

Nucleic Acid Sequence-Based Amplification Assays for Rapid Detection of West Nile and St. Louis Encephalitis Viruses

ROBERT S. LANCIOTTI* AND AMY J. KERST

Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Fort Collins, Colorado

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The development and application of nucleic acid sequence-based amplification (NASBA) assays for the detection of West Nile (WN) and St. Louis encephalitis (SLE) viruses are reported. Two unique detection formats were developed for the NASBA assays: a postamplification detection step with a virus-specific internal capture probe and electrochemiluminescence (NASBA-ECL assay) and a real-time assay with 6-carboxyfluorescein-labeled virus-specific molecular beacon probes (NASBA-beacon assay). The sensitivities and specificities of these NASBA assays were compared to those of a newly described standard reverse transcription (RT)-PCR and TaqMan assays for SLE virus and to a previously published TaqMan assay for WN virus. The NASBA assays demonstrated exceptional sensitivities and specificities compared to those of virus isolation, the TaqMan assays, and standard RT-PCR, with the NASBA-beacon assay yielding results in less than 1 h. These assays should be of utility in the diagnostic laboratory to complement existing diagnostic testing methodologies and as a tool in conducting flavivirus surveillance in the United States.

West Nile (WN) and St. Louis encephalitis (SLE) viruses are arthropod-borne viruses (family *Flaviviridae*, genus *Flavivirus*) within the Japanese encephalitis virus serocomplex (15). As with other members of this complex, WN and SLE viruses possess a single-stranded plus-sense RNA genome of approximately 11,000 nucleotides. Both WN and SLE viruses circulate in natural transmission cycles involving primarily *Culex* species mosquitoes and birds; humans and other mammals are thought to be incidental hosts (14). Severe human disease caused by both WN and SLE viruses has been reported and is commonly associated with old age (14). Endemic SLE virus transmission in nature is silent, with no reports of avian mortality, whereas in the Western Hemisphere and Israel WN virus infections have been reported to cause high rates of mortality among domestic and wild birds as well as equines (9, 14).

Historically, WN virus has circulated primarily in Africa, Asia, southern Europe, and Australia and has been responsible for several significant epidemics, notably, in Israel (1950s), France (1962), South Africa (1974), and Romania (1996) (6, 17, 21). In 1999 and 2000, WN virus was responsible for epidemics and epizootics in the northeastern United States, in which there were human fatalities and extensive avian mortality (1, 3, 4, 11). SLE virus is endemic throughout the United States and has also been isolated from several South American countries (14). Over the past 70 years, SLE virus has been responsible for numerous epidemics throughout the United States; the largest occurred in 1975, with approximately 2,000 cases reported (14). The appropriate public health responses for both WN and SLE virus epidemics are identical and involve public education and mosquito control programs. In both instances, however, timely implementation of these interventions

is critical to reduce the risk to humans; therefore, surveillance programs for WN and SLE viruses must ensure rapid detection of virus activity. Typical means of surveillance for these viruses have involved the testing of field-collected mosquitoes and, in the case of WN virus, the testing of dead birds for the presence of virus by isolation in cell culture. However, virus isolation followed by identification through immunofluorescence assays can take over a week to complete. TaqMan assays for the rapid detection of WN virus from mosquito pools and avian tissues have been described, but no such approach exists for SLE virus (10, 13, 18).

In the diagnostic laboratory, human WN and SLE virus infections can be inferred by immunoglobulin M (IgM) capture and IgG enzyme-linked immunosorbent assays (ELISAs); however, confirmation of the type of infecting virus is possible only by detection of a fourfold or greater rise in virus-specific neutralizing antibody titers in either cerebrospinal fluid (CSF) or serum by performing the plaque reduction neutralization assay (PRNT) with several flaviviruses (7, 13). Virus isolation in cell culture from CSF or serum has generally been unsuccessful, likely due to the low level and short-lived viremia associated with infections with these viruses (14, 20). Recently, several investigators have reported on TaqMan assays for the detection of WN virus from human CSF specimens for which cell culture assays were negative, suggesting that nucleic acid-based assays hold greater promise for the detection of these viruses in human specimens (2, 10).

Nucleic acid sequence-based amplification (NASBA) is a robust amplification technology that has been used to detect a number of pathogens, including RNA viruses (5, 8, 12, 16, 19, 22). The amplification methodology involves the use of three enzymes, reverse transcriptase, T7 RNA polymerase, and RNase H; and the final amplification product is single-stranded RNA with a polarity opposite that of the target. The amplified RNA product can be detected through the use of a target-specific capture probe bound to magnetic particles in

* Corresponding author. Mailing address: Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, CDC, Rampart Rd., Fort Collins, CO 80521. Phone: (970) 221-6440. Fax: (970) 221-6476. E-mail: rsl2@cdc.gov.

