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Short communication

Detection of the dengue non-structural 1 antigen in cerebral spinal fluid samples using a commercially available enzyme-linked immunosorbent assay

F.M.C. Araújo[a,b,c,*], R.S.N. Brilhante[c,d], L.P.G. Cavalcanti[b], M.F.G. Rocha[d], R.A. Cordeiro[c,d], A.C.B. Perdigão[a,e], I.S. Miralles[a,b], L.C. Araújo[f], R.M.C. Araújo[f], E.G. Lima[a,e], J.J.C. Sidrim[c,d]

[a] Central Public Health Laboratory of Ceará, Brazil
[b] State Health Secretariat of Ceará, Brazil
[c] Postgraduate Program in Medical Sciences, Federal University of Ceará, Brazil
[d] Specialized Center of Medical Mycology, Ceará, Brazil
[e] Northeast Biotechnology Network, State University of Ceará, Brazil
[f] Federal University of Ceará, Brazil

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A B S T R A C T

The involvement of the central nervous system in dengue infections has been reported in countries where the disease in endemic. The purpose of this study was to determine whether an enzyme-linked immunosorbent assay kit designed to detect the dengue NS1 antigen in serum was able to detect this antigen in cerebral spinal fluid (CSF) samples from patients with fatal outcomes. To evaluate the sensitivity of the kit, 26 dengue-positive CSF samples were used. The Pan-E Dengue Early kit was able to detect the NS1 antigen in 13 of 26 dengue-positive CSF samples, resulting in a sensitivity of 50% (95% confidence interval, 29.9–70.1%) and specificity of 100% (95% confidence interval, 75.3–100%). The kit was able to detect the NS1 antigen in CSF of individuals who had died of dengue. When used in combination with IgM, the detection rate rose to 92.3%. This study reports a method for rapidly detecting the dengue virus in CSF, thereby increasing the diagnosis of dengue fever cases with unusual neurological manifestations.

Dengue fever is a mosquito-borne disease caused by one of four dengue viruses (DENV), DENV-1, DENV-2, DENV-3 and DENV-4, which belong to the Flavivirus genus of the Flaviviridae family (Gubler, 2002; ICTV, 2006). Its prevalence has been increasing in recent decades. Currently it is endemic in over 100 countries in Africa, the Americas, Eastern Mediterranean, Southeast Asia and Western Pacific (WHO, 2009).

In Brazil, dengue epidemics have been described since 1986, initially with involvement of DENV-1, followed by DENV-2 in 1990, and DENV-3 after 2000 (Nogueira et al., 2007). In the state of Ceará in Northeast Brazil, the disease is endemic and is caused by serotypes 1, 2 and 3, with cases reported every year and periodic epidemics. The highest number of cases was observed in 1987, 1994, 2001 and 2008. In 2003, a severe DENV-3 epidemic occurred, and dengue hemorrhagic fever (DHF) incidence was high among adults (Cavalcanti et al., 2010).

Dengue's symptoms can vary from mild fever, the most common form, to potentially fatal forms, such as dengue DHF fever and dengue shock syndrome (DSS). Unusual manifestations, such as myocardiopathy, hepatic insufficiency, fulminant hepatitis, encephalopathy and encephalitis, have also become common (Nogueira et al., 2007). These findings are due perhaps to the increase of dengue diagnosis. The involvement of the central nervous system (CNS) in dengue patients has been reported in countries where the disease is endemic. There is no antiviral therapy or vaccine approved for use against dengue, so patient management requires good laboratory and clinical support. Specific and rapid diagnosis can help directing the proper treatment to patients (WHO, 2009).

The DENV has three genes that encode for structural proteins: envelope (E), membrane (M) and capsid (C), and seven genes that encode for non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The glycoprotein NS1 is highly conserved, secreted by cells infected with DENV in vitro and in vivo, but its biological activity is still not well understood. During in vitro infection, NS1 protein is expressed in a form associated with the intracellular membrane, which is essential for viral replication, or associated with the cell surface, which can be involved in signal transduction (Mackenzie et al., 1996). NS1 protein, in solution, circulates and accumulates in the plasma of patients infected with DENV throughout the clinical phase, and can be correlated with the development of more serious forms of the disease (Libraty et al., 2002).

