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Evaluation of Commercially Available Assays for Diagnosis of Acute Dengue in Schoolchildren during an Epidemic Period in Medellin, Colombia

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Abstract. During an active surveillance study in school children in Medellin, we assessed the performance of two diagnostic strategies for dengue virus. A total of 41 patients with suspected dengue acute infection were evaluated. Diagnostic strategies consisted of one combining Panbio® Dengue virus IgM and IgG Capture ELISAs (enzyme-linked immunosorbent assays) with reverse transcriptase polymerase chain reaction (RT-PCR) and another using a commercial rapid SD Bioline Dengue Duo (IgG/IgM + NS1 Ag) test. These two strategies were compared with the enzyme-linked immunospot microneutralization test (ELISPOT-MNT). The sensitivity and specificity were 53.9% and 80.0% for the combination of Panbio® ELISAs and RT-PCR tests, and 30.8% and 73.3% for the SD Bioline Duo test, respectively. ELISPOT-MNT detected 16.4% additional cases and revealed the presence of neutralizing antibodies in all the acute samples, evidencing that they were all secondary infections. In contrast, Panbio® and SD Dengue Duo rapid tests only classified 23.0% and 26.9% of the cases as secondary dengue infections, respectively. Cohen's kappa coefficient and McNemar's association tests demonstrated a significant disagreement between the two diagnostic strategies and ELISPOT-MNT. Overall, these results evidence the relatively poor performances of commercial assays for the diagnosis of acute and secondary dengue infections, compared with ELISPOT-MNT, and raise concern about the accuracy of these assays for the diagnostic of dengue in endemic areas.

INTRODUCTION

Dengue is an increasing problem in tropical and subtropical regions worldwide. It was estimated that about 390 million (95% confidence interval [CI] = 284–528) cases of dengue infections occurred in 2010, of which 96 million (95% CI = 67–136) manifest apparently.[1] Dengue displays a wide range of clinical manifestation from unapparent, mild fever to severe and fatal disease.[2] In Colombia, 147,257 dengue fever cases and 9,755 cases of severe dengue were reported in 2010.[3]

Dengue virus (DENV) is a positive-stranded RNA virus of the genus *Flavivirus*. There are four antigenically distinct DENV serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) that display high degree of antigenic cross-reactivity with other mosquito and tick-borne flaviviruses.[4] In Americas, DENVs are mainly transmitted to humans by *Aedes aegypti*, a highly domestic mosquito whose females display a strong anthropophilia.[5] All the four serotypes are present in the country.[6] After infection with any of DENV serotype, a long-lasting serotype-specific immune response is induced. Secondary infection with another serotype has been associated with more severe disease, because of antibody-dependent enhancement by cross-reactive antibodies and cross-reactive T cells. Nevertheless, most of the secondary infections are asymptomatic or result in non-severe disease.[7]

There is no specific treatment of DENV infection. However, early diagnosis and good supportive care can improve patient management and decrease dengue mortality.[8] Thus, diagnostic assays with good sensitivity and specificity are needed to differentiate DENV infections from other febrile illness such as leptospirosis, malaria, chikungunya, Zika, influenza, and others.

Some laboratory tests can be used to confirm dengue infection, such as detection of DENV nonstructural (NS1) antigen, detection of anti-DENV IgM and IgG antibodies, real-time polymerase chain reaction (RT-PCR), and viral isolation. In general practice, detection of anti-DENV antibodies is the most widely used test.[9] In Colombia routine diagnosis of dengue is performed by detection of anti-dengue IgM antibodies and, in few cases viral isolation for epidemiological purposes.