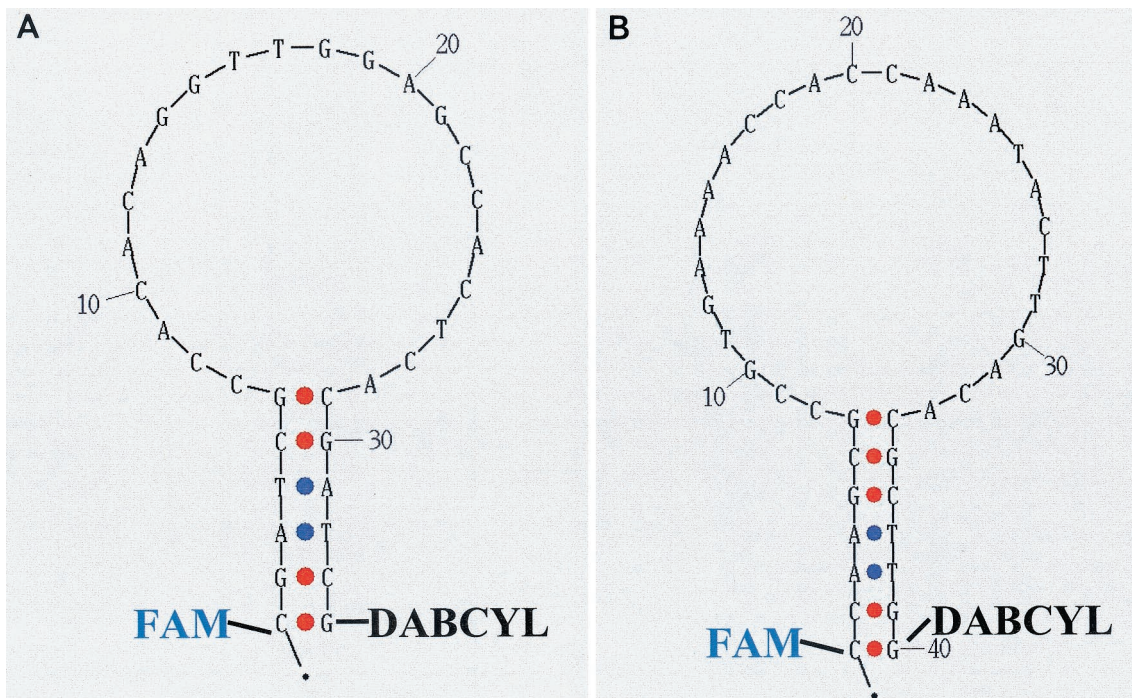


FIG. 1. Primary nucleotide sequence and predicted secondary structure of the WN (A) and SLE (B) virus molecular beacon probes. The folding algorithm used to generate the figure was designed by D. Stewart and M. Zucker, and the software program is available at the Zucker Group's website (RNA mfold; <http://bioinfo.math.rpi.edu/~zukerm/rna/>).

conjunction with a ruthenium-labeled detector probe and an instrument (NucliSens Reader; bioMérieux) capable of measuring electrochemiluminescence (ECL) (5). Alternatively, RNA amplified by NASBA can specifically be detected in real time through the use of molecular beacon probes included in the amplification reaction (16). Molecular beacon probes possess a 5' fluorescent dye and a 3' quencher molecule (typically, 4-dimethylaminophenylazobenzoyl [DABCYL]) and are designed to form stem-loop structures that bring into close proximity the 5' and 3' ends of the probe, resulting in minimal fluorescence (Fig. 1). In the presence of a complementary target sequence, the probe will hybridize to the target, separating the reporter dye from the quencher, resulting in a measurable increase in fluorescence.

We report here on the development of NASBA assays for the detection of WN and SLE viruses that use both ECL and molecular beacon detection technologies. We compared the sensitivities and specificities of these assays to those of virus isolation, TaqMan assay, and standard reverse transcription (RT)-PCR. These newly developed NASBA assays display sensitivities similar to or even greater than the sensitivity of our previously developed TaqMan assay. In addition, the NASBA assays provide a more rapid means of amplification and detection, with positive results available in less than 1 h.

MATERIALS AND METHODS

Virus strains. All virus strains were obtained from the reference collection maintained at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC). WN virus strain NY99 (flamingo 382-99) and SLE virus strain TBH 28 were titrated in Vero cells by a standard plaque assay.

RNA extraction. Viral RNA was isolated from virus seeds, mosquito pools, homogenized avian tissues, and human CSF by using the QIAamp viral RNA kit (Qiagen, Valencia, Calif.). Mosquito pools and avian tissues were first homogenized as described previously (10), and total RNA was extracted from 100 μ l (virus seeds) or 140 μ l (mosquito and avian samples). RNA was eluted from the Qiagen columns in a final volume of 100 μ l of elution buffer and was stored at -70°C until use.