* Corresponding author at: Av. Barão de Studart 2405, Postal Code: 60120-002, Fortaleza, CE, Brazil. Tel.: +55 85 31011498; fax: +55 85 31011493.
E-mail address: fernanda.montenegro@lacen.ce.gov.br (F.M.C. Araújo).

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Table 1
Detection rate for the methods used to diagnose dengue according to the available samples of patients with DENV infection.

Clinical sample	RT-PCR[a] P/S (%)	Virus Isolation P[b]/S[c] (%)	Serotype detected	IgM[d] P/S (%)	NS1 Ag[e] P/S (%)
CSF[g]	9/26 (34.6)	3/26 (11.5)	3 DENV-2 4 DENV-3 2 DENV-1	19/26 (73.1)	13/26 (50)
Blood or serum	2/20 (10.0)	2/26 (7.7)	2 DENV-3 1 DENV-1	4/11 (36.4)	–[f]
Total	11/46 (23.9)	5/52 (9.6)	3 DENV-2 6 DENV-3 3 DENV-1	23/37 (62.2)	13/26 (50)

[a] Reverse transcriptase-polymerase chain reaction (RT-PCR).
[b] Positive (P).
[c] Studied (S).
[d] Immunoglobulin M (IgM).
[e] Non-structural antigen (NS1 Ag).
[f] Not done (–).
[g] Cerebral spinal fluid (CSF).

Several new commercial assays for detection of the NS1 antigen have been developed. The employment of the enzyme-linked immunosorbent assay (ELISA) to detect NS1 protein from DENV in serum or plasma of patients with acute disease serves as a supplementary method for use in association with other diagnostic tests (Dussart et al., 2008; Blacksell et al., 2008). However, there are no reports of the use of these tests on the cerebro spinal fluid (CSF). This application would be very useful to diagnose cases of non-classical dengue.

The purpose of this study was to evaluate the performance of the immunosorbent assay Pan-E Dengue Early ELISA (Panbio Diagnostics, Brisbane, Australia) (NS1 Early), in detecting the presence of the NS1 antigen (NS1 Ag) in CSF samples obtained during autopsies.

Epidemiological and clinical data were obtained from a national database system that provides information on age and sex of each patient, CSF collection and the date when symptoms occurred. Clinical data and CSF samples were collected from patients who died of a dengue-like illness, and were autopsied at the municipal coroner's office in the context of the dengue surveillance activity of the Ceará State Health Secretariat. CSF specimens from people with other diagnosed diseases (HIV infection, leptospirosis, visceral leishmaniasis, pneumonia, fungal meningitis, meningococcal meningitis and other bacterial forms of meningitis) were used to evaluate the specificity of the assay kit. Contamination of CSF with blood was an exclusion criterion for the samples.

CSF samples were tested for the presence of DENV by viral isolation in C6/36 cells, as previously described (Gubler et al., 1984); genome detection was by RT-PCR (Lanciotti et al., 1992); and IgM detection was by IgM-capture with the Enzyme-Linked Immunosorbent Assay (ELISA) (Panbio, Brisbane, Australia). All assays were conducted according to the manufactures 'instructions, using a 1:2 sample dilution (Soares et al., 2006). Cases were considered positive for DENV infection when CSF samples were positive according to one or more of these tests and patients presented dengue-like syndrome. Cases were considered negative when CSF samples were negative for all of these tests and were diagnosed as suffering from another disease.

The study was approved by the ethics and research committee of São José Infectious Disease Hospital (protocol 005/2009, number CAAE:0005.0.042.000-09).

The NS1 Early system is an antigen-capture ELISA that provides qualitative, non-serotype-specific detection of DENV NS1 Ag. All tests were performed in accordance with the manufacturers' instructions. Briefly, 100 μL of diluted (1:2) samples, positive control, negative control and calibrator were added to microwells that were pre-coated with a polyclonal capture anti-NS1 antibody and then incubated for 1 h, at 37 °C. Each plate was washed six times and incubated for 1 h at, 37 °C, following the addition of HRP-conjugated anti-NS1 Mab. After six washes, antibody complexes were detected by adding TMB and incubating the plate for 10 min at room temperature. The reaction was stopped by adding a stop solution, and the plate was read at 450 nm, with a 630 nm reference filter. A sample ratio was determined for each sample by dividing the average optical density (OD) of the test sample by the average OD of the cutoff control. Sample ratios of <0.5, 0.5 to <1.0, and ≥1 were indicative of negative, inconclusive and positive results, respectively. Inconclusive samples were considered negative after the tests were repeated and remained inconclusive.

