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Detection of North American Eastern and Western Equine Encephalitis Viruses by Nucleic Acid Amplification Assays

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We have developed nucleic acid sequence-based amplification (NASBA), standard reverse transcription PCR (RT-PCR), and TaqMan nucleic acid amplification assays for the rapid detection of North American eastern equine encephalitis (EEE) and western equine encephalitis (WEE) viral RNAs from samples collected in the field and clinical samples. The sensitivities of these assays have been compared to that of virus isolation. While all three types of nucleic acid amplification assays provide rapid detection of viral RNAs comparable to the isolation of viruses in Vero cells, the TaqMan assays for North American EEE and WEE viral RNAs are the most sensitive. We have shown these assays to be specific for North American EEE and WEE viral RNAs by testing geographically and temporally distinct strains of EEE and WEE viruses along with a battery of related and unrelated arthropodborne viruses. In addition, all three types of nucleic acid amplification assays have been used to detect North American EEE and WEE viral RNAs from mosquito and vertebrate tissue samples. The sensitivity, specificity, and rapidity of nucleic acid amplification demonstrate the usefulness of NASBA, standard RT-PCR, and TaqMan assays, in both research and diagnostic settings, to detect North American EEE and WEE viral RNAs.

Eastern equine encephalitis (EEE) and western equine encephalitis (WEE) viruses are arthropodborne viruses (arboviruses) that are associated with both human and equine encephalitis throughout the Americas (6, 14, 15). EEE and WEE viruses are members of the family *Togaviridae*, genus *Alphavirus* (23). Members of the genus *Alphavirus* have a spherical, enveloped virion of 60 to 65 nm in diameter and possess a single-stranded, positive-sense RNA genome of over 11,000 nucleotides in length. The natural transmission cycles of EEE and WEE viruses involve a variety of mosquito and avian species (6, 14, 15). Under unique ecological conditions, EEE and WEE viruses are transmitted from avian hosts to equines and humans, which are presumed to be dead-end hosts (6, 14, 15). Historically, in the United States, there has been a difference in the geographic distribution of these two antigenically and ecologically distinct viruses. The majority of EEE virus activity has occurred in the eastern United States within the geographic range of *Culiseta melanura*, the primary mosquito vector of North American EEE virus (6, 14). The majority of WEE virus activity has occurred in the western United States, where *Culex tarsalis* is the primary mosquito vector of WEE virus (6, 15). It is also important to note the distinction between the two major antigenic groups of EEE virus; the North American group includes isolates of EEE virus from the United States, Canada, and the Caribbean, while the South American group includes isolates of EEE virus from Central and South America (1, 2, 3, 13, 14, 16, 22). North American strains of EEE virus are considered to be more virulent than South American strains of EEE virus, which are rarely associated with human illness (18).

Surveillance for the presence of North American EEE and WEE viruses in vector mosquitoes is used to assess the risk of epizootic and epidemic activity. Public health efforts include mosquito control programs and education campaigns that can be implemented to decrease the likelihood of virus transmission to vulnerable vertebrate hosts. The timing of these interventions is critical, requiring the use of rapid diagnostic assays for surveillance. Traditionally, the identification of alphaviruses from mosquitoes and vertebrate tissues has been achieved by inoculation of cell culture or suckling mouse brain followed by identification of an isolate by immunofluorescence assays. However, while these methods are reliable, they are also time-consuming and cannot be used in laboratories that do not have cell culture or animal use capabilities.

Nucleic acid amplification assays are ideally suited for surveillance as highly sensitive, time-efficient alternatives to standard methods of detecting virus (5, 8, 9, 10). In particular, the introduction of novel nucleic acid amplification assays, NASBA and TaqMan, as rapid, powerful diagnostic tools has provided a platform for the development of additional assays for the detection of North American EEE and WEE viral RNAs (4, 7, 9, 10, 11, 12, 17, 20).

