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Use of Capillary Blood Samples as a New Approach for Diagnosis of Dengue Virus Infection[▽]

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We evaluated the use of capillary blood samples stored on filter papers for diagnosis of dengue virus infection. Venous and capillary blood samples were collected from 130 patients suspected of having dengue fever. We compared the performances of standard reference methods using capillary blood samples absorbed onto filter papers versus venous blood samples. The resulting sensitivity, specificity, and positive predictive value of tests performed on filter paper compared to those performed on venous blood samples were 81.6% (62/76; 95% confidence interval [CI], 74.9% to 88.3%), 90.7% (49/54; 95% CI, 85.7% to 95.7%), and 92.5% (62/67; 95% CI, 86.2% to 98.8%), respectively. During the acute phase of dengue virus infection (day 1 to day 4), the tests performed on capillary blood samples had a sensitivity of 88.5% (95% CI, 82.0% to 95.0%) and a specificity of 93.8% (95% CI, 88.9% to 98.7%). During the convalescent phase of infection, this method allowed the viral serotype to be determined for 4 of 15 (27%) dengue virus-infected patients for whom virological diagnosis using venous samples was negative. Capillary blood samples could therefore be a good alternative for the diagnosis of dengue virus infection in tropical areas. Indeed, these samples are convenient for storage and transport without the need for a cold chain and simplify the collection of samples from children. Moreover, our results suggest that viral particles persist longer in capillary blood than in peripheral blood. Analysis of the viability of viral particles under these conditions may give new insights into the physiopathology of dengue virus infection and the transmission of dengue virus during outbreaks.

Dengue virus, a group of four closely related viruses of the *Flaviviridae* family (dengue virus serotypes 1 to 4), is the most important flavivirus in terms of human morbidity. Dengue viruses are transmitted to humans by *Stegomyia aegypti* mosquitoes, formerly known as *Aedes aegypti*. It is estimated that every year there are more than 250,000 cases of severe dengue virus infection, called dengue hemorrhagic fever, and at least 100 million cases of uncomplicated dengue fever (12, 16). Dengue fever is characterized by fever, very often associated with headache, myalgia, arthralgia, rash, and occasionally hemorrhagic manifestations. Dengue hemorrhagic fever involves acute fever associated with a hemorrhagic diathesis and a tendency to develop shock (dengue shock fever) (7, 21).

Two virological techniques are routinely used for diagnosis of dengue virus infection: reverse transcriptase PCR (RT-PCR) and/or isolation in cell culture (5, 17). These techniques allow detection of the presence of dengue virus in venous blood samples taken during the early phase of illness (day 0 to day 4) (4, 11, 20). After day 5, during defervescence, serological diagnosis by μ chain affinity capture enzyme-linked immunosorbent assay (ELISA) can detect dengue virus-specific immunoglobulins M (IgM antibody capture [MAC]-ELISA) (5, 9). The increase of IgM titers appears especially in primary dengue virus infection cases, but in the case of secondary dengue virus infections, IgM can be detected earlier (21). For purposes of dengue diagnosis, the fifth day of fever is the day of transition from viral detection to testing for increased antibody titers (11).

All current techniques for the diagnosis of dengue virus infection require a venous blood sample, which can be difficult to obtain, especially from young children or for cultural reasons. Virological diagnosis also requires the transport of samples under cold conditions for the maintenance of viral integrity (6). The use of saliva samples has been considered as an alternative to venous blood samples for the detection of dengue virus-specific IgM and IgA (3). In addition, blotting paper has been used as a blood support for the diagnosis of human immunodeficiency virus and hepatitis C viruses. This approach has been valuable for epidemiological studies in the field and helps conserve biological samples (1, 2). Filter paper has also been proposed for the serodiagnosis of dengue virus infection, but the method used did not allow the identification of the viral serotype (13, 19). However, Prado et al. showed that strains of dengue virus stored on blotting paper were stable (14).