Levels of anti-DENV IgM antibodies are detected in serum samples by enzyme-linked immunosorbent assay (ELISA). Anti-DENV IgM antibodies are detectable from day 3 to 5, and in 99% of the patients by day 10 postinfection. A positive result in patients with clinical manifestations may be indicative of either active primary or secondary dengue infections. However, during secondary infections, IgM antibodies may not be detected.[2,10] In contrast, the detection of anti-DENV IgG antibodies in serum is then useful for determination of secondary infections since high levels of anti-DENV IgG antibodies can be detected in the acute phase and can persist for long periods.[2] The standard method for the differentiation of primary and secondary dengue infection is the hemagglutination inhibition (HI) test, which is based on the ability of dengue antigens to agglutinate animal or human red blood cells.[11]

Typing anti-DENV antibodies is difficult due to cross-reactivity between the different DENV serotypes and with others mosquito and tick-borne flavivirus. Russell and Nisalak[12] developed an in vitro assay using plaque reduction to measure DENV neutralizing antibodies and identify the infecting serotype. This assay is known as the plaque reduction neutralization test (PRNT).[12] It is still used to measure DENV serotype-specific neutralizing antibodies and to make inference on the DENV serotype[13] (despite no universal definition has been developed for the interpretation of the PRNT for this purpose).[14]

However, standard methods such as HI and PRNT are labor intensive, time consuming and require acute and convalescent sera with an interval of more than 7 days, which makes them inadequate for routine early diagnosis.[15] Thus,

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HI test has been gradually replaced by capture IgM and IgG ELISAs,[11] and several microneutralization tests (MNT) have been developed to overcome the limitations of PRNT. Enzyme-linked immunospot (ELISPOT) MNT is a test performed in a 96-well format, where viral plaques develop after 2–4 days of culture and viral antigen is detected in infected cells by an indirect immunostaining method. Infected cells are turned into spots that are detected automatically with an ELISPOT reader.[16]

In 2010, Colombia experienced the worst dengue outbreak in its history. During this epidemic year and the following post-epidemic period, an active surveillance study of dengue cases in schoolchildren was performed in the Colombian City of Medellin.[17]

We used samples from this study to evaluate the performances of commercially available assays in comparison to ELISPOT-MNT used as a reference method. The specific aims of this study were 1) to evaluate the accuracy of the combination of Panbio® IgM and IgG Capture ELISAs plus RT-PCR and the accuracy of the SD Bioline Duo rapid test (NS1 Ag and IgM/IgG) for the diagnosis of acute dengue infection in pediatric patients with febrile syndrome and 2) to determine the accuracy and agreement of Panbio® IgM and IgG Capture ELISAs and SD Bioline Duo rapid test to classify dengue infection in pediatric patients as primary or secondary.

MATERIALS AND METHODS

Full description of the study has already been published.[17] In brief, from May 2010 to December 2011, school-aged children from grade 5 to 11 were enrolled in a prospective longitudinal study conducted in three schools in Medellin, Colombia. During the study period, dengue cases were identified by school absenteeism due to a febrile episode in the previous 7 days. Two blood samples were collected in the acute and in the convalescent phases (14–21 days after the date of the onset of symptoms). A total of 299 samples were obtained and were evaluated by RT-PCR (as described elsewhere),[18] and with Panbio® Dengue IgM and IgG Capture ELISAs. Among these samples 41 (of each positive and negative samples) were also evaluated by SD Bioline Dengue Duo test and with ELISPOT-MNT.

Panbio® Dengue IgM Capture ELISA and Dengue IgG Capture ELISA (Alere, Australia) were used to qualitatively determine IgM and IgG antibodies to DENV, respectively, following manufacturer's instructions. Acute DENV infection was defined by IgM or IgG seroconversion and/or detection of virus genome by RT-PCR. Positive cases were classified as primary dengue infection if IgG test was negative in the acute serum sample or secondary DENV infection if the test was positive. SD Bioline Dengue Duo test (Standard Diagnostics, Kyonggi-do, South Korea) was used according to the manufacturer's instruction to detect the DENV NS1 antigen, IgM and IgG anti-DENV antibodies. Only acute samples were tested with this method. Results of this test were interpreted as dengue positive if NS1antigen and/or IgM were positive. Positive cases were classified as primary or secondary dengue infection when IgG test was negative or positive, respectively.