Primer design. The WN virus RT-PCR and the TaqMan assay primer-probe design methodology and sequences have been published previously (10). SLE virus RT-PCR primers were designed for the present study by using the PrimerSelect software program (DNASTAR Inc., Madison, Wis.) and the published sequence of the Mississippi 1975 SLE strain-MSI.7 (GenBank accession number M16614). The sequences of the PrimerSelect-derived primer pairs were compared to an alignment of 13 SLE virus structural region sequences, and two primer pairs that demonstrated maximum homology to all SLE virus strains were selected (Table 1). The two SLE virus-specific RT-PCR primer pairs performed equally in the sensitivity and specificity experiments; therefore, only data for primer pair 727c-1119c are shown (Table 2). The SLE virus-specific TaqMan assay primers and probe were designed with the PrimerExpress software package (PE Applied Biosystems, Foster City, Calif.), and the selection of the two primer-probe sets was based upon homology to aligned SLE virus structural region sequences as described above. Both SLE virus-specific TaqMan assay primer-probe sets performed equivalently, and only data for the 834-905c set are shown. The SLE virus-specific TaqMan assay probes were 5' labeled with the reporter dye 6-carboxyfluorescein (FAM) and labeled at the 3' end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). WN virus- and SLE virus-specific primers and probes for the NASBA assays were designed by following the primer design guidelines described in the NucliSens Basic Kit Application Manual (bioMérieux, Durham, N.C.). The reverse primers for the NASBA assays 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 virus-specific capture probes for the NASBA-ECL assay were labeled with biotin at the 5' end and were immobilized onto avidin-coated magnetic particles by following the protocol described in the NucliSens Basic Kit Application Manual. Molecular beacon probes for the NASBA assays were designed with the help of the NucliSens Basic Kit World Wide Web-hosted help

TABLE 1. Oligonucleotide primers and probes in the NASBA, RT-PCR, and TaqMan assays^a

Primer	Genome position	Sequence (5'-3')	Product size (bp)
SLE virus-specific standard RT-PCR primers			
SLE727	727-750 ^b	GTAGCCGACGGTCAATCTCTGTGC	393
SLE1119c	1119-1096	ACTCGGTAGCCTCCATCTTCATCA	
SLE1637	1637-1659	GACGAGCCCTGCCACAACCTGATT	495
SLE2131c	2131-2108	GTGCCTCTCCGACGACGATGTAA	
SLE virus-specific TaqMan assay primers and probes			
SLE2420	2420-2439	CTGGCTGTCGGAGGGATTCT	68
SLE2487c	2487-2468	TAGGTCAATTGCACATCCCG	
SLE2444-probe	2444-2466	TCTGGCGACCAGCGTGCAAGCCG	
SLE834	834-852	GAAACTGGGTTCTGCGCA	72
SLE905c	905-889	GTTGCTGCCTAGCATCCATCC	
SLE857-probe	857-880	TGGATATGCCCTAGTTGCGCTGGC	
SLE virus-specific NASBA assay primers and probes			
SLE708	708-729	<i>gatgcaaggtgcatatgag-CGCATGGGACATTCCGAGGCCGTA^c</i>	234
SLE941c	941-919	<i>aattctaatacgaactactataggagaagg-CATAAGCATGATCACAAAGACCA</i>	
SLE802-ECL probe	802-827	<i>CCGTGAAAACCACCAAATACTTGACA</i>	
SLE802-Beacon probe	802-827	<i>ccaagcg-CCGTGAAAACCACCAAATACTTGACA-cgcttgg</i>	
WN virus-specific NASBA assay primers and probes			
WN 1333	1333-1354	<i>gatgcaaggtgcatatgag-ACCAAGGCAATAGGAAGAACCA</i>	162
WN1494c	1494-1472	<i>aattctaatacgaactactataggagaagg-GTATGAAGGCCGCCGAGGAGTGA</i>	
WN1432-ECL probe	1432-1456	<i>TCCACACAGGTTGGAGCCACTCAGG</i>	
WN1433-Beacon probe	1433-1454	<i>cgatcg-CCACACAGGTTGGAGCCACTCA-cgatcg</i>	

^a WN virus-specific standard RT-PCR and TaqMan assay primers have been published previously (10).

^b SLE virus-specific primer genome positions are according to SLE MSI.7 sequence in GenBank (accession number M16614).

^c NASBA forward primers have a 5' ECL sequence (lowercase, bold, and italic); reverse primers have a T7 promoter sequence (lowercase, bold, and italic) beacon probes have stem sequences shown in lowercase-bold.

desk. The virus-specific capture probes for the NASBA-ECL assays were flanked with a sequence of seven (SLE virus) or six (WN virus) nucleotides capable of forming a self-complementary stem, such that the beacon probes would assume a stem-loop structure (Fig. 1). Molecular beacon probes were synthesized with a FAM fluorophore label at the 5' end and a DABCYL molecule as a quencher at the 3' end.

RT-PCR and TaqMan assays. Standard RT-PCRs were performed with the TITAN One-Tube RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, Ind.) by using 5 µl of RNA and 50 pmol of each primer in a total reaction volume of 50 µl as described previously (10). After the RT-PCR was performed, a 5-µl portion was analyzed by agarose gel electrophoresis on a 3% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, Maine), and the DNA was visualized with ethidium bromide staining. The TaqMan assays for WN and SLE viruses were performed as described previously with 5 µl of RNA per 50-µl reaction mixture by using the TaqMan RT-PCR Ready-Mix kit (PE Applied Biosystems) (10). The samples were subjected to 45 cycles of amplification in an ABI Prism 7700 sequence detection system instrument (PE Applied Biosystems) by the manufacturer's protocol for TaqMan RT-PCR cycling conditions. Positive results by the TaqMan assays were calculated by taking into account the real-time cycle number at which fluorescence increases above the threshold value (C_T ; threshold fixed at 0.1) and the relative increase in fluorescence (Rn) calculated by the end-point plate read function of the instrument. A sample was interpreted as positive if both the C_T value was ≤ 37 and the Rn value was two or more times the average of the Rn values for the eight negative control wells. The results for samples that met one of the two criteria for positivity were interpreted as equivocal.

