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Identification of Dengue Virus in Respiratory Specimens from a Patient Who Had Recently Traveled from a Region Where Dengue Virus Infection Is Endemic▽

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Dengue is the most important arthropod-borne viral disease, and it is a major public health problem in subtropical and tropical regions. The virus is transmitted to humans by the bite of infected female mosquitoes of the genus *Aedes*. The global resurgence of dengue is thought to be due to failure to control the *Aedes* populations, uncontrolled urbanization, population growth, climate change, and increased airplane travel. In this paper we describe the methods used to detect dengue virus infection in a patient who presented to a hospital in New York State. The patient was a 21-year-old female who had recently traveled from Thailand. Serologic testing, viral culture, and molecular methods, including reverse transcription-PCR (RT-PCR) and real-time RT-PCR, were tools used for diagnosis. Enzyme-linked immunosorbent assay and immunofluorescence assay of serum specimens indicated flavivirus infection. Conventional RT-PCR and sequencing, in addition to real-time RT-PCR of serum samples and nasal and throat swabs from the patient, confirmed dengue virus 1 (DEN-1) infection. A cytopathic effect was observed in virus cultures of the acute-phase serum samples and nasal swabs. DEN-1 was subsequently detected by RT-PCR from cell culture supernatants and by direct fluorescent-antibody assay staining of the cell culture monolayer. We show that a multipronged approach to the laboratory diagnosis of dengue infections can be used to successfully diagnose and differentiate the dengue virus serotypes. In addition, we show that both dengue viral RNA and infectious virus can be detected in respiratory specimens from an infected patient.

Dengue virus is a mosquito-borne flavivirus belonging to the family *Flaviviridae*. The virus causes human disease ranging from dengue fever to dengue hemorrhagic fever and dengue shock syndrome. An estimated 50 million people are infected with dengue virus every year, mainly in tropical and subtropical countries, with approximately 2.5 billion people living in at-risk areas (22). The virus is enveloped and has a positive-strand RNA genome of approximately 11,000 bases, which encodes three structural proteins and seven nonstructural proteins. There are four serologically related but genetically and antigenically distinct strains of dengue viruses, named DEN-1 through DEN-4. Infection by a specific dengue virus usually induces lifelong protective immunity to that strain but only partial and transient protection against the other strains. Typing the infecting dengue virus is crucial for diagnosis and prognosis, especially since a second infection by a different dengue virus serotype increases the risk factor for dengue hemorrhagic fever and dengue shock syndrome (3).

Dengue infections are rare in individuals living in the United States but do occur in persons who have traveled to regions of the world where dengue is endemic. The patient discussed in this paper had traveled from Thailand, where dengue virus is endemic. Dengue fever has been reported in an increasing number of international travelers and is thought to be the second most frequent cause of hospitalization after malaria (17, 20). Acute infection by dengue virus can cause dengue fever, which is characterized by biphasic fever, headache, myalgia, eye pain, arthralgia, rash, prostration, lymphadenopathy, and leukopenia. In general, clinical findings include neutropenia followed by lymphocytosis, presence of atypical lymphocytes, and occasionally elevated levels of aspartate aminotransferase in serum (19). Virus can be detected in blood for 4 or 5 days after the onset of symptoms and then disappears as antibody (immunoglobulin M [IgM]) production increases. In primary infections, IgG antibody appears within a few days, whereas in secondary infections, the IgG level rises immediately after the onset of symptoms and remains high in most patients (18). In the early phases of the disease, because the symptoms are usually nonspecific, it is hard to distinguish the disease from West Nile, Japanese, and Saint Louis encephalitis and influenza, yellow fever (YF), measles, scrub typhus, malaria, and leptospirosis. This problem is exacerbated by the serologic cross-reactivity among flaviviruses, including West Nile virus (WNV), Japanese encephalitis (JE) virus, Saint Louis encephalitis (SLE) virus, dengue virus, and YF virus.

In the absence of either a vaccine or an effective therapy for dengue fever, timely diagnosis is critical to prevent the spread of disease, enhance preparedness, and exclude other viral etiologies of infection. Current methods for diagnosis of dengue infection and typing of dengue virus include virus isolation, serology, antigen detection, and molecular detection (4, 7).