To perform ELISPOT-MNT, Vero cells ATCC®CCL-81™ (American Type Culture Collection, Manassas, VA) were seeded in 96-well polystyrene plates. Serum samples were inactivated at 56°C for 30 minutes, and 4-fold serial dilutions were carried out starting at 1:20 in BA-1 medium. Working stocks of virus that contained 100–200 plaque-forming unit of DENV were prepared. Prototype viruses used for this test were DENV-1 16007, DENV-2 16681, DENV-3 16562, and DENV-4 1036. Of each serum dilution, 50 μL was combined with 50 μL of each virus and incubated for 16 hours at 4°C. After incubation, 50 μL of the virus–serum mixture was transferred to 90% confluent VERO cells. After an additional incubation for 2–3 days, the cells were fixed with 3.7% formaldehyde. Immunostaining was performed by incubation of the plates (overnight at 4°C) with polyclonal rabbit antibodies against each serotype of DENV, followed by incubation (2 hours at 37°C) with goat anti-rabbit IgG (H+L) antibodies coupled to horseradish peroxidase (Thermo Fisher Scientific Inc., Waltham, MA). Revelation was performed with the substrate-chromogen 3-amino-9-ethylcarbazole, and the number of foci in each well was counted with an automatized C.T.L ImmunoSpot® analyzer (Cellular Technology Ltd., Shaker Heights, OH).

The ELISPOT-MNT_{50} titer was defined as the dilution of serum that reduced the number of plaques by at least 50% when compared with the virus-only control. When ELISPOT-MNT_{50} values were < 1:20, serum was considered containing no detectable neutralizing antibodies.

Acute infection was defined serologically by a 4-fold rise in neutralizing antibody titers between the acute and convalescent samples of a single patient, as determined using ELISPOT-MNT, whatever the serotype of DENV. Cases that were negatives in the acute sample but positives to any DENV serotype in the convalescent sample were considered as primary DENV infection. Cases with neutralizing antibodies to two or more DENV serotypes in the acute sample were defined as secondary DENV infection. Nonreactive cases were determined by absence of neutralizing antibodies in both samples. Non-recent infections were determined by the presence of neutralizing antibodies in both acute and convalescent samples and no changes/reductions in the ELISPOT-MNT_{50} titer. Homotypic or heterotypic immune response were defined as positive responses against single or multiple serotypes, respectively.[14]

Statistical analysis was performed using EPIDAT version 3.1 (Pan American Health Organization, Xunta of Galicia, Spain). Cross-tabulation was conducted to assess the sensitivity, specificity, positive and negative predictive values (PPV/NPV), and positive and negative likelihood ratios (LR−/LR+) of the different tests. The agreement between the different methods and ELISPOT-MNT was assessed by Cohen's kappa coefficient (κ) and McNemar's test. Significance was assigned at $P \leq 0.05$. Uncertainty was expressed by 95% confidence intervals (95% CI).

Ethical human subjects protocol approval. Only schoolchildren whose parents or legal guardians provided written informed consent were enrolled in the study. This research was approved by the Ethical Review Committee of Instituto Colombiano de Medicina Tropical, Universidad CES.

RESULTS

The ages of the 41 selected patients ranged from 6 to 18 years (mean = 12.1, SD = ±3.0). Acute samples were drawn 3.4 ± 1.4 days and convalescent samples were drawn 14.0 ± 3.8 days after the onset of symptoms.

Using the combination of RT-PCR and Panbio® IgM and IgG Capture ELISAs, 41.5% (17/41) were classified as positive for DENV infection. Two of them were found positives simultaneously by RT-PCR, IgM and IgG detections; five were found positives simultaneously by IgM and IgG detection; two were found positives by detection of IgM antibodies only; one was found positive only by seroconversion of IgG antibodies; and seven were found positives by RT-PCR only. The serotypes found were DENV-1 (five cases), DENV-2 (three cases), and DENV-3 (one case). Among these cases, 47.1% (8/17) were classified as secondary infections (high levels of anti-DENV IgG) using Panbio® IgG Capture ELISA. These secondary infections were detected in five acute specimens (29.4%) and three convalescent specimens (17.6%, although this test is usually used in the acute specimen). The other positive (9/17) samples were classified as primary dengue infections. All dengue cases were classified as non-severe or non-differentiated fever.