NASBA-ECL and NASBA-beacon assays. All NASBA amplification reactions were performed with the NucliSens Basic Kit amplification reagents (bio-

Mérieux). For the ECL detection assay, amplification reactions were set up by combining 5 µl of RNA with 50 pmol of each primer in a 10-µl amplification cocktail. The mixture was heated to 65°C for 5 min and then placed in a 41°C water bath for 5 min, followed by the addition of the enzyme mixture. After the 90-min amplification reaction, a 5-µl portion was removed and was diluted to 100 µl with the detection diluent (1:20 dilution) supplied with the NucliSens kit. The diluted amplification product was combined with the virus-specific capture probe bound to magnetic beads and the generic ECL detection probe, and the mixture was then incubated at 41°C for 30 min by the manufacturer's protocol. Samples were read in a NucliSens reader (bioMérieux), which detects the amplified RNA-capture probe complex through the electrochemiluminescence emitted by the bound generic ECL probe. The NucliSens reader calculates positive results on the basis of the values for the positive and negative controls included in the assay. For the real-time molecular beacon assay (NASBA-beacon assay), the NASBA reactions used the same amplification reagents described above, with the addition of the molecular beacon probe at a final concentration of 0.2 µM and 0.6 µM 5-6-carboxy- α -rhodamine as a reference dye. The amplification mixture was heated to 65°C for 5 min, followed by cooling to 41°C, and enzyme was added as described above. The amplification and real-time detection were performed at 41°C for 120 min in an ABI Prism 7700 sequence detection system instrument (PE Applied Biosystems). The results for the samples were interpreted as positive if they met the two criteria described in the interpretation of the TaqMan assay results, with the exception that time to positivity (T_p ; in minutes) was used in the interpretation instead of C_T values. T_p values ≤ 60 min were considered positive.

Avian tissues, mosquito pools, and clinical specimens. Avian tissues and mosquito pool specimens that had been collected during the 1999 WN virus outbreak and previously tested for WN virus by virus isolation, RT-PCR, and

TABLE 2. Sensitivities and specificities of the SLE virus NASBA assays compared to those of Vero cell culture, TaqMan assay, and standard RT-PCR^a

Sample	Quantity (no. of PFU)	RT-PCR with 727-1119c	TaqMan assay ^b		NASBA-ECL assay ^c		NASBA-beacon assay ^d	
			C _T	Int.	ECL units	Int.	Beacon (min)	Int.
Titrated SLE virus (TBH 28) seed ^e								
SLE-1	150,000	POS	13.2	POS	5,280,120	POS	17.5	POS
SLE-2	15,000	POS	17.7	POS	4,437,851	POS	19.8	POS
SLE-3	1,500	POS	20.6	POS	2,484,647	POS	23.7	POS
SLE-4	150	POS	24.2	POS	2,199,690	POS	26.4	POS
SLE-5	15	POS	28.1	POS	2,292,637	POS	27.5	POS
SLE-6	1.5	POS	32.0	POS	625,447	POS	34.1	POS
SLE-7	0.15	POS	36.5	POS	4,506	POS	59.0	POS
SLE-8	0.015	NEG	38.7	NEG	260	NEG	90.0	NEG
SLE-9	0.0015	NEG	45.0	NEG	118	NEG	90.0	NEG
SLE-10	0.00015	NEG	45.0	NEG	ND	NEG	ND	NEG
SLE virus strains								
Maryland 1975	ND	POS	12.3	POS	307,577	POS	11.3	POS
Guatemala 1969	ND	POS	10.6	POS	18,995	POS	24.9	POS
Panama 1973	ND	POS	20.2	POS	460,798	POS	15.0	POS
Ecuador 1976	ND	POS	11.8	POS	381,882	POS	11.6	POS
Texas 1966	ND	POS	16.7	POS	688,404	POS	9.9	POS
Florida 1979	ND	POS	14.4	POS	1,209,079	POS	9.1	POS
Illinois 1979	ND	POS	14.1	POS	1,154,617	POS	9.3	POS
Mississippi 1975	ND	POS	14.2	POS	1,494,960	POS	8.4	POS
California 1963	ND	POS	12.3	POS	530,166	POS	9.4	POS
Texas 1999-29a	ND	POS	25.2	POS	1,113,905	POS	9.1	POS
Texas 1999-29b	ND	POS	16.6	POS	37,173	POS	29.3	POS
Texas 1999-30a	ND	POS	23.4	POS	623,510	POS	10.7	POS
Texas 1999-30b	ND	POS	16.0	POS	338,634	POS	19.8	POS
Other viruses								
EEE	ND	NEG	45	NEG	99	NEG	90	NEG
WEE	ND	NEG	45	NEG	88	NEG	90	NEG
VEE	ND	NEG	45	NEG	114	NEG	90	NEG
HJ	ND	NEG	45	NEG	101	NEG	90	NEG
LAC	ND	NEG	45	NEG	85	NEG	90	NEG
DEN-2	ND	NEG	45	NEG	101	NEG	90	NEG
YF	ND	NEG	45	NEG	104	NEG	90	NEG
POW	ND	NEG	45	NEG	106	NEG	90	NEG
JE	ND	NEG	45	NEG	73	NEG	90	NEG
WNV	ND	NEG	45	NEG	78	NEG	90	NEG