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Serological evidence of virus infection is based on capture IgM and IgG enzyme-linked immunosorbent assay (ELISA) but has little impact on patient management and control measures because it requires documentation of a rise in antibody concentration from an acute-phase sample to a convalescent-phase sample. A further disadvantage of these tests is that there is a strong cross-reaction with other flavivirus-specific IgG antibodies. The plaque reduction neutralization technique, although it allows typing of the virus, is costly and difficult to perform. It is most effective in testing for primary dengue infections and less effective in testing for secondary dengue infections. An ELISA based on changes in the avidity of IgG during the infectious period has been developed (13). This test discriminates between primary and secondary dengue virus infections during the acute phase of dengue infection. Virus isolation by cell culture remains the gold standard for virus detection but has the disadvantage of taking over 7 days to complete. Both conventional and real-time reverse transcription-PCR (RT-PCR) methods have been used for molecular detection (2, 5, 10, 11). These methods are rapid, specific, and sensitive. In addition, under some conditions when virus infectivity is compromised and the virus does not grow in cell culture, viral RNA can still be detected by these methods. While blood specimens are traditionally used for diagnostic testing, epidemiologic and primate studies suggest that dengue virus is present in the respiratory tract (1, 8, 12), and therefore, respiratory specimens may be alternative specimens for diagnostic testing. Here, we used conventional RT-PCR, real-time RT-PCR, ELISA, a microsphere immunoassay (MIA), and viral culture for detection of the viral agents in the patient's blood and respiratory tract specimens.

CASE REPORT

A 21-year-old woman presented to a hospital in New York State with a 5-day history of fevers, myalgias, anorexia, nausea, vomiting, and dry cough. She was a native of Bangkok, Thailand, and had entered the United States 10 days earlier. She had previously been in excellent health and had no known exposure to live poultry or ill contacts. Upon admission to the hospital, the patient was afebrile and hemodynamically stable. She appeared generally weak and mildly dehydrated. Her lungs were clear, and she lacked a rash and lymphadenopathy. The remainder of the findings of the examination were unremarkable. Laboratory data on blood obtained from the patient revealed a white blood cell count of 1.3×10^3 cells/μl (with 27% bands, 45% neutrophils, and 8% atypical lymphocytes), a hemoglobin level of 15.0 g/dl, and a platelet count of 45×10^3 platelets/μl. Blood chemistry examination revealed normal serum electrolytes and renal profile; the total bilirubin level was 0.6 mg/dl, and the alanine aminotransferase level was 182 U/liter. Chest radiographs revealed faint perihilar opacities. Thin and thick blood smears were devoid of intraerythrocytic parasites. Bacterial blood cultures were sterile. In addition to blood samples, cotton-tipped swabs of nasal, pharyngeal, and nasopharyngeal (NPS) posterior naris mucosae were obtained. The nasal swab was obtained the same day the patient was admitted to the hospital, whereas the other specimens were collected on the following day. The swabs were immediately placed in viral transport medium and sent to the New York State Department of Health for diagnostic evaluation. In addition, convalescent-phase serum samples were collected 3 weeks after the acute-phase serum specimen and sent to the New York State Department of Health.

(This work was presented in part at the 54th Annual Meeting of the American Society of Tropical Medicine and Hygiene in December 2005.)

MATERIALS AND METHODS

Serological testing. Sera were evaluated by IgM antibody capture ELISA (MACELISA) and indirect IgG ELISA to inactivated dengue virus produced in tissue culture at the CDC in San Juan, Puerto Rico. Sera were also evaluated in WNV IgM antibody capture and MIAs to WNV recombinant envelope protein (rWNV-E) and recombinant nonstructural protein 5 (NS5) (rWNV-NS5) as previously described (21). The MIA for rWNV-NS5 can discriminate between WNV and dengue virus infections.