We present the development and application of NASBA, standard reverse transcription (RT)-PCR, and TaqMan nucleic acid amplification assays designed to detect North American EEE and WEE viral RNAs from a variety of vertebrate and mosquito samples. The sensitivities and specificities of these assays are compared. While all three types of nucleic acid amplification assays have been found to be specific for target RNAs, the TaqMan assays for the detection of North Ameri-

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TABLE 1. Oligonucleotide primers and probes for NASBA,[a] standard RT-PCR, and TaqMan assays

Primer	Coding location	Sequence (5′–3′)	Product size (bp)
EEE NASBA			
EEE 9597	9597–9616	*gatgcaaggtcgcatatgag*CACATGGATGGCCGCACGAA	208
EEE 9804c	9804–9783	*aattctaatacgactcactatagggagaagg*CAGCAAAGTAACGCCAGGAGTA	
EEE 9619probe	9619–9643	GGTAGTCTATTACTACAACAGATAC	
EEE RT-PCR			
EEE 5640	5640–5659	CGGCAGCGGAATTTGACGAG	433
EEE 6072C	6047–6072	ACTTTGACGGCCACTTCTGCTGATGA	
EEE TaqMan			
EEE 9391	9391–9411	ACACCGCACCCTGATTTTACA	69
EEE 9459c	9459–9439	CTTCCAAGTGACCTGGTCGTC	
EEE 9414probe	9414–9434	TGCACCCGGACCATCCGACCT	
WEE NASBA			
WEE 9336	9336–9356	*gatgcaaggtcgcatatgag*CGAGCAGACGCAACAGCAGAA	233
WEE 9566C	9566–9545	*aattctaatacgactcactatagggagaagg*CAGGATAGCAAGAGCGACACCA	
WEE 9390probe	9390–9414	GTGGGGCGAGAAGGGCTGGAGTACG	
WEE RT-PCR			
WEE 5100	5100–5122	GTTTGGCGGCGTCTCGTTCTCTA	338
WEE 5437c	5437–5414	TCCGTGGTGCTGGTACTGGTCTGT	
WEE TaqMan			
WEE 10,248	10,248–10,267	CTGAAAGTCGGCCTGCGTAT	67
WEE 10,314c	10,314–10,295	CGCCATTGACGAACGTATCC	
WEE 10,271probe	10,271–10,293	ATACGGCAATACCACCGCGCACC	

[a] NASBA forward primers have 5′ECL sequences (lowercase, italic); reverse primers have T7 promoter sequences (lowercase, italic).

can EEE and WEE viral RNAs are the most sensitive. Additionally, the NASBA and TaqMan assays are the most rapid of the assays compared, providing results in less than 4 h.

MATERIALS AND METHODS

Viruses. Viruses were obtained from the Arbovirus Diseases Branch of the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC). The prototype EEE virus strain NJ/60 and WEE virus strain Fleming were titrated in Vero cells by plaque assay.

Field-collected and clinical samples. Mosquito pools and avian tissues were collected and tested as a part of arboviral surveillance programs at both state and federal levels. Equine and human tissues were submitted to the CDC for confirmation of EEE virus infection previously inferred by clinical history and diagnostic testing performed at another laboratory. Mosquito pools were processed as previously described (9). Avian, equine, and human tissues were homogenized in Ten Broeck grinders with 1 ml of BA-1 diluent. Mosquito pool and vertebrate tissue homogenates were clarified by centrifugation at 20,000 × *g* for 3 min; the resultant supernatants were subjected to RNA extraction.

RNA extraction. Viral RNA was extracted from virus seed, as well as from mosquito pool and vertebrate tissue homogenate supernatants processed as described above by using the QIAamp viral RNA minikit, following the manufacturer's instructions (QIAGEN, Valencia, Calif.). A minimum of two aliquots of BA-1 diluent were processed as negative extraction controls along with each group of samples subjected to RNA extraction. Extractions were performed with samples ranging in volume from 70 to 140 μl. RNA was eluted in a volume equal to the volume of starting sample. Eluted RNA was stored at −70°C until used.

NASBA assay. As previously described, NASBA assays were performed with 5 μl of RNA and 50 pmol of each primer (Table 1) by using the NucliSens basic kit (bioMerieux, Durham, N.C.) with the enhanced chemiluminescence (ECL) detection format; samples were read, and positive results were determined by the Nuclisens reader (bioMerieux) (10). Two negative amplification controls were included with each group of samples processed. In the negative amplification control reactions, 5 μl of RNase- and DNase-free water was substituted for 5 μl of extracted RNA.