To evaluate the performance of the test statistical measures of sensitivity and specificity, a confidence interval (CI) of 95% was used.

CSF samples from 26 patients who presented fatal dengue infection and 60 negative for dengue were selected to assess the performance of the NS1 Early kit. All these 26 positive samples were positive for dengue in CSF previously by at least one other diagnostic method. The kit showed 73 negative and 13 positive results for the presence of NS1 Ag. The 13 positive samples were previously positive for DENV in CSF. The detection rate for the methods used to diagnose dengue, according to the samples of patients with DENV infection, is shown in Table 1.

The results of sensitivity and specificity in relation to the detection of NS1 Ag in CSF can be seen in Table 2. Two CSF samples that gave ambiguous results were retested and considered negative. The percentage of detection of the NS1 Early test in CSF of patients with laboratory diagnosis of dengue fever, according to the IgM detection methodology, is shown in Table 3. The detection rate of the combination of IgM antibodies and NS1 Ag was 92.3% (Table 4).

In patients with dengue, the average between the onset of symptoms to death was six days, ranging from 1 to 14 days. The signs and symptoms presented were: fever (77%); headache (42.3%); vomiting (30.7%); asthenia (26.9%); myalgia (26.9%); dyspnea (19.2%); mental confusion (19.2%); abdominal pain (15.4%); agitation (11.5%); hemorrhage (11.5%); somnolence (7.7%); splenomegaly (7.7%); chills (7.7%); cough (7.7%); diarrhea (7.7%); coma (7.7%); and, at a lower percentage (3.8%), anorexia, abdominal bloating,

Table 2
Diagnostic accuracy score for the NS1 detection by ELISA kit in CSF samples.

Diagnosis	Positive	Negative	Total
Dengue	13	13	26
Non dengue	0	60	60
Total	13	73	86
Sensitivity	50% (95% CI[a]-29.9–70.1)		
Specificity	100% (95% CI-94.0–100)		

[a] Confidence interval (CI).

Table 3
Detection rate of NS1 Ag according to the presence of IgM in 26 CSF samples.

	Presence of IgM[a]	Absence of IgM	Total
NS1+[b]	9	4	13
NS1−[c]	10	3	13
Total	19	7	26
Detection (%)	9/26 (34.6)	4/26 (15.4)	13/26 (50)

[a] Immunoglobulin M (IgM).
[b] Non-structural 1 antigen positive (NS1+).
[c] Non-structural 1 antigen negative (NS1−).

limb stiffness, ear infection, cyanosis, nausea, chest pain, pancytopenia, hypotension, sweating, acute renal failure, septic shock, intra-orbital swelling, dizziness, paresthesia, hallucinations and convulsion.

Of the 26 dengue patients, 9 had co-morbidities: one had a respiratory infection, three had bacterial meningitis, one had Wilson's disease, one had infectious enteritis, one had pneumonia, one had an ear infection and one had a varicella-zoster virus infection. Six patients were classified as DHF, one as DSS and the other 19, who did not meet the World Health Organization criterion for DHF classification, were considered as having severe dengue.

The mean age of patients was 27 years, ranging from 2 months to 84 years, and 26.9% (7/24) of them were younger than 7 years old. Females accounted for 62.5% of the cases and males for the other 37.5%.

The increasing transmission of the dengue virus continues to be a global public health problem, especially in developing countries, where access to preventive and diagnostic resources is limited. Since the appearance of this virus in Brazil, specifically in the state of Ceará, in 1986, the number of serious cases of the disease has been increasing steadily in line with other tropical and subtropical regions of the world, where the disease has become endemic, with cyclical variation: years of substantial epidemics followed by non-epidemic years (WHO, 2009).

The severe forms of the disease have caused unusual manifestations of dengue, such as neurological signs. DENV virus, though not considered neurotropic, has been isolated or its viral antigens have been observed in human CNS (Miagostovich et al., 1997; Ramos et al., 1998). This also has been demonstrated by viral isolation and detection of viral RNA in CSF and demonstration of intrathecal synthesis of specific dengue antibodies (Chen et al., 1991; Nogueira et al., 2002; Puccioni-Sholer et al., 2009). However, Rosen et al. (1999), could not evidence virus replication in the brain in fatal human infections.