^a Abbreviations: POS, positive; NEG, negative; Int., interpretation; ND, not determined; EEE, eastern equine encephalitis virus; WEE, western equine encephalitis virus; VEE, Venezuelan equine encephalitis virus; HJ, Highlands J virus; LAC, La crosse virus; DEN-2, dengue virus type 2; YF, yellow fever virus; POW, Powassan virus; JE, Japanese encephalitis virus.

^b C_T value of <37 was positive; for an explanation of TaqMan assay data interpretation, see Materials and Methods.

^c NASBA-ECL assay units >300 are interpreted as positive.

^d The values shown are the times at which fluorescence crosses the threshold; values <60 min are interpreted as positive. For a complete explanation of real-time NASBA-beacon assay data interpretation, see Materials and Methods.

^e All TaqMan C_T, NASBA-ECL, and NASBA-beacon values were calculated by averaging the values for samples tested in duplicate.

TaqMan assays were coded for blind testing and tested by the WN virus NASBA assays. CSF specimens were obtained from patients presenting with fever and/or viral encephalitis during the time frame of the WN virus epidemic in New York State. Patient positivity for WN virus by serology was determined by a positive IgM capture ELISA and the presence of detectable WN virus-specific neutralizing antibody, as measured by the plaque reduction neutralization assay. These specimens were also previously tested by virus isolation and RT-PCR assays. No similar panel of field-collected or human specimens was available for testing by the SLE virus assays.

RESULTS

Sensitivities and specificities of NASBA assays. To ascertain the detection limits of the WN and SLE virus NASBA assays, we tested 10-fold dilutions of seed viruses that had previously been quantitated by plaque titration. For comparison, these same virus seed dilutions were also tested by standard RT-

PCR and TaqMan assays (Fig. 2; Tables 2 and 3). Both formats of the SLE NASBA assay (ECL and molecular beacon) detected less than 1 PFU of SLE virus (0.15 PFU), which was the same level of detection achieved in the standard RT-PCR and TaqMan assays (Table 2). The WN virus NASBA-ECL assay was 10-fold more sensitive than the NASBA-beacon assay and the TaqMan assay, detecting 0.01 PFU of WN virus, whereas the other assays detected 0.1 PFU of WN virus (Table 3).

The SLE virus-specific primer pairs shown in Table 1 were tested for their specificities by performing the NASBA, TaqMan, and RT-PCR assays with viral RNAs extracted from 13 geographically and temporally distinct SLE virus strains, including both North and South American isolates (Table 2). The primer pairs were also evaluated for their specificities by performing the assays with RNAs extracted from five serologically related