Standard RT-PCR. Standard RT-PCR assays were performed with 5 μl of RNA and 50 pmol of each primer (Table 1) in a 50-μl total reaction volume by using the Titan one-tube RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, Ind.), following the manufacturer's protocol. The following cycling conditions were used: 1 cycle of 45°C for 1 h and 94°C for 3 min and 45 cycles of 94°C for 30 s, 55°C for 1 min, and 68°C for 3 min. After amplification, a 5-μl volume of RT-PCR product was analyzed by gel electrophoresis with 3.5% NuSieve 3:1 agarose (FMC Bioproducts, Rockland, Maine). DNA was visualized by ethidium bromide staining. Two negative amplification controls were included with each group of samples processed. In the negative amplification control reactions, 5 μl of RNase- and DNase-free water was substituted for 5 μl of extracted RNA.

TaqMan RT-PCR. TaqMan assays were performed with 5 μl of RNA, 50 pmol of each primer (Table 1), and 10 pmol of probe in a total volume of 50 μl by using the TaqMan RT-PCR ready mix kit (PE Applied Biosystems, Foster City, Calif.), according to the manufacturer's instructions. Amplification and fluorescence detection were performed with the ABI Prism 7700 sequence detection system instrument (PE Applied Biosystems); 45 cycles of amplification were performed according to the manufacturer's recommendations for TaqMan RT-PCR cycling conditions. Positive results were determined according to the amplification cycle at which fluorescence increased above the threshold value (C_T; fixed at 0.1) along with the relative change in fluorescence (Rn), determined by performing a plate read function at the end of amplification. As previously described, a sample was determined to be positive if the C_T value was ≤37 and the Rn value was two or more times the average of eight negative wells (9). A sample was determined to be equivocal if it met one of the two above criteria for positivity. Eight negative amplification controls were included with each group of samples processed. In the negative amplification control reactions, 5 μl of RNase- and DNase-free water was substituted for 5 μl of extracted RNA.

Primer design. All EEE and WEE virus primers and/or probes were designed by using the published sequences of the North American EEE virus strain 82V2137 (GenBank accession number U01034) and the WEE virus strain 71V_1658 (GenBank accession number AF214040). The EEE and WEE virus standard RT-PCR primers were designed by using the PrimerSelect software program (DNASTAR Inc., Madison, Wis.). The EEE and WEE virus TaqMan primers and probes were designed with the PrimerExpress software package (PE Applied Biosystems). The PrimerExpress-derived TaqMan primer pairs and probes were compared to an alignment of multiple EEE and WEE virus sequences, and primer pairs and probes that demonstrated maximum homology to all virus strains (Table 1) were selected. The EEE and WEE virus TaqMan

TABLE 2. Sensitivities and specificities of North American EEE virus NASBA, standard RT-PCR, TaqMan, and Vero cell culture assays[a]

Sample	Quantity (PFU)	NASBA[c]		Standard RT-PCR	TaqMan[d]	
		ECL units	Int.		C_T	Int.
Titrated EEE NJ/60 seed (New Jersey 1960)						
EEE-1	770,000	1,086,839	POS	POS	11.7	POS
EEE-2	77,000	1,004,872	POS	POS	14.4	POS
EEE-3	7,700	918,511	POS	POS	17.7	POS
EEE-4	770	87,634	POS	POS	20.8	POS
EEE-5	77	647,862	POS	POS	23.8	POS
EEE-6	7.7	543,737	POS	POS	26.3	POS
EEE-7	0.77	550,841	POS	POS	30.2	POS
EEE-8	0.077	721,557	POS	EQUIV[e]	33.7	POS
EEE-9	0.0077	146	NEG	NEG	37.2	EQUIV
EEE-10	0.00077	163	NEG	NEG	45	NEG
EEE virus strains						
EEE Michigan 1989	ND	255,654	POS	POS	14.9	POS
EEE New Jersey 1980	ND	10,894	POS	POS	14.3	POS
EEE Texas 1989	ND	91,898	POS	POS	15.1	POS
EEE Dominican Republic 1987	ND	254,396	POS	POS	14.5	POS
EEE Delaware 2001	ND	109,511	POS	POS	13.4	POS
EEE Virginia 2001 a	ND	107,749	POS	POS	14	POS
EEE Virginia 2001 b	ND	124,663	POS	POS	15.8	POS
EEE Virginia 2001 c	ND	113,366	POS	POS	14	POS
EEE Virginia 2001 d	ND	115,768	POS	POS	15.1	POS
EEE Virginia 2001 e	ND	53,367	POS	POS	16.2	POS
EEE Ecquador 1974[b]	ND	546	POS	NEG	43.3	NEG
EEE Brazil 1956[b]	ND	156	NEG	NEG	45	NEG
EEE Brazil 1976[b]	ND	169	NEG	NEG	45	NEG
EEE Panama 1981[b]	ND	167	NEG	NEG	45	NEG
Other viruses						
WEE McMillan	ND	147	NEG	NEG	45	NEG
Highlands J B230	ND	171	NEG	NEG	45	NEG
VEE TC83	ND	142	NEG	NEG	45	NEG
LaCrosse Original	ND	147	NEG	NEG	45	NEG
Dengue-2 New Guinea	ND	126	NEG	NEG	45	NEG
Powassan LB	ND	135	NEG	NEG	45	NEG
Yellow Fever 17-D	ND	135	NEG	NEG	45	NEG