To overcome the problems of obtaining venous blood samples and of their conservation, especially in tropical regions, we evaluated the value of filter paper as a support for capillary blood samples in the diagnosis of dengue virus infection. We also looked at the possibility of detecting viral particles in capillary blood samples after the acute phase, in particular, at the fifth day of fever, when diagnosis of dengue virus using venous samples is difficult.

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MATERIALS AND METHODS

Sample collection. Sera and capillary blood samples were collected during serotype 3 and serotype 2 dengue (DEN-3 and DEN-2, respectively) virus outbreaks occurring in French Guiana between March 2005 and July 2006. The study included 130 patients presenting symptoms compatible with dengue fever, including fever, myalgia, and arthralgia, referred to the Institut Pasteur de la Guyane for biological diagnosis of dengue virus infection. The first day of fever was defined as day 1 of disease.

Ninety-three samples were obtained during the acute phase of infection (days 1 to 4), and 37 were obtained during the convalescent phase (day 5 onward).

For each patient, serum obtained by intravenous puncture was used for viral diagnosis (RT-PCR and/or isolation on *Aedes pseudoscutellaris* cells [AP61]) and serological diagnosis (detection of IgM antibodies to dengue virus). After informed consent was obtained from each patient, capillary blood samples were obtained from a finger and absorbed on filter paper for analysis by molecular methods (RT-PCR) and serological methods (ELISA) when enough biological material was available. The drop of collected capillary blood was deposited on a strip of Whatman filter paper (Schleicher and Schuell) and immediately placed in a tube at room temperature (20 to 25°C and 85 to 90% relative humidity) until it was taken to the laboratory for analysis (14).

Detection of dengue virus by RT-PCR analysis of capillary blood samples and venous blood samples. All venous and capillary blood samples were analyzed by RT-PCR. RT-PCRs with venous blood and blotting paper samples were carried out separately to avoid contamination. Filter papers containing capillary blood samples were cut into strips and placed in 1.8-ml tubes. Viral RNA was extracted from a 25-μl aliquot of serum and from the filter paper of each patient using TRIzol reagent (Invitrogen Life Technologies, Paisley, Refrewshire, United Kingdom), according to the manufacturer's recommendations. Dengue viruses were detected and typed according to Lanciotti et al. (10). The extracts were precipitated with isopropanol and 1 μl of glycogen (5 μg/μl) (Roche Diagnostics, Mannheim, Germany). Air-dried RNA pellets were suspended in 20 μl of water, and 5-μl aliquots were mixed with 200 ng of random hexamer primers. A SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) was used for first-strand cDNA synthesis according to the manufacturer's recommendations. The initial RT-PCR and subsequent seminested PCR were carried out as previously described (15).

Virus isolation from venous blood samples. All sera obtained during this study were used for virus isolation in tissue culture as described by Reynes et al. (15). Sera were diluted 1:10 in tissue culture medium (Leibovitz; Sigma) and applied to a confluent monolayer of AP61 cells. The cultures were incubated at 28°C for 1 h, and fresh tissue culture medium was added. The plates were then incubated at 28°C for 7 days. The cells were harvested, and the dengue virus serotype was identified using an indirect immunofluorescence assay with monoclonal antibodies specific to DEN-1, -2, -3, and -4 viruses (provided by the CDC, Fort Collins, CO).

Serological diagnosis. The best dilution for the MAC-ELISA reagents was determined in preliminary tests with positive and negative reference samples. The method used has been described by Talarmin et al. (18).

Each well of flat-bottomed microplates was coated with 100 μl of goat anti-human IgM diluted 1:500 in phosphate-buffered saline (PBS; Sigma Laboratories, l'Isle d'Abeau Chesnes, France). The microplates were incubated at 37°C for 2 h and then washed with PBS containing 0.05% Tween 20 (PBS-T).