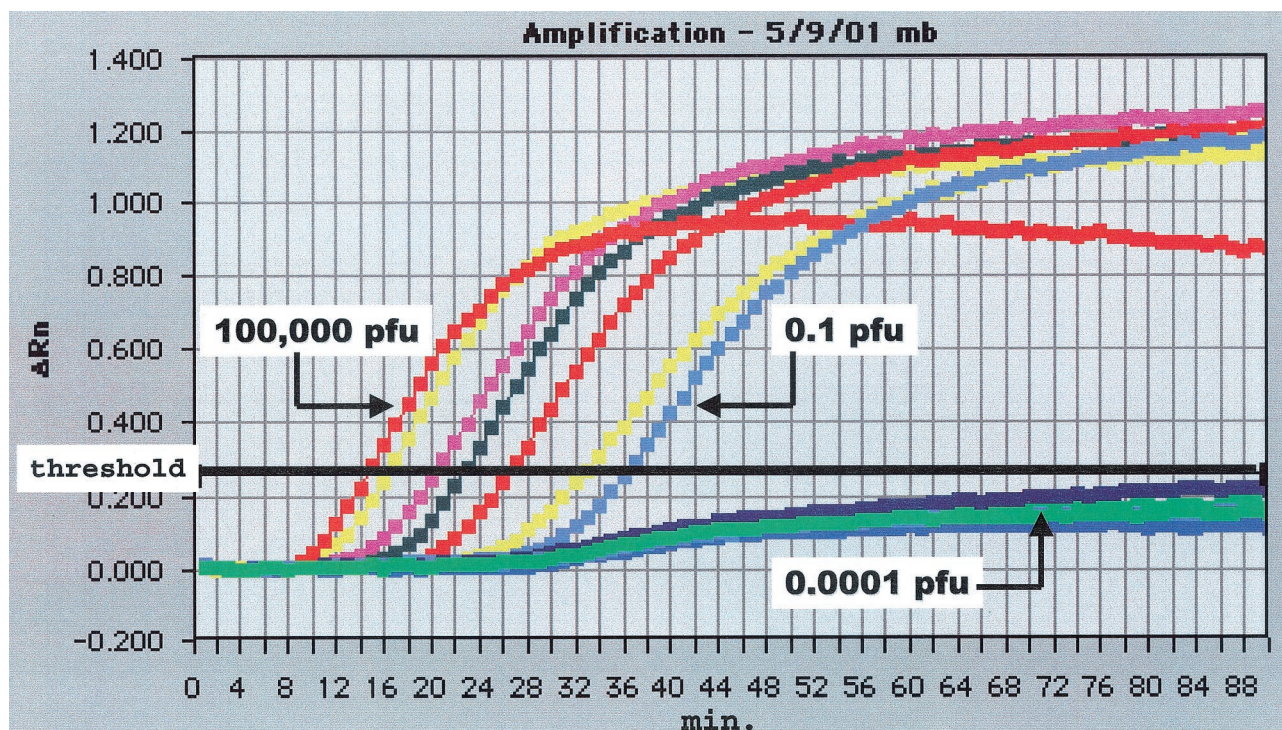


FIG. 2. NASBA amplification with real-time molecular beacon detection of dilutions of WN NY 1999 virus. The amplification plot was generated in an ABI Prism 7700 sequence detection system instrument (PE Applied Biosystems). The x axis is the time from the initiation of amplification; the y axis is the increase in fluorescence (ΔRn); threshold fluorescence is shown as the bold horizontal line. Tenfold virus dilutions (Table 3), ranging from 100,000 to 0.0001 PFU, were tested.

flaviviruses (Japanese encephalitis, WN, dengue type 2, yellow fever, and Powassan viruses) and five arthropod-borne viruses that circulate in North and South America (eastern equine encephalitis, western equine encephalitis, Venezuelan equine encephalitis, Highlands J, and La Crosse viruses). All of the SLE virus-specific primer pairs were highly specific for SLE virus strains; they detected all of the SLE virus strains and yielded negative results for all of the arthropod-borne flaviviruses or other Western Hemisphere arthropod-borne viruses (Table 2). A similar strategy was used to evaluate the specificities of the WN virus-specific primers; the primers were tested by using RNAs extracted from various WN virus strains and other arthropod-borne viruses. The WN virus-specific primers used for the TaqMan and RT-PCR assays have previously been evaluated for their specificities, and the data are reproduced in Table 3 for comparison (10). Both NASBA assays for WN virus demonstrated a high degree of specificity for WN virus strains, detecting all WN strains tested (with one exception; see below) and yielding negative results for all other viruses tested. Kunjin virus was not detected by the primers used for the NASBA assays; however, these results are not unexpected. Kunjin virus, which has been detected only in Australia, is taxonomically classified as a subtype of WN virus, yet it demonstrates only 87% nucleotide identity with the WN virus strains that are circulating in the United States and Europe.

NASBA assay detection of WN virus in field-collected mosquito pools and avian tissues. A coded panel of 68 specimens consisting of a random combination of mosquito pool specimens and avian tissues obtained from collections retrieved in

New York and New Jersey during the 1999 WN epidemic-epizootic (September to November 1999) were tested by virus isolation in Vero cell culture and by the NASBA, TaqMan, and RT-PCR assays. Due to sample depletion, the NASBA-beacon assay was performed with only a subset of these samples (32 samples), with the results being identical to those obtained by the ECL detection assay. WN virus was isolated from 32 of the 68 samples (Table 4). All 32 of these culture-positive specimens were also positive by the NASBA assay. Two NASBA assay-positive specimens were culture negative, and these specimens had equivocal results by both the TaqMan and the RT-PCR assays, suggesting that these samples contained low levels of WN virus. The TaqMan assay detected WN virus RNA in 31 of the 32 culture-positive samples; the single TaqMan assay-negative, culture-positive specimen was also positive by the NASBA assay. As stated above, the two samples with equivocal results by the TaqMan assay also had equivocal results by RT-PCR and positive results by the NASBA assay. The RT-PCR assay detected WN virus RNA in 24 of the 32 culture-positive specimens. Five of the samples with equivocal results by RT-PCR (faint bands) were positive by all other methods.

NASBA assay detection of WN virus in human specimens. Twenty CSF specimens from patients classified as being either non-WN virus infected or confirmed to be infected with WN virus, as determined by serological testing (IgM ELISA and PRNT assay), were tested by the NASBA, TaqMan, and RT-PCR assays (Table 5). Virus isolation was performed with most of these specimens, and no WN virus was isolated (data not shown). Seven of the 10 CSF samples from patients with se-

TABLE 3. Sensitivities and specificities of WN virus NASBA assays compared to those of Vero cell culture, TaqMan assay, and standard RT-PCR^a