[a] Abbreviations: POS, positive; NEG, negative; EQUIV, equivocal; ND, not determined; Int., interpretation.
[b] EEE South American antigenic group strain.
[c] NASBA ECL units of >300 are interpreted as positive.
[d] TaqMan C_T values of ≤37 are interpreted as positive (Rn values not included).
[e] Equivocal standard RT-PCR result determined by the presence of a faint DNA band.

probes were 5′ labeled with the FAM reporter dye and labeled at the 3′ end with the quencher molecule BHQ1. EEE and WEE virus NASBA primers and probes were designed by following the primer design guidelines described in the Nuclisens basic kit application manual (bioMerieux). The NASBA reverse primers incorporate the T7 promoter sequence at the 5′ end of the primer, and the forward primers contain a generic capture sequence complementary to the ruthenium-labeled detection probe (generic ECL probe) at the 5′ end of the primer (Table 1). The NASBA-ECL virus-specific capture probes were 5′ biotin labeled and immobilized onto avidin-coated magnetic particles by following the protocol described in the Nuclisens basic kit application manual.

Vero cell culture. Supernatants taken in 100-μl volumes from field-collected and clinical sample homogenates were inoculated onto Vero cells grown in 25-cm^2 flasks. Inoculated flasks were incubated at 37°C, 5% CO_2 for 10 days and reviewed for signs of cytopathic effect daily. Positive results were determined by the presence of cytopathic effect and were confirmed by immunofluorescence or TaqMan assays.

RESULTS

Comparison of NASBA, standard RT-PCR, and TaqMan assay sensitivities. To evaluate the sensitivities of NASBA, standard RT-PCR, and TaqMan assays, RNAs extracted from dilutions of previously titrated EEE virus strain NJ/60 and WEE virus strain Fleming were tested. The standard RT-PCR assay for the detection of North American EEE viral RNA detected <1 PFU of virus, whereas the NASBA and TaqMan assays were 10-fold more sensitive, detecting <0.1 PFU of virus (Table 2). The standard RT-PCR assay for the detection of WEE viral RNA detected <100 PFU of virus, whereas the NASBA assay was 10-fold more sensitive, detecting <10 PFU of virus (Table 3). The TaqMan assay for the detection of WEE viral RNA was 100-fold more sensitive than the NASBA assay and 1,000-fold more sensitive than the standard RT-PCR assay, detecting <0.1 PFU of WEE virus (Table 3).

Comparison of NASBA, standard RT-PCR, and TaqMan assay specificities. To evaluate the specificities of the North American EEE virus NASBA, standard RT-PCR, and TaqMan assays, 14 isolates of EEE virus including representatives of both North American and South American antigenic groups were tested (Table 2). We also tested related alphaviruses (Highlands J, Venezuelan equine encephalitis [VEE], and WEE

TABLE 3. Sensitivities and specificities of WEE virus NASBA, standard RT-PCR, TaqMan, and Vero cell culture assays[a]