The results of this study show that NS1 Ag can be detected in CSF by using a commercially available kit: NS1 Early, even though this kit was not standardized for testing CSF. There is no kit available designed to detect IgM antibodies in CSF either. Instead, this detection is generally done using a kit with standardized serum. Soares et al. (2006) found this antibody functioning with CSF in a 1:2 dilution. However, to detect IgM the patient must have had the disease for more than five days, whereas viral antigens can be detected only during viremia, thus enabling diagnosis.

Table 4
Detection of IgM and NS1 Ag in CSF samples.

Test	Positive	Negative	Detection (%)
IgM[a]	19	7	73.1
NS1[b]	13	13	50
IgM-NS1 (combined)[c]	24	2	92.3

[a] Immunoglobulin M (IgM).
[b] Non-structural antigen (NS1 Ag).
[c] IgM and/or NS1 Ag positive (combined).

NS1 Ag was detected in 50% of the cases where CSF had been found positive by other methods. The sensitivity of serum NS1 Ag detection by this kit ranges from 60.4% (Dussart et al., 2008) to 91.6% (Serakan et al., 2007). It is known that the quantity of IgM in CSF is lower than in serum (Chen et al., 1991; Puccioni-Sholer et al., 2009). Maybe the same phenomenon occurs in the presence of NS1 Ag in CSF. Taking into account that there are no published parameters for comparing this finding of NS1 Ag in CSF, it should be considered one more method for the diagnosis of dengue in this type of material, thus opening the discussion on its applicability in this type of sample. However, the use of NS1 Ag combined with IgM detection increased dengue diagnosis in CSF to 92.3%, consistent with a recent assessment of these two kits in serum reported by Blacksell et al. (2008).

The specificity values of 100% for the NS1 Early kit found when testing CSF were similar to other reports in the literature with the use of serum (Blacksell et al., 2008; Dussart et al., 2008; Lima et al., 2010). However, Guzman et al. (2010) found 90% specificity when tested in healthy blood donors and patients with other confirmed diagnoses.

A study using the NS1 Early kit showed that when IgM was present in the serum, the sensitivity of NS1 Ag detection fell from 91.6% to 48.3% (Serakan et al., 2007). This also happened in the CSF samples studied here, but despite the average of six days between the onset of the disease and the sample collection, the detection rate decreased slightly in relation to the presence of IgM, from 57.1% to 47.4% for the NS1 Early kit. This fact is probably due to the type of patient, since all the samples were taken from patients with fatal outcomes. They could have had higher levels of the NS1 glycoprotein circulating in the plasma and higher levels of viral RNA, which could be one of the reasons for the development of serious forms of the disease, as suggested by Libraty et al. (2002), even though many patients with high levels of viremia never develop clinical complications. However, the severe syndrome has been observed in patients who present circulating heterotypic dengue antibodies at high concentrations (Halstead, 2009).

This evaluation shows that the NS1 Early kit for detection of NS1 Ag in serum can be used on CSF samples from patients diagnosed with dengue. Serological tests are simple, rapid and easy to perform, but the average lifespan of IgM antibodies, which can be up to two months, confuses the diagnosis in cases where the date of disease onset is unknown, leaving questions about whether the infection is acute or recent (Innis, 1997). The detection of the NS1 antigen combines the accuracy and speed of RT-PCR with the practicality of the ELISA technique, providing a reliable result that facilitates clinical management of patients with neurological manifestations.

The use of tests to detect the NS1 Ag allows studying the involvement of the CNS in patients suspected of being infected with DENV, enabling better support for patients with severe forms of the disease. Therefore, the results of this study suggest the use of the Pan-E Dengue Early ELISA assay to detect viral NS1 Ag in CSF, to diagnose severe forms of dengue that present neurological manifestations in endemic regions for dengue. However, it should be used in association with DENV IgM antibody detection in CSF, in order to increase the sensitivity of the diagnosis. Besides this, more studies, including during non-epidemic periods and prospective studies, are needed to assess better the accuracy of the kit on CSF samples.

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