Sample	Quantity (no. of PFv)	RT-PCR with 233-640	TaqMan assay ^b		NASBA-ECL assay ^c		NASBA-beacon assay ^d	
			C _T	Int.	ECL units	Int.	Beacon (min)	Int.
Titrated WN virus (NY99) seed ^e								
WNV-1	100,000	POS	17.9	POS	1,653,417	POS	15.2	POS
WNV-2	10,000	POS	20.9	POS	1,187,613	POS	16.6	POS
WNV-3	1,000	POS	24.2	POS	1,810,790	POS	20.2	POS
WNV-4	100	POS	27.8	POS	1,666,084	POS	22.8	POS
WNV-5	10	POS	31.2	POS	1,211,426	POS	28.0	POS
WNV-6	1	POS	34.1	POS	1,209,491	POS	31.7	POS
WNV-7	0.1	NEG	36.8	POS	326,954	POS	35.6	POS
WNV-8	0.01	NEG	45.0	NEG	5,782	POS	90.0	NEG
WNV-9	0.001	NEG	45.0	NEG	110	NEG	90.0	NEG
WNV-10	0.0001	NEG	45.0	NEG	91	NEG	ND	NEG
WN virus strains								
WNV-Romania-1996M	ND	POS	29.02	POS	313,605	POS	32.8	POS
WNV-Egypt-1951	ND	POS	25.54	POS	437,541	POS	19.0	POS
WNV-Italy 1998	ND	POS	23.82	POS	237,753	POS	18.1	POS
WNV-Kenya 1998	ND	POS	21.38	POS	226,175	POS	26.1	POS
Kunjia	ND	POS	20.58	POS	109	NEG	90	NEG
Other viruses								
DEN-2	ND	NEG	45	NEG	27	NEG	90	NEG
YF	ND	NEG	45	NEG	8	NEG	90	NEG
SLE	ND	NEG	45	NEG	1	NEG	90	NEG
JE	ND	NEG	45	NEG	7	NEG	90	NEG
MVE	ND	NEG	45	NEG	2	NEG	90	NEG
EEE	ND	NEG	45	NEG	1	NEG	90	NEG
WEE	ND	NEG	45	NEG	12	NEG	90	NEG
POW	ND	NEG	45	NEG	29	NEG	90	NEG
LAC	ND	NEG	45	NEG	1	NEG	90	NEG

^a Abbreviations: POS, positive; NEG, negative; Int., interpretation; ND, not determined; DEN-2, dengue virus type 2; YF, yellow fever virus, JE, Japanese encephalitis virus; MVE, Murray Valley encephalitis virus; EEE, equine encephalitis virus; WEE, western equine encephalitis virus; POW, Powassan virus; LAC, La Crosse virus.

^b A C_T value of <37 was positive; for an explanation of TaqMan assay data interpretation, see Materials and Methods.

^c NASBA-ECL assay units >300 are interpreted as positive.

^d Values shown are the times at which fluorescence crosses the threshold; values <60 min are interpreted as positive. For a complete explanation of real-time NASBA-beacon assay data interpretation, see Materials and Methods.

^e All TaqMan C_T, NASBA-ECL, and NASBA-beacon assay values were calculated by averaging the values from samples tested in duplicate.

rologically confirmed WN virus infections were positive by the NASBA-ECL assay, and none of these were positive by RT-PCR (Table 5). Interestingly, three specimens had equivocal results by the TaqMan assay, but all of these specimens were positive by the NASBA assay. Finally, one specimen positive for WN virus by the TaqMan assay was negative by the NASBA assay.

TABLE 4. Detection of WN virus in mosquito pools and avian tissues by Vero cell culture, NASBA assay, TaqMan assay, and RT-PCR

Result	No. of samples			
	Vero cell culture	NASBA-ECL assay ^a	TaqMan assay	RT-PCR
Positive	32	34	31	24
Negative	36	34	35	37
Equivocal ^b	0	0	2	7

^a The real-time NASBA-beacon assay was performed with a subset (n = 32) of these coded specimens and yielded results identical to those of the NASBA-ECL assay; the remaining samples were not tested due to sample depletion.

^b Equivocal indicates that the samples satisfied only one of two required criteria for positive interpretation; for complete explanation of equivocal results, see Materials and Methods.

DISCUSSION

This report describes the development of NASBA assays for the rapid detection of WN and SLE viral RNAs. The NASBA assays used two formats for the detection of virus-specific amplification: either the postamplification ECL detection system or a real-time system with virus-specific molecular beacon probes. The NASBA assays demonstrated a level of detection similar to or greater than those of virus isolation and TaqMan assays. The NASBA assay for SLE virus, in both detection formats, was able to detect 0.15 PFU of SLE virus, the same level of detection achieved by the TaqMan and standard RT-PCR assays (Table 2). The NASBA-ECL assay for WN virus was consistently 10-fold more sensitive than either the TaqMan assay or the NASBA-beacon assay, detecting 0.01 PFU of WN virus (Table 3). The NASBA assays also demonstrated a high degree of specificity; no false-positive results were obtained with any of the serologically related flaviviruses tested or with any of the other domestic arthropod-borne viruses tested (Tables 2 and 3).