Sample (location and of collection)	Quantity (PFU)	NASBA[b]		Standard RT-PCR	TaqMan[c]	
		ECL units	Int.		C_T	Int.
Titrated WEE Fleming seed (California, unknown)						
WEE-1	3,500,000	1,387,131	POS	POS	9	POS
WEE-2	350,000	1,309,269	POS	POS	12.1	POS
WEE-3	35,000	1,259,877	POS	POS	15.7	POS
WEE-4	3,500	1,403,760	POS	POS	19.1	POS
WEE-5	350	1,023,508	POS	POS	22.5	POS
WEE-6	35	874,991	POS	POS	26.4	POS
WEE-7	3.5	575	POS	EQUIV[d]	29.7	POS
WEE-8	0.35	174	NEG	EQUIV[d]	32.3	POS
WEE-9	0.035	119	NEG	NEG	36.9	POS
WEE-10	0.0035	106	NEG	NEG	45	NEG
WEE virus strains						
WEE (Cuba, 1971)	ND	2,046,014	POS	POS	9.2	POS
WEE (Tex., 1973)	ND	2,072,176	POS	POS	9.4	POS
WEE (Argentina, 1982)	ND	1,089,793	POS	POS	29.5	POS
WEE (S.D., 1983)	ND	1,244,439	POS	POS	10.7	POS
WEE (N.Mex., 1984)	ND	2,258,732	POS	POS	14.1	POS
WEE (Tenn., 1987)	ND	1,814,717	POS	POS	16.7	POS
WEE (Canada, 1941)	ND	1,629,668	POS	POS	9.3	POS
WEE (Brazil, 1966)	ND	2,318,310	POS	POS	14.6	POS
WEE (Neb., 1997a)	ND	1,989,971	POS	POS	14	POS
WEE (Neb., 1997b)	ND	2,101,613	POS	POS	15	POS
WEE (Neb., 1997c)	ND	1,960,286	POS	POS	12	POS
WEE (Neb., 1997d)	ND	833,799	POS	POS	21	POS
Other viruses						
EEE NJ/60	ND	95	NEG	NEG	45	NEG
Whataroa M78	ND	117	NEG	NEG	45	NEG
Kyzylgach LIEV65A	ND	106	NEG	NEG	45	NEG
Fort Morgan CM4-146(A)	ND	150	NEG	NEG	45	NEG
Highlands J B230	ND	85	NEG	NEG	45	NEG
Buggy Creek 81V1822	ND	86	NEG	NEG	45	NEG
Aura Bear 10135	ND	140	NEG	NEG	45	NEG
VEE TC83	ND	97	NEG	NEG	45	NEG
LaCrosse Original	ND	128	NEG	NEG	45	NEG
Dengue-2 New Guinea	ND	104	NEG	NEG	45	NEG
Powassan LB	ND	35	NEG	NEG	45	NEG
Yellow Fever 17-D	ND	118	NEG	NEG	45	NEG

[a] Abbreviations: POS, positive; NEG, negative; EQUIV, equivocal; ND, not determined; Int., interpretation.
[b] NASBA-ECL units of >300 are interpreted as positive.
[c] TaqMan C_T values of ≤37 are interpreted as positive (Rn values not included).
[d] Equivocal standard RT-PCR result determined by the presence of a faint DNA band.

viruses) and unrelated arboviruses that circulate throughout the Americas (La Crosse, dengue 2, Powassan, and yellow fever viruses) (Table 2). The NASBA, standard RT-PCR, and TaqMan assays detected all of the North American EEE virus isolates tested (Table 2). These assays were specific for North American EEE virus and did not detect South American EEE virus, related alphaviral, or unrelated arboviral RNAs (with the exception of the NASBA assay detection of one South American EEE virus strain, Ecuador 1974) (Table 2).

To evaluate the specificities of the WEE virus NASBA, standard RT-PCR, and TaqMan assays, we tested 12 geographically and temporally distinct strains of WEE virus (Table 3). As with specificity testing of the North American EEE viral RNA assays, a battery of related alphaviruses, including six members of the WEE virus serocomplex (Aura, Whataroa, Kyzylgach, Fort Morgan, Highlands J, Buggy Creek), VEE and EEE viruses and unrelated arboviruses were tested (Table 3). The NASBA, standard RT-PCR, and TaqMan assays detected all WEE virus strains tested (Table 3). The assays were specific for WEE viral RNA and detected none of the related alphaviral or unrelated arboviral RNAs tested (Table 3).

Detection of North American EEE viral RNA in field-collected mosquitoes and vertebrate tissues. A panel of 20 field-collected, veterinary, and human diagnostic samples were tested by isolation of virus in Vero cell culture as well as by NASBA, standard RT-PCR, and TaqMan assays for the presence of North American EEE viral RNA (Table 4). Virus was isolated from 4 of the 20 samples. The standard RT-PCR assay detected North American EEE viral RNA from the four Vero cell culture-positive samples (Table 4). The TaqMan assay detected viral RNA from 9 of the 20 samples tested, including the 4 Vero cell culture-positive samples (Table 4). The NASBA assay yielded positive results from 7 of the 20 samples,