Virus isolation and identification. Virus isolation was attempted on the acute-phase serum and respiratory samples (nasal, pharyngeal, and the two NPS swabs) from the patient. A 0.1-ml portion of each sample was added to duplicate wells of Vero (African green monkey kidney cells) and C6/36 (*Aedes albopictus*) cell monolayers. Vero and C6/36 cells were maintained at 37°C and 28°C, respectively. Cultures were blind passaged onto a second round of Vero and C6/36 cells after 7 days. Dengue virus does not cause a cytopathic effect (CPE) in C6/36 cells; therefore, all C6/36 cultures grown were passaged onto Vero cell culture monolayers for evidence of CPE. Immunofluorescence assays (IFAs) were performed on the infected-cell culture monolayers from all samples. Briefly, 10-well slides were spotted with 0.025 ml of the cell suspension of each sample culture and allowed to air dry. DEN-1, -2, -3, and -4-specific mouse monoclonal antibodies (MAbs) were as follows: DEN-1-specific MAb (Chemicon International Inc., Temecula, CA), DEN-2-specific MAb (Chemicon International Inc.), DEN-3-specific MAb (U.S. Biological, Swampscott, MA), and DEN-4-specific MAb (Chemicon International Inc.). The DEN-1, -2, -3, and -4-specific MAbs were diluted 1:100, added to duplicate wells for each sample, and incubated for 1 h at 37°C. After incubation, the slides were washed for 15 min in phosphate-buffered saline. A secondary antibody conjugated to fluorescein isothiocyanate (KPL, Gaithersburg, MD) was then added to each well, and slides were incubated for 1 h at 37°C. After incubation, the slides were again washed for 15 min in phosphate-buffered saline, and coverslips were added. Fluorescence was evaluated using a Zeiss Axiovert microscope equipped with a fluorescein isothiocyanate filter set.

Nucleic acid extraction and RT-PCR. Nucleic acid was extracted using the NucliSens miniMAG extraction method (bioMérieux, Marcy l'Etoile, France). Two hundred fifty microliters of each specimen was extracted and resuspended in a total volume of 50 μl. Flavivirus heminested RT-PCR using universal primers was modified from the method of Scaramozzino et al. (15) as follows. After amplification, 5 μl of the total nucleic acid was the template for RT-PCR, with 0.5 μM of primers CFD2 and MAMD and the QIAGEN one-step RT-PCR kit (QIAGEN Inc., Valencia, CA). The amplification reaction for the first round was performed under the following conditions: 30 min at 50°C; 15 min at 95°C; 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C; and a final extension step (10 min at 72°C). Five microliters of the product from the first round of PCR was amplified in a second round of PCR using primers CFD2 and FS778 (1× PCR buffer with a final [$MgCl_2$] of 1.5 mM, 0.5 μM of each primer, 10 μM deoxynucleoside triphosphate mix, 0.25 μl HotStarTaq DNA polymerase [QIAGEN Inc.]). Second-round thermocycler conditions were 15 min at 95°C; 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; and a final extension step (10 min at 72°C). Following RT-PCR, the product was run on a 2% agarose gel. The first-round PCR should generate a product of approximately 250 bp, and the second-round PCR should generate a product of approximately 220 bp. Bands of the expected size were cut from the gel and purified using an Ultrafree-DA centrifugal filter unit (Millipore, Billerica, MA). Sequencing reactions on the PCR products were performed using 3.2 pmol of the CFD2 and FS778 primers at the Wadsworth Center Molecular Genetics Core facility on an automated DNA sequencer model 3100 (Applied Biosystems, Foster City, CA).

Real-time RT-PCR detection. The real-time RT-PCR assay for DEN-1 is adapted from the method of Houng et al. (6). The forward primer for DEN-1 was modified to 5′-GGGAAGCTGTATCCTGGTGGTAA-3′. For this method, the assay was optimized to run as a one-step real-time RT-PCR using the SuperScript III platinum one-step quantitative RT-PCR system (Invitrogen, Carlsbad,

TABLE 1. Serological results of acute- and convalescent-phase sera from the patient[a]

Sample	MACELISA WNV P/N	MIA		SLEV IgG by IFA	Virus titer by PRNT	
		WNV-E	WNV-NS5		JEV	DENV
Acute-phase	3.7 (IND)	NR	NR	<16	<10	10
Convalescent-phase	6.4 (IND)	NR	NR	256	10	40

[a] Abbreviations: E, envelope protein; WNV, West Nile virus; SLEV, Saint Louis encephalitis virus; MACELISA, IgM antibody capture enzyme-linked immunosorbent assay; MIA, microsphere immunoassay; IFA, immunofluorescence assay; PRNT, plaque reduction neutralization technique; JEV, Japanese encephalitis virus; DENV, dengue virus; IND, indeterminate; NR, nonreactive.