Each serum sample was diluted 1:100 in PBS containing 0.5% Tween 20 and 5% nonfat dried milk (PBS-T-NDM), and 100-μl aliquots were transferred to each well of the microplates in duplicate. For capillary blood absorbed on filter paper, human antibodies were eluted as follows. The filter paper was incubated in 400 μl in PBS-T-NDM for 30 min at room temperature. The sample was centrifuged at 3,000 rpm for 10 min at 4°C, and 100 μl of the supernatant was transferred to microplates. Six negative and two positive reference sera were included on each plate as controls. The plates were then incubated at 37°C for 1 h and washed with PBS-T, and 100 μl (16 hemagglutination units) of DEN-2 virus antigen, prepared with sucrose acetone, or an uninfected control sample diluted in PBS-T-NDM was added to one of each pair of wells. The plates were incubated at 4°C overnight and washed with PBS-T, and bound antigens were detected with anti-dengue virus mouse ascitic fluid prepared in our laboratory and diluted 1:10,000 in PBS-T-NDM. Then, 100 μl of conjugated goat anti-mouse IgG peroxidase (Sigma Laboratories) diluted 1:1,000 in PBS-T-NDM was added to the wells, and the plates were incubated at 37°C for 1 h. The plates were washed three times, and tetramethylbenzidine was used as a substrate (100 μl per well) to reveal bound antibody. Positive reference sera appeared blue and negative reference sera remained clear. Fifty microliters of 0.5 N sulfuric acid was added to each well to block the reaction. The optical density at 450 nm (OD_{450})

was read using an ELISA reader (model LP 300; Sanofi Diagnostics Pasteur, Marnes la Coquette, France). For MAC-ELISA, the mean OD values ± standard deviations for the negative controls were determined. A serum was considered negative when the OD values were less than the mean value for the negative control plus 2 standard deviations, indeterminate if the OD value was between 2 and 3 standard deviations from the negative control value, and positive when the OD values were more than 3 standard deviations higher than the negative control value.

Statistical analysis. The sensitivity and specificity of the assays and the positive and negative predictive values were measured using two-by-two tables, which are commonly employed for the evaluation of diagnostic methods. The sensitivity and specificity of the capillary blood sample tests were determined by comparison of the results with those obtained with venous blood samples using routine diagnostic tests (viral isolation or/and RT-PCR and serology).

RESULTS

During the last two dengue outbreaks in French Guiana, 130 patients presenting clinical symptoms of dengue virus infection were included in the study. For each patient, a venous blood sample and a capillary blood sample absorbed into filter paper were obtained. The data obtained from capillary blood samples (RT-PCR and serology) were compared to those obtained from venous samples (viral isolation, RT-PCR, and serology) (Table 1). The serological analysis on capillary blood samples was performed when enough biological material was available: nine patients with a positive serological result on venous samples. Among the 130 analyzed capillary blood samples, 67 scored positive and 63 scored negative for dengue virus, whereas for the 130 corresponding venous blood samples, 76 scored positive and 54 scored negative. Identical results between capillary and venous samples were obtained for 111 patients (85.4%), while the other 19 gave unmatched results (14.6%). Of these 111 capillary blood samples with identical results, 62 scored positive for dengue virus infection and 49 scored negative. The sensitivity and the specificity of tests performed on filter paper were 81.6% (62/76; 95% confidence interval [CI], 74.9% to 88.3%) and 90.7% (49/54; 95% CI, 85.7% to 95.7%), respectively (Table 1). The positive predictive value was 92.5% (62/67; 95% CI, 86.2% to 98.8%) during this study.