TABLE 4. Detection of North American EEE virus and viral RNA from field-collected mosquitoes and vertebrate tissues by Vero cell culture, NASBA, standard RT-PCR, and TaqMan assays[a]

Sample (location and yr of collection)	Vero cell culture result	NASBA[b]		Standard RT-PCR	TaqMan[c]		EEE virus infection inferred by[e]:
		ECL units	Int.		C_T	Int.	
Field-collected mosquitoes							
Mosquito pool 1 (Va., 2001)	POS	107,036	POS	POS	23.2	POS	TaqMan
Mosquito pool 2 (Va., 2001)	POS	83,055	POS	POS	24.1	POS	TaqMan
Mosquito pool 3 (Va., 2001)	POS	32,274	POS	POS	25.3	POS	TaqMan
Mosquito pool 4 (Va., 2001)	POS	127,581	POS	POS	24.2	POS	TaqMan
Mosquito pool 5 (Va., 2001)	NEG	33,117	POS	NEG	34.8	POS	TaqMan
Mosquito pool 6 (N.J., 2000)	NEG	107	NEG	NEG	45	NEG	NT
Mosquito pool 7 (N.J., 2000)	NEG	103	NEG	NEG	45	NEG	NT
Mosquito pool 8 (N.J., 2000)	NEG	175	NEG	NEG	45	NEG	NT
Mosquito pool 9 (N.J., 2000)	NEG	187	NEG	NEG	45	NEG	NT
Mosquito pool 10 (N.J., 2000)	NEG	164	NEG	NEG	45	NEG	NT
Vertebrate tissues							
Equine cortex (N.C., 2000)	NEG	79,899	POS	EQUIV[d]	31.3	POS	Histopathology
Equine cerebellum (N.C., 2000)	NEG	56,367	POS	NEG	37.6	EQUIV	Histopathology
Equine brain stem (N.C., 2000)	NEG	200	NEG	EQUIV[d]	30.2	POS	Histopathology
Avian heart (Maine, 2001)	NEG	233	NEG	EQUIV[d]	32.4	POS	Isolation; FA
Human brain (Mich., 2001)	NEG	230	NEG	NEG	32.8	POS	Serology
Avian brain 1 (N.J., 2000)	NEG	113	NEG	NEG	45	NEG	NT
Avian brain 2 (N.J., 2000)	NEG	156	NEG	NEG	45	NEG	NT
Avian brain 3 (N.J., 2000)	NEG	140	NEG	NEG	45	NEG	NT
Avian brain 4 (N.J., 2000)	NEG	137	NEG	NEG	45	NEG	NT
Avian brain 5 (N.J., 2000)	NEG	127	NEG	NEG	45	NEG	NT

[a] Abbreviations: POS, positive; NEG, negative; EQUIV, equivocal; NT, not tested.
[b] NASBA-ECL units of >300 are interpreted as positive.
[c] TaqMan C_T values of ≤37 are interpreted as positive (Rn values not included).
[d] Equivocal standard RT-PCR result determined by the presence of a faint DNA band.
[e] Sample submitted to CDC molecular diagnostic laboratory for confirmation of EEE virus infection inferred by testing at another laboratory.

including the 4 samples from which North American EEE virus was isolated (Table 4).

Detection of WEE viral RNA in field-collected mosquitoes and vertebrate tissues. A panel of 13 field-collected mosquito pool and avian tissue samples were tested by isolation of virus in Vero cell culture as well as by NASBA, standard RT-PCR, and TaqMan assays for the presence of WEE viral RNA (Table 5). All of the samples tested were negative by isolation of virus by Vero cell culture. The standard RT-PCR and TaqMan assays detected WEE viral RNA from 3 of the 13 samples

TABLE 5. Detection of WEE virus and viral RNA from field-collected mosquitoes and vertebrate tissues by Vero cell culture, NASBA, standard RT-PCR, and TaqMan assays[a]

