENV in mosquito pools by virus isolation, RNA detection, and NS1 antigen detection*

No. of days dried	Pool size	Titer (log_{10} PFU/mL) by day PI					RT-PCR by day PI					Nested PCR by day PI					Mean A_{450} by day PI				
		Uninfected	1	7	14	21	Uninfected	1	7	14	21	Uninfected	1	7	14	21	Uninfected	1	7	14	21
1	1	neg	0	1.94	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	6.88	6.96	6.92	6.73
1	10	neg	0	1.64	0	0	neg	pos	pos	pos	pos	neg	pos	pos	neg	pos	neg	6.90	6.92	6.85	6.62
1	100	neg	0	0	0	0	neg	neg	neg	pos	pos	neg	pos	pos	pos	neg	neg	6.33	6.91	6.88	6.70
3	1	neg	0	1.64	1.94	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	2.19	4.44	4.55	4.54
3	10	neg	0	1.12	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	4.66	4.61	4.56	4.53
3	100	neg	0	0	0	0	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	neg	4.08	4.60	4.54	4.57
14	1	neg	0	0	0	0	neg	neg	pos	pos	pos	neg	pos	pos	pos	pos	neg	1.33	5.98	5.66	4.70
14	10	neg	0	0	0	0	neg	neg	neg	pos	pos	neg	pos	pos	pos	pos	neg	1.82	5.71	5.55	4.68
14	100	neg	0	0	0	0	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	neg	1.16	5.62	5.54	4.69
30	1	neg	0	0	0	0	neg	neg	pos	neg	pos	neg	pos	pos	neg	pos	neg	1.44	6.27	6.16	6.11
30	10	neg	0	0	0	0	neg	neg	pos	pos	pos	neg	neg	pos	pos	pos	neg	2.06	6.12	6.12	6.00
30	100	neg	0	0	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	0.94	6.17	6.08	6.06

*Mosquitoes at 1, 7, 14, or 21 days post-DENV infection were subjected to 1, 3, 14, or 30 days of drying at room temperature and pools of 1 infected mosquito in the total pool size shown were constructed and assayed by virus titration, RT-PCR/nested PCR or NS1 Ag detection for DENV infection.
DENV = dengue virus; PI = post-infection; PFU = plaque-forming units; RT-PCR = reverse transcription-polymerase chain reaction; pos = positive, neg = negative.

Table 4

Detection of infectious DENV, DENV RNA, or NS1 antigen in mosquito pools for which the infected mosquito had been subjected to both drying and freeze-thaw cycles*

Pool size	Titer ($\log_{10}$ PFU/mL)	RT-PCR/ Nested PCR	Mean A_{450}
1	ND	pos/pos	6.39
10	ND	pos/pos	6.37
100	ND	pos/pos	6.33
1,000	n/a	neg/neg	neg

*At 21 days post-DENV infection, mosquitoes were subjected to 30 days of drying at room temperature followed by 5 cycles of freezing and thawing. Pools of 1 infected and treated mosquito in the total pool size shown were constructed and assayed by virus titration, RT-PCR/nested PCR or NS1 Ag detection for DENV infection.

DENV = dengue virus; PFU = plaque-forming units; RT-PCR = reverse transcription-polymerase chain reaction; ND = titer below the level of detection; n/a = pool not assayed; pos = positive, neg = negative.

The Platelia Dengue NS1 Ag kit proved to be the most sensitive test for detecting DENV in mosquito pools in which the infected mosquitoes had been subjected to drying, as could occur in field surveillance programs.

Detection of DENV in pools containing DENV-infected mosquitoes subjected to repeated freezing and thawing. To address the ability of the assays to detect DENV in mosquitoes in another potentially field-relevant situation, DENV-infected mosquitoes at 1, 7, 14, and 21 days PI were subjected to 1 or 5 cycles of 1 hour of freezing and 1 hour of thawing. The treated mosquitoes were used to construct pools of 1, 10, or 100 mosquitoes, which were assayed for DENV by virus isolation, RT-PCR/nested PCR, and NS1 antigen detection (Tables 1 and 5).

The DENV was isolated from all pools (8 of 8) with a single infected mosquito subjected to 1 or 5 freeze-thaw cycles and from 88% of pools (7 of 8) with a total of 10 mosquitoes, the lone exception being a pool subjected to 5 freeze-thaw cycles and where the infected mosquito was harvested 21 days PI (Table 5). None of the pools containing 100 mosquitoes yielded a virus isolate. Overall, virus was isolated from 62% (15 of 24) of the pools containing DENV-infected mosquitoes. Virus titers were not reduced following multiple freeze-thaw cycles (Table 5).

The DENV RNA was detected in 96% (23 of 24) of mosquito pools by either RT-PCR or nested PCR following 1 or 5 freeze-thaw cycles (Table 5), showing the stability of DENV RNA in the mosquito during freezing and thawing.