Sixty-two patients were diagnosed with dengue virus infection by both specimen types (capillary and venous samples); 87.1% (54/62) of these patients gave samples during the acute phase, and 12.9% (8/62) gave samples during the convalescent phase of infection. The dengue virus was detected by molecular methods in 50 capillary blood samples and confirmed with venous blood samples tested by virological methods; 37 were DEN-2 serotype virus, and 13 were DEN-3 serotype virus. Seven of these 62 positive capillary blood samples were de-

TABLE 1. Sensitivity and specificity of tests performed on capillary blood specimens for the diagnosis of dengue virus infection[a]

Virus detection by RT-PCR or serology of capillary blood samples	Result of standard methods of virus detection with venous blood samples (no. of specimens)[b]	
	Positive	Negative
Positive	62	5
Negative	14	49

[a] Sensitivity, 81.6% (62/76 [95% CI, 74.9% to 88.3%]); specificity, 90.7% (49/54 [95% CI, 85.7% to 95.7%]).
[b] Isolation, RT-PCR, or serology.

TABLE 2. Performance of capillary blood sample testing according to the time of sample collection during the acute phase of infection

Day after the onset of DF[a]	No. of patients tested	No. of positive capillary blood samples[b]	No. of positive venous blood samples[c]	No. of double-positive patients[d]	Sensitivity (% [95% CI])	Specificity (% [95% CI])
1	27	21	21	21	100.0 (86.8–100.0)	100.0 (86.8–100.0)
2	29	19	18	18	94.7 (81.0–99.5)	90.0 (72.7–97.8)
3	22	9	12	9	75.0 (53.0–91.0)	100.0 (84.6–100.0)
4	15	7	9	6	66.7 (38.4–88.2)	83.0 (55.0–96.0)
Total	93	56	60	54	88.5 (82.0–95.0)	93.8 (88.9–98.7)

[a] DF, dengue fever.
[b] Patients with a positive diagnosis for dengue virus infection by capillary blood sample testing.
[c] Patients with a positive diagnosis for dengue virus infection by testing venous blood samples.
[d] Patients with a positive diagnosis for dengue virus infection by testing both their capillary and their venous blood samples.

tected by RT-PCR (five with DEN-2 serotype and two with DEN-3 serotype) and were confirmed only by IgM serological analysis of their venous samples. Three of the capillary samples from these seven patients were obtained during acute dengue virus infections, and four others were obtained during the convalescent phase of infection. Finally, dengue virus infection in the remaining five patients (of the 62) was detected by only serological methods from their respective capillary and venous samples. The virological analyses were negative for these patients regardless of the specimen type used.

Venous and capillary blood samples from 93 patients collected between day 1 and day 4 of dengue virus infection were analyzed. Of these 93 patients, 54 presented a positive dengue virus infection (Table 2). By comparison with results obtained for venous samples analyzed by reference techniques, testing capillary blood collected onto filter paper between day 0 and day 4 of dengue virus infection showed a sensitivity of 88.5% (95% CI, 82.0% to 95.0%) and a specificity of 93.8% (95% CI, 88.9% to 98.7%). Table 2 shows the performance of capillary blood sample testing according to the timing of the test (number of days after onset of fever). Dengue viruses of all serotypes were detected by testing capillary blood samples absorbed onto filter paper. Among the selected population, 37 samples were collected after the acute phase of infection, on day 5 or later. Venous blood sample testing detected IgM against dengue virus in 40.5% (15/37) of these patients. Corresponding capillary blood samples from these 15 patients were tested for viral sequences by RT-PCR. The test was positive for four patients, who gave samples on days 5, 6, 8, and 12 after the onset of clinical symptoms (Table 3). Two of these patients had DEN-2 serotype infections, whereas the two others had DEN-3 serotype infections. The IgM serology of capillary samples of these four patients also confirmed their recent infection by dengue virus. In conclusion, it was possible to

detect viral particles in capillary samples absorbed into filter paper by RT-PCR during the convalescent phase of infection in 4 of the 15 cases (27%) although virological diagnosis using venous blood samples was negative.