Sample (location and yr of collection)	Vero cell culture result	NASBA[b]		Standard RT-PCR	TaqMan[c]		Wee virus infection inferred by[d]:
		ECL units	Int.		C_T	Int.	
Field-collected mosquitoes							
Mosquito pool 1 (N.Mex., 2001)	ND	4,733	POS	POS	34	POS	Standard RT-PCR
Mosquito pool 2 (N.Mex., 2001)	NEG	51,943	POS	POS	29.7	POS	Standard RT-PCR
Mosquito pool 3 (N.Mex., 2001)	NEG	244	NEG	POS	30	POS	Standard RT-PCR
Mosquito pool 4 (N.J., 2000)	NEG	79	NEG	NEG	45	NEG	NT
Mosquito pool 5 (N.J., 2000)	NEG	177	NEG	NEG	45	NEG	NT
Mosquito pool 6 (N.J., 2000)	NEG	149	NEG	NEG	45	NEG	NT
Mosquito pool 7 (N.J., 2000)	NEG	122	NEG	NEG	45	NEG	NT
Mosquito pool 8 (N.J., 2000)	NEG	131	NEG	NEG	45	NEG	NT
Vertebrate tissues							
Avian brain 1 (N.J., 2000)	NEG	186	NEG	NEG	45	NEG	NT
Avian brain 2 (N.J., 2000)	NEG	121	NEG	NEG	45	NEG	NT
Avian brain 3 (N.J., 2000)	NEG	137	NEG	NEG	45	NEG	NT
Avian brain 4 (N.J., 2000)	NEG	135	NEG	NEG	45	NEG	NT
Avian brain 5 (N.J., 2000)	NEG	87	NEG	NEG	45	NEG	NT

[a] Abbreviations: POS, positive; NEG, negative; ND, not determined; NT, not tested.
[b] NASBA-ECL units of >300 are interpreted as positive.
[c] TaqMan C_T values of ≤37 are interpreted as positive (Rn values not included).
[d] Sample submitted to CDC molecular diagnostic laboratory for confirmation of WEE virus infection inferred by testing at another laboratory.

tested, and the NASBA assay detected WEE viral RNA from 2 of the 13 samples tested.

DISCUSSION

This report describes the development and application of NASBA, standard RT-PCR, and TaqMan assays for the detection of North American EEE and WEE viral RNAs. These assays offer sensitivities of detection similar to that of the isolation of viruses in Vero cells, with the TaqMan assays providing the most sensitive detection of <0.1 PFU of North American EEE virus strain NJ/60 and WEE virus strain Fleming (Tables 2 and 3). These assays demonstrated a high level of fidelity for target RNAs; no related or unrelated arboviral RNAs were detected (with the exception of EEE virus NASBA assay detection of one South American EEE virus strain, Ecuador 1974) (Tables 2 and 3).

In the testing of field-collected mosquitoes, the NASBA and TaqMan assays for the detection of North American EEE virus have been shown to be highly sensitive, detecting viral RNA from five mosquito pool samples, four of which were Vero cell culture positive (Table 4). Additionally, the standard RT-PCR assay for the detection of North American EEE virus detected viral RNA from the four mosquito pool samples from which virus was isolated (Table 4). Of the WEE viral RNA assays, the standard RT-PCR and TaqMan systems detected WEE viral RNA from three mosquito pool samples; the NASBA assay was shown to be less sensitive, detecting viral RNA from two of the three standard RT-PCR- and TaqMan-positive pools (Table 5). All of the North American EEE and WEE virus nucleic acid amplification assays compared have been shown to have sensitivities of detection in mosquito pools similar to or greater than that of the isolation of virus in Vero cells. These data indicate the utility of NASBA, standard RT-PCR, and TaqMan assays for the surveillance of North American EEE and WEE viruses.

With respect to the detection of viral RNA from avian, equine, and human tissues, the TaqMan assay has demonstrated exceptional sensitivity for the detection of North American EEE viral RNA, yielding positive results from 4 of the 10 vertebrate tissues tested, none of which were Vero cell culture positive (Table 4). These data support the use of the TaqMan assay for the detection of North American EEE viral RNA from veterinary and human clinical samples, as well as from mosquito pools. As WEE virus-infected vertebrate tissues were not available, the WEE virus assays compared here could not be applied to veterinary or human clinical samples. However, the ability of the WEE virus TaqMan assay to detect <0.1 PFU of WEE Fleming virus, combined with its ability to detect viral RNA from field-collected mosquito pools, has demonstrated a level of sensitivity conducive to diagnostic application (Tables 3 and 5). Additionally, the ability of the North American EEE virus TaqMan assay to detect viral RNA from vertebrate tissue samples suggests that the WEE virus TaqMan assay will also be useful with similar tissues (Table 4).