The DENV NS1 antigen was detected by use of the Platelia Dengue NS1 Ag kit in every pool containing a DENV-infected mosquito (Table 5). Mean A_{450} values ranged from 4.60 to 6.78, all of which greatly exceeded the cutoff value. Multiple freeze-thaw cycles did not adversely affect NS1 antigen detectability.

Freezing and thawing samples had little effect on the ability to detect NS1 antigen in infected mosquito pools using the Platelia Dengue NS1 Ag kit or the ability to detect DENV RNA by RT-PCR/nested PCR. Similarly, there was little effect of freezing and thawing on DENV isolation when assaying mosquito pools of 1 or 10 mosquitoes (Table 5). The sensitivity of the Platelia Dengue NS1 Ag kit exceeded the sensitivity of virus isolation and slightly exceeded RT-PCR/nested PCR for detection of DENV in pools subjected to freeze-thaw cycles (Tables 1 and 5).

Detection of DENV in pools containing DENV-infected mosquitoes subjected to both drying and freezing-thawing. We also examined the ability of the assays to detect DENV in mosquitoes at 21 days PI that were subjected to a "worst case" field scenario of 5 cycles of freezing and thawing followed by 30 days of drying. The treated mosquitoes were used to construct pools of 1, 10, 100, and 1,000 mosquitoes, which were assayed for DENV by virus isolation, RT-PCR/nested PCR, and NS1 antigen ELISA (Tables 1 and 4). None of the pools yielded a virus isolate. In contrast, DENV RNA was detected by RT-PCR/nested PCR in pools containing up to 100 mosquitoes, but not in the one containing 1,000 mosquitoes. The DENV NS1 antigen was detected using the Platelia Dengue NS1 Ag kit (Table 4) in similar pool sizes. Interestingly, the NS1 assay A_{450} values did not differ from those obtained with pools constructed with mosquitoes subjected to either drying or freezing-thawing alone (Tables 3 and 5), revealing the stability of the NS1 analyte under simulated field conditions.

DISCUSSION

Our findings strongly suggest that NS1 antigen detection is a promising approach for DENV surveillance in vector mosquitoes and confirm previous studies in this regard. We were able to detect DENV NS1 antigen, by use of the Platelia Dengue NS1 Ag kit, in pools containing one infected mosquito and up to 999 uninfected mosquitoes, as well as in pools constructed with infected mosquitoes that had been subjected to field-relevant handling conditions including drying for up to 30 days and/or multiple cycles of freezing and thawing. Overall, detection of DENV infection by use of the Platelia Dengue NS1 Ag kit was more sensitive than RT-PCR and virus isolation in our laboratory and simulated field condition-pools of mosquitoes (Table 1). Virus isolation was the least sensitive test for detection of DENV in pools containing ≥ 100 mosquitoes and in pools that had been subjected to field relevant collecting and processing conditions. The overall sensitivity

Table 5

Effects of freezing and thawing infected mosquitoes on detection of DENV in mosquito pools by virus isolation, RNA detection, and NS1 antigen detection*

No. of freeze-thaw cycles	Pool size	Titer ($\log_{10}$ PFU/mL) by day PI					RT-PCR by day PI					Nested PCR by day PI					Mean A_{450} by day PI				
		Uninfected	1	7	14	21	Uninfected	1	7	14	21	Uninfected	1	7	14	21	Uninfected	1	7	14	21
1	1	neg	1.94	4.49	3.64	3.64	neg	pos	pos	pos	pos	neg	pos	pos	pos	neg	neg	6.75	6.65	6.63	6.54
1	10	neg	1.00	4.25	3.64	3.11	neg	neg	pos	pos	pos	neg	neg	pos	neg	pos	neg	6.66	6.65	6.60	6.51
1	100	neg	0	0	0	0	neg	pos	pos	pos	pos	neg	pos	pos	neg	pos	neg	6.78	6.57	6.58	6.40
5	1	neg	1.94	4.41	3.64	2.94	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	neg	4.60	6.64	6.63	6.56
5	10	neg	1.64	4.11	3.64	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	6.38	6.55	6.65	6.55
5	100	neg	0	0	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	4.94	6.57	6.60	6.56

*Mosquitoes at 1, 7, 14, or 21 days post-DENV infection were subjected to 1 or 5 freeze-thaw cycles and pools of 1 infected mosquito in the total pool size shown were constructed and assayed by virus titration, RT-PCR/nested PCR or NS1 Ag detection for DENV infection.