DISCUSSION

New dengue virus infection diagnosis techniques are required to overcome the constraints associated with collecting venous blood samples for dengue virus infection diagnosis in children. Moreover, it would be useful to develop a method able to identify the circulating serotypes of dengue virus involving simplified logistics for the chain from the field to the laboratory (with no necessity for a cold chain). We therefore evaluated the performance of a new technique for the diagnosis of dengue virus infections: collection of capillary samples onto filter paper and analysis of capillary viremia during dengue virus infections. Our study showed that for diagnosis of dengue virus infection, capillary blood samples taken from the finger might be a satisfactory alternative. This specimen type had a sensitivity of 81.6% (95% CI, 74.9% to 88.3%) and a specificity of 90.7% (95% CI, 85.7% to 95.7%). By comparing capillary blood samples versus venous samples analyzed only by RT-PCR, the sensitivity was found to be better than or equal to 90.7% (95% CI, 85.7% to 95.7%) but with some loss of specificity, equal to 82.9% (95% CI, 76.4% to 89.4%). This new approach has two advantages for diagnosis of dengue virus infection. First of all, these capillary blood samples are convenient for storage, transportation, and sample collection, particularly from children. Secondly, this specimen type allows identification of the virus serotype involved in the infection and the detection of dengue virus-specific IgM antibodies.

During the acute phase of infection, the sensitivity was 88.5% (95% CI, 82.0% to 95.0%), and the specificity was

TABLE 3. Analysis of the four patients with capillary samples collected during the convalescent phase and positive by RT-PCR

Patient no.	Day postinfection	Virus detection in venous blood samples by:			Virus detection in capillary blood samples by:	
		Cell isolation	RT-PCR	IgM serology	RT-PCR (serotype)	IgM serology
1	5	Negative	Negative	Positive	Positive (DEN-3)	Positive
2	6	Negative	Negative	Positive	Positive (DEN-2)	Positive
3	8	Negative	Negative	Positive	Positive (DEN-3)	Positive
4	12	Negative	Negative	Positive	Positive (DEN-2)	Positive

93.8% (95% CI, 88.9% to 98.7%). Therefore, in view of the simplicity of the capillary collection method and its performance (positive predictive value during the epidemic, 92.5%; diagnosis specificity, 90.7%), testing this type of sample could be very useful for epidemiological studies in the field. This approach may be particularly valuable for studies at the beginning of an epidemic of suspected dengue fever in a region of endemicity, as it can detect asymptomatic dengue virus infection without the need of venous puncture.

Moreover, in 4 of 15 cases (27%), this method allowed detection of viral particles in capillary blood samples collected after the acute dengue virus infection, at a stage when tests for dengue virus in the corresponding venous samples were negative. This is very important for assessing the epidemic risk as the epidemic starts. This method seems to be more sensitive than virological tests on venous samples. Four patients who gave samples 5, 6, 8, and 12 days after the onset of the disease scored positive for dengue virus in tests with capillary blood, and infection was suspected by the occurrence of clinical symptoms and the presence of dengue virus-specific IgM in their sera. To confirm these findings, sequential capillary blood samples from dengue virus-infected patients should be studied to follow viremia in the capillary blood system. If the viremia in capillary samples can be detected longer than venous viremia, as already indicated by Kuno (8), it would be interesting to investigate the nature of the viral particles detected by testing their capacity to infect cell culture lines, for example. This would be informative about the pathogenesis of dengue virus infection. Chikungunya virus, an alphavirus, and another arbovirus have viremia kinetics similar to those of dengue virus, characterized by the detection of viral particles between day 1 and day 4 using virological methods. However, in patients infected by this alphavirus during the Reunion Island outbreak, viral particles were detected by RT-PCR in some patients until 12 days after the onset of the disease (F. Staikowski, Intensive Care Unit, Saint Pierre Hospital Reunion Island, personal communication). In conclusion, the use of capillary blood samples taken from the finger and stored on blotting paper appears to be an alternative to venous samples for the diagnosis of dengue virus infections. This specimen type could be a very useful tool for dengue surveillance. Moreover, these preliminary results suggest that it would be interesting to investigate the late capillary viremia observed in patients during the convalescent phase of infection.

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