The NASBA assays for WN virus were able to detect WN virus in mosquito pools, avian tissue specimens, and human CSF specimens with sensitivities similar to or greater than that

TABLE 5. Detection of WN virus in human CSF specimens by NASBA, TaqMan, and RT-PCR assays^a

Sample no.	WN virus serology ^b	NASBA-ECL assay result		TaqMan assay		RT-PCR result
		ECL units	Interpretation ^c	C _T value	Interpretation ^d	
1	POS	578,934	POS	35.7	POS	NEG
2	POS	65,745	POS	36.2	POS	NEG
3	POS	39	NEG	37	POS	NEG
4	POS	219,583	POS	38.3	EQUIV	NEG
5	POS	2,176	POS	39.3	EQUIV	NEG
6	POS	222,392	POS	45	NEG	NEG
7	POS	189,660	POS	39.67	EQUIV	NEG
8	POS	18,395	POS	35.2	POS	NEG
9	POS	35	NEG	45	NEG	NEG
10	POS	10	NEG	45	NEG	NEG
11–20	All NEG	All <50	All NEG	All 45	All NEG	All NEG

^a Abbreviations: POS, positive; NEG, negative; EQUIV, equivocal.

^b WN virus serology positive is defined as positivity by IgM and PRNT assay.

^c NASBA-ECL assay units >300 are interpreted as positive.

^d C_T values <37 and Rn values greater than two times the average fluorescence for negative control samples is interpreted as a positive TaqMan assay result. For complete explanation of TaqMan assay: data interpretation, see Materials and Methods.

of a previously described TaqMan RT-PCR assay for WN virus (10). The NASBA-ECL assay for WN virus detected WN virus in two specimens that were Vero cell culture negative, and these two specimens had equivocal results by the TaqMan and RT-PCR assays. Our accumulated experience with the TaqMan assay for WN virus strongly suggests that in most cases specimens with equivocal results by the TaqMan assay actually possess low levels of viral RNA. We have consistently observed that equivocal results are reproducible with other primer-probe combinations and that increasing the cycle number reveals sustained amplification. In this instance, the results for two culture-negative, NASBA assay-positive specimens were equivocal by a TaqMan assay with an independent primer-probe set, suggesting that these samples had low levels of WN viral RNA rather than false-positive NASBA assay results. The NASBA assay for WN virus was also able to detect WN virus in three human CSF specimens that had equivocal results by the TaqMan assay. Again, it is likely that these samples had low levels of WN viral RNA rather than false-positive results. Alternatively, one TaqMan assay-positive CSF specimen (Table 5, sample 3) was NASBA assay negative. Unfortunately, these samples were depleted and no further testing by the NASBA or TaqMan assay was possible. The lack of a corresponding panel of human and/or field-collected specimens infected with SLE virus abrogated the ability to use the NASBA assays for SLE virus with real-world specimens. However, the sensitivity and specificity data generated with laboratory SLE virus-infected specimens give a clear indication that the test should perform similarly to the NASBA assay for WN virus for the detection of SLE virus in field-collected and clinical specimens.

The introduction of WN virus into the northeastern United States creates the distinct possibility that two serologically related flaviviruses will cocirculate in the same geographical region. Therefore, rapid and accurate surveillance assays for detection of these viruses are needed throughout the Western Hemisphere. Rapid detection of these viruses in field-collected specimens can accelerate appropriate public education and mosquito control measures that could prevent transmission and disease among humans. The ability of the NASBA assay to

rapidly detect WN virus in human clinical specimens is also significant, given the nonspecificity of the IgM ELISA and the time required to serologically confirm WN virus infection by the PRNT assay. The difficulty in isolating WN virus from human specimens in tissue culture also necessitates the need for a reliable virus detection assay. The high cost of instrumentation capable of performing real-time TaqMan assays (\$50,000 to \$100,000) is, in many cases, prohibitive to the establishment of these assays in the clinical laboratory. The NASBA-ECL assay uses instrumentation that costs much less than TaqMan assay instruments (NASBA-ECL assay instruments are approximately \$20,000), or alternatively, the NucliSens reader can also be leased from the manufacturer. In addition, in-house NASBA assays are easily developed with the NucliSens Basic Kit (bioMérieux), which contains standardized reagents for nucleic acid isolation, NASBA, and ECL detection. The NASBA-beacon assay can be performed with any instrument capable of maintaining a constant temperature while measuring fluorescence, and such instruments are also generally less costly than real-time TaqMan assay instruments (NASBA-beacon assay instruments cost approximately \$20,000). As reported here, the NASBA-beacon assay can also be performed in real-time TaqMan assay instruments (i.e., the ABI Prism 7700 instrument or the Bio-Rad iCycler instrument). For laboratories that use TaqMan assays, the NASBA-beacon assay could thus be a primary or confirmatory test by a unique amplification method with the same instrument.

Of particular importance is the substantial reduction in time required for the confirmation of results by NASBA assays: less than 1 h. The NASBA-beacon assay data reveal that the NASBA assay is an inherently more rapid amplification technology than the TaqMan RT-PCR. The accumulation of amplified RNA, as detected by fluorescence values that exceed a threshold, can be detected as early as 14 min after the addition of enzyme, and for most samples amplification is essentially complete in approximately 45 min (Fig. 2). Taken together, the data reported here indicate that the NASBA assays are extremely rapid, highly sensitive, and specific and could be used along with TaqMan assays and/or virus isolation for a compre-

hensive WN virus detection system in the diagnostic laboratory.

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