The North American EEE virus nucleic acid amplification assays are generally more sensitive than the WEE virus assays (Tables 2 and 3). We have considered several potential explanations for this discrepancy. (i) The North American EEE virus assays' primers could be more sensitive than the WEE virus assays' primers. When alternative North American EEE and WEE virus NASBA, standard RT-PCR, and TaqMan primer-probe set sensitivities were tested, results similar to those presented here were obtained (data not included). This reduces the likelihood that primer design contributes to the difference in sensitivities. (ii) A difference in the secondary structures of EEE and WEE viral RNAs could cause a difference in primer binding reflected in the reduced sensitivities of the WEE virus assays. At the beginning of amplification, the NASBA assays for the detection of EEE and WEE viral RNAs incorporate an incubation step at 65°C for 5 min. During this step, the secondary structure of RNA becomes denatured, reducing potential primer binding inhibition. If WEE viral RNA secondary structure was the cause of the reduced sensitivity, it would be expected that the WEE virus NASBA assay be more comparable in sensitivity to the EEE virus NASBA and significantly more sensitive than the WEE virus standard RT-PCR and TaqMan assays, because of secondary structure denaturation. In fact, the WEE virus NASBA assay data does not indicate enhanced sensitivity when compared to the EEE virus NASBA and WEE virus standard RT-PCR and TaqMan assays (Tables 2 and 3). These findings contradict the idea that a difference in the secondary structures of EEE virus and WEE viral RNAs is responsible for the differences in sensitivities between the EEE and WEE virus assays. (iii) There could be a difference in the ratio of noninfectious to infectious viral particles for these two viruses in the Vero cell culture system. If the EEE virus strain NJ/60 has a greater ratio of noninfectious to infectious viral particles than the WEE virus strain Fleming, the North American EEE virus assays would appear to be more sensitive than the WEE virus assays when compared to standard plaque assay, due to the ability of nucleic acid amplification assays to detect both noninfectious and infectious viral particles. We have not performed experiments to determine the difference in noninfectious and infectious viral particles for these two viruses; however, these findings warrant further investigation. As nucleic acid amplification technologies become more routinely used for viral RNA detection, we suspect that differences in the sensitivities of assays contingent upon the type of viral RNAs being detected will become more evident when compared to live virus culture systems.

We have developed the North American EEE and WEE virus nucleic acid amplification assays to expedite mosquito control and public health interventions in response to an outbreak of EEE or WEE viruses, as well as to shorten the processing time for veterinary and human diagnostic sample results. One advantage of nucleic acid amplification is the rapid, high-throughput capability that these assays provide, particularly when coupled with an automated RNA extraction system (19). As mentioned previously, the NASBA and TaqMan assays are the most rapid of the assays compared, providing results in less than 4 h. Additionally, the standard RT-PCR assays offer a turnaround time of less than 8 h. In contrast, traditional methods of detecting alphaviruses by cell culture or mouse inoculation followed by immunofluorescence assays can take up to, and in excess of, 1 week to complete. Also, the kit-based format of the NASBA, standard RT-PCR, and TaqMan assays allows for ease of standardization from lab to lab, which is a key component of effective surveillance and diagnosis. In terms of assay reproducibility, we have found that the

NASBA, standard RT-PCR, and TaqMan assays provided consistent sensitivities when multiple preparations of EEE and WEE viral dilutions were tested (data not included). A newly elucidated advantage of nucleic acid amplification assays has been demonstrated in recent studies indicating that viral RNA can be detected in the absence of cold chain handling when Vero cell plaque assay fails to recover live virus (8, 21). These findings contribute to the importance of the use of North American EEE and WEE virus NASBA, standard RT-PCR, and TaqMan assays in situations where sample integrity may have been compromised, rendering diagnostic techniques that require live virus recovery no longer useful. It is a concern that nucleic acid amplification assay-positive and live virus assay-negative results could be the product of laboratory contamination rather than evidence of infection. In this study, significant measures including the use of negative controls at the RNA extraction and amplification levels of sample processing as well as a physical separation between pre- and postamplification steps were used to confirm the reliability of such results.

This study has demonstrated that while the NASBA and standard RT-PCR assays have been shown to be sufficiently rapid, specific, and sensitive in their detection of North American EEE and WEE viral RNAs, the TaqMan assays for the detection of North American EEE and WEE viral RNAs provide unparalleled sensitivities. These findings support the use of these TaqMan assays not only for surveillance of virus in field-collected samples, but also for the detection of North American EEE and WEE viral RNAs in veterinary and human diagnostic samples.

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