CA) on an ABI 7500 sequence detection system instrument (Applied Biosystems). The 25-μl reaction mixture contained 0.5 μl of SuperScript III reverse transcriptase/platinum *Taq* mix, 12.5 μl of 2× reaction mix, 0.3 μM of each primer, 0.2 μM of probe, 0.5× Rox reference dye, 5.45 μl diethyl pyrocarbonate-treated H_2O (Ambion, Austin, TX), and 5 μl of template. RT-PCR amplification, which includes an initial RT step, was performed as follows: 30 min at 48°C, followed by 45 cycles, with 1 cycle consisting of 10 s at 95°C, 15 s at 95°C, and 1 min at 60°C. Data were collected each cycle after the 1-min step at 60°C. Results were analyzed using the ABI 7500 software.

RESULTS

An acute-phase serum specimen was collected from the patient. This specimen was IgM positive in the dengue MACELISA but negative in the dengue IgG ELISA at the CDC in San Juan, Puerto Rico. A serum specimen collected 3 weeks later was both IgM and IgG positive. The acute-phase serum specimen exhibited borderline reactivity in the West Nile virus MACELISA with a positive/negative ratio (ratio of the optical density of a test serum to the optical density of a known negative serum sample) (P/N) of 3.7 (Table 1). The polyvalent rWNV-E MIA and the rWNV-NS5 MIA were nonreactive. The convalescent-phase serum sample, collected 3 weeks later, also tested in the borderline region with a P/N of 6.4 in the WNV MACELISA. Plaque reduction neutralization tests, including JE virus and dengue virus tests, were performed on the paired sera. The titer against dengue virus rose from 10 to 40, whereas the titer against JE virus rose from <10 to 10 in the convalescent-phase specimen. These results are consistent with a primary dengue virus infection.

Nucleic acid was extracted from the nasal and pharyngeal swabs, both NPS specimens, and the serum specimens. Samples were tested using the conventional heminested RT-PCR assay for the detection of flaviviruses. As stated above, the expected size of the amplified DNA is approximately 250 bp in the first round of RT-PCR and 220 bp in the second round. The serum sample, nasal swab, and throat swab extracts produced a band of the expected size for the flavivirus-specific RT-PCR (Table 2). WNV was ruled out with a WNV-specific RT-PCR assay. The PCR products from the flavivirus-specific RT-PCR assay were sequenced, and a BLASTn search of the NCBI website was performed. A 171-bp sequence had 100% identity with the sequence of DEN-1 strain Myanmar. Extracts from the nose and throat swabs were positive for DEN-1 by real-time RT-PCR (Table 2).

Dengue virus grows slowly in culture. It can take up to 3 weeks before CPE become evident. The specimens were put in culture and passaged. CPE was observed in the acute-phase serum culture following one C6/36 passage and one Vero passage and in the nasal swab culture after one C6/36 passage and two Vero passages (Table 2). Virus was not isolated from the other respiratory samples. Positive IFA reactions (specific fluorescence) were observed in the acute-phase serum and nasal swab culture by using the DEN-1 MAb. All other samples were negative. DEN-1 virus was confirmed by real-time RT-PCR, conventional RT-PCR, and sequencing from the cell culture supernatants.

TABLE 2. Results of RT-PCR and culture for various specimens from the patient

Specimen	Conventional RT-PCR result[a]	Real-time RT-PCR result[b]	Culture result[c]
Serum sample	+	Undet	C1V1
NPS swab (first swab)	Undet[d]	Undet	Undet
NPS swab (second swab)	Undet	Undet	Undet
Nasal swab	+	36.4	C1V2
Pharyngeal swab	+	39.5	Undet

[a] A + sign indicates that a band of the expected size was obtained in the conventional RT-PCR assay.
[b] The numbers are the threshold cycle values obtained in the reaction. In this particular assay, values below 45 are positive.
[c] C1V1 is CPE first observed on or after one C6/36 cell passage and one Vero cell passage, and C1V2 is after one C6/36 cell passage and two Vero cell passages.
[d] Undet, undetected.

DISCUSSION

To enable timely diagnosis, etiological investigation, and disease control efforts, there is a need for rapid detection of dengue virus in the acute phase of illness. Therefore, molecular techniques are preferred methods of diagnosis, since they are rapid, sensitive, and specific. The disadvantages of molecular methods are that they require high-precision instruments and their results are highly dependent on the existence of nucleic acid sequence homologies among variant strains. Primer-template mismatches can lead to false-negative results.