DENV = dengue virus; PI = post-infection; PFU = plaque-forming units; RT-PCR = reverse transcription-polymerase chain reaction; pos = positive, neg = negative.

of virus isolation for detection of DENV in the infected pools assayed was 29%. The RT-PCR and nested PCR assays yielded overall sensitivities of 79% and 86%, respectively. The overall sensitivity of the Platelia Dengue NS1 Ag kit ELISA was 98%; the only false negative results were obtained with one pool with an A_{450} value of 0.94, which is borderline positive, and one pool of 1,000 mosquitoes for which the infected mosquito had been subjected to 30 days of drying and 5 cycles of freezing-thawing (Tables 3 and 4). Detection of DENV NS1 antigen by use of the Platelia Dengue NS1 Ag kit was more efficacious than detection of DENV RNA by RT-PCR/nested PCR in very large pools and in pools that had been subjected to simulated field conditions, especially drying of mosquitoes (Table 3).

The false negative results with the RT-PCR/nested PCR tests may be attributable to problems in extracting the RNA analyte from homogenates of the concentrated mosquito suspensions in the larger pools and from dried tissues in mosquitoes subjected to drying. Similarly, virus isolation was compromised by large pool sizes (Tables 2, 3, and 5). There might have been proteases and/or RNases in the concentrated mosquito suspensions that could inactivate virus or degrade RNA. We included fetal bovine serum and HEPES buffer in the trituration diluent to inhibit trypsin and other protease activity and to preserve virus integrity, however we did not add RNase inhibitors during homogenization. Very importantly for mosquito-based arbovirus surveillance, sensitivity of NS1 antigen detection was not compromised in the larger pools (Tables 2, 3, and 5).

We infected the mosquitoes by intrathoracic inoculation. Although this had the advantage of providing mosquitoes of known infection status and virus titer, mosquitoes collected in the field will be infected orally and will likely exhibit variable virus titers and analyte accumulation caused by differences in time of extrinsic incubation, permissiveness to DENV infection and replication, and environmental conditions including temperature.[23,32,33] Viral tissue tropisms and load also may be different in intrathoracically infected mosquitoes as compared with orally infected mosquitoes and could affect the efficacy of the respective tests. However, the previous study by Tan and others,[28] which used Bio-Rad's Dengue NS1 Ag Strip test, showed that detection of DENV NS1 antigen was successful for both orally infected and field collected specimens. Interestingly, in our studies DENV NS1 could be detected using the Platelia NS1 Ag kit in nearly all of the pools that tested false negative by RT-PCR/nested PCR. The role of virus load in these results needs to be investigated in both laboratory and field studies.

We determined the sensitivity of the Platelia Dengue NS1 Ag tests using only DENV-2. Similar studies need to be conducted with DENV-1, -3, and -4. The monoclonal antibody used in the test has been shown to detect the NS1 protein of all 4 DENV serotypes, albeit with some differences in sensitivity.[37] Moreover, Tan and others[28] showed that all 4 DENV serotypes could be readily detected in infected mosquitoes using the Dengue NS1 Ag Strip test from the same manufacturer as the Platelia Dengue NS1 kit. Nonetheless, it would be prudent to determine the ability to detect DENV-1, -3, and -4 NS1 in mosquitoes subjected to the simulated field collection conditions by use of the Platelia Dengue NS1 Ag kit. In this regard, a new NS1 detection kit that differentiates DENV serotypes and that has excellent sensitivity and specificity has been developed using serotype-specific anti-NS1 monoclonal antibodies.[38,39] In future studies, we will determine the diagnostic efficacy of the Platelia Dengue NS1 Ag kit for surveillance for DENV in vector populations in dengue endemic areas and will address the issues of detection of the NS1 antigen in field-collected mosquitoes infected with different DENV serotypes.

In summary, our studies suggest that NS1 is an excellent analyte for DENV surveillance in mosquito vectors. Detection of DENV-infected mosquitoes with the Platelia Dengue NS1 Ag kit was more sensitive than either virus isolation or RT-PCR/nested PCR. Clearly, the Platelia Dengue NS1 Ag kit or other NS1 assays could be fruitfully applied to DENV surveillance in *Ae. aegypti* and could become a significant addition to the armamentarium of vector surveillance and control programs as previously suggested by Tan and others[28] and Muller and others[29] The Platelia Dengue NS1 Ag kit proved to be a simpler, more rapid, and less laborious test for detection of DENV-infected mosquitoes than either virus isolation or RT-PCR/nested PCR. Theoretically, the Platelia Dengue NS1 Ag kit could be more cost-effective than the other tests because up to 92,000 mosquitoes could be tested with a single plate using pools of 1,000. A cost analysis comparing the three types of tests would be most informative in this regard.[40] Of importance, the Platelia Dengue NS1 Ag kit could be readily and rapidly applied by public health agencies not versed in mosquito-based arbovirus surveillance in the face of emergence of DENV in new geographic areas such as in the southern United States or southern Europe.

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Authors' addresses: Natalia V. Voge, Irma Sánchez-Vargas, Carol D. Blair, Lars Eisen, and Barry J. Beaty, Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO, E-mails: Natalia.Voge@colostate.edu, Irma.Sanchez-Vargas@colostate.edu, Lars.Eisen@colostate.edu, Carol.Blair@colostate.edu, and Barry.Beaty@colostate.edu.

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