The conventional RT-PCR method targets a conserved region of the NS5 gene, using a primer pair proposed by Kuno (9) and adapted by Scaramozzino et al. (15). We further adapted the assay and performed it as a one-step RT-PCR. The assay is a heminested, genus-specific RT-PCR which amplifies genomes from the pathogenic flaviviruses: YF virus, DEN1 to -4, JE virus, SLE virus, and WNV, among others. We determined that the PCR assay detects Powassan virus, a North American tick-borne encephalitis virus (data not shown). Sequencing of the PCR product and comparison of the amplified NS5 sequence with the sequences currently in the databases will aid in the identification of the flavivirus. Sequencing will also aid in eliminating false-positive results arising from generation of nonspecific PCR products of the expected size. The procedure can be used as a first-line diagnostic PCR screening method for pathogenic flaviviruses.

The real-time RT-PCR procedure is a 5′ nuclease assay, which targets the 3′ noncoding sequence (6). The 3′ noncoding region contains the most conserved sequence in the four dengue virus serotypes and is thought to be involved in viral replication, transcription, and virulence (16, 23). The original pro-

tocol for the real-time assay entailed a two-step assay requiring separate RT and PCRs. We adapted this procedure to a one-step reaction, and we redesigned the forward primer. A number of real-time RT-PCR master mixes were compared, and the one that gave the most efficient reaction and best sensitivity and specificity was selected. Modification of the assay made it less prone to cross-contamination and also decreased the turnaround time for obtaining results.

Serum IgM against the virus envelope protein is generally produced within 5 days of onset of infection, with subsequent production of IgG (14). However, identification of the specific serotype, via IgG and IgM ELISAs, can be problematic. Cross-reactivity is frequently observed among all four serotypes of dengue virus, when ELISA using whole-virus antigen for the detection of dengue virus-specific antibody is used; this is especially true for secondary dengue infection. Although culture is the gold standard for identification of dengue infections, the virus grows slowly, and timely results are not available. In addition, growth in culture is dependent on the recovery of viable virus from a specimen from the patient.

In the case reported here, laboratory diagnosis was based on IgM and IgG ELISAs, virus isolation, and RT-PCR. The ELISAs exhibited cross-reactivity but indicated flavivirus infection. Viral culture showed CPE indicative of virus growth, and the conventional RT-PCR, sequencing, and real-time RT-PCR confirmed and clarified the subtype of dengue virus that was present. This multimethod analysis led to the reliable detection of dengue virus in the patient's specimens. Interestingly, virus was detected by conventional RT-PCR, real-time RT-PCR, and culture in the nasal specimen collected 1 day prior to the other specimens (Table 2). This may indicate the importance of timing in the collection of specimens during the course of the disease, or it may indicate that nasal swabs are better specimen types for testing. Follow-up studies are warranted to determine the timing and applicability of nasal swabs for dengue patients.

Our interest in investigating the presence of dengue virus in the respiratory tract was severalfold. NPS specimens are relatively easy to obtain and transport and are the specimens of choice for rapid detection of influenza virus. Indeed, illness from influenza and dengue infections may be difficult to distinguish on clinical grounds, and the differentiation has major epidemiologic implications. Wesselsbron virus, which is a flavivirus of the YF virus subgroup, has been reported to be found in the throat wash fluid of a laboratory worker (8). Studies of dengue virus infections in monkeys have shown virus to be present in the upper respiratory tract at the end, or just after termination, of viremia (12). An outbreak of dengue virus infection in health care workers raised the possibility that aerosol transmission can occur (V. Gupta, S. Bhoi, and P. Aggarwal, presented at the 54th Annual Meeting of the American Society of Tropical Medicine and Hygiene, December 2005).

We have shown that a multipronged laboratory approach can be used to diagnose dengue infection and differentiate dengue virus serotypes. Furthermore, the modified real-time RT-PCR assay that we use has the advantage of being less prone to cross-contamination while displaying reduced turnaround time for results. To our knowledge, ours is the first study to have identified dengue virus in respiratory tract specimens. Whether NPS specimens represent a useful alternative to blood specimens for the diagnosis of dengue infection will require additional study.

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ADDENDUM IN PROOF

Since the acceptance of this manuscript, we have detected dengue virus 1 by PCR and sequencing in a serum sample and an NPS specimen from a patient with a diagnosis of dengue fever who had recently returned to the United States from Nicaragua. This second case adds support to the utility of testing respiratory specimens for the diagnosis of dengue virus infections.

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