Using SD Bioline Dengue Duo rapid test, 29.3% (12/41) of the acute samples were classified as positive for DENV infection. One was found positive by simultaneous detection of NS1 antigen, IgM and IgG antibodies; two of them were found positives by the simultaneous detection of NS1 antigen and IgM antibodies; eight were found positives by the simultaneous detection of IgM and IgG antibodies; and one was found positive by the detection of IgM antibodies only. Of these cases, 75.0% (9/12) were classified as secondary infections using IgG of SD Bioline Dengue Duo rapid test. The other positive samples (3/12) were classified as primary dengue infections.

In contrast to the results obtained with the combination of RT-PCR and Panbio® IgM and IgG Capture ELISAs (41.5% of positive cases) and SD Bioline Dengue Duo rapid test (29.3% of the positive cases), ELISPOT-MNT evidenced acute dengue infection in 63.4% (26/41) of the cases. All the cases displayed neutralizing antibody titers in the acute samples and were classified therefore as secondary infections. Figure 1 shows the acute and convalescent neutralization titers for each DENV serotype.

Among these 26 cases determined to be acute secondary infections using ELISPOT-MNT, Panbio® IgG Capture ELISA detected high IgG levels in 11.5% (3/26) of the cases in the acute samples and three more IgG seroconversion in the convalescent specimen. It was found that three cases were positive in the acute sample but negative in the convalescent sample, indicating that those cases were not recent infection. The IgG SD Bioline detected 27.0% (7/26) of the cases as secondary infections.

It was determined using ELISPOT-MNT that 14/26 cases had 4-fold increase in neutralizing antibody titers to a single DENV serotype (DENV-1: 19.2% [5/26], DENV-2: 15.4% [4/26], DENV-3: 7.7% [2/26] and DENV-4: 11.5% [3/26]). The 12 other cases were found to had 4-fold increase in neutralizing antibody titers to multiple DENV serotypes (DENV-1/DENV-4: 19.2% [5/26], DENV-2/DENV-4: 3.8% [1/26], DENV-3/DENV-4: 3.8% [1/26], DENV-1/DENV-2/DENV-3: 3.8% [1/26], DENV-1/DENV-3/DENV-4: 11.5% [3/26]), and to all four serotypes, 3.8% (1/26).

Interestingly, all cases (36.6%, 15/41) that were not determined to be acute dengue infection by ELISPOT-MNT (defined as 4-fold increase in neutralizing antibody titers between acute and convalescent samples) had high neutralizing antibody titers and were therefore classified as non-recent infections. In all ELISPOT-MNT-positive cases, we found high antibody titers to more than one serotype in the convalescent samples.

Using ELISPOT-MNT as gold standard, statistical analysis was conducted to determine the sensitivity and the specificity of the two diagnostic strategies (combination of RT-PCR plus IgM and IgG ELISAs and SD Bioline duo test) as well as their agreement with the results of ELISPOT-MNT (Table 1). The combination of RT-PCR plus IgM and IgG ELISAs correctly identified 14 of the 26 acute cases determined using ELISPOT-MNT. Of the 15 infections determined as non-recent using ELISPOT-MNT, the combination of the tests identified 12 cases as negatives and three cases as positives DENV infections. The overall sensitivity and specificity of the combination of RT-PCR plus Panbio® IgM and IgG ELISAs were determined to be 53.8% (95% CI = 32.8–74.9%) and 80.0% (95%CI = 56.4–100.0%), respectively. The positive predictive value was 82.3% (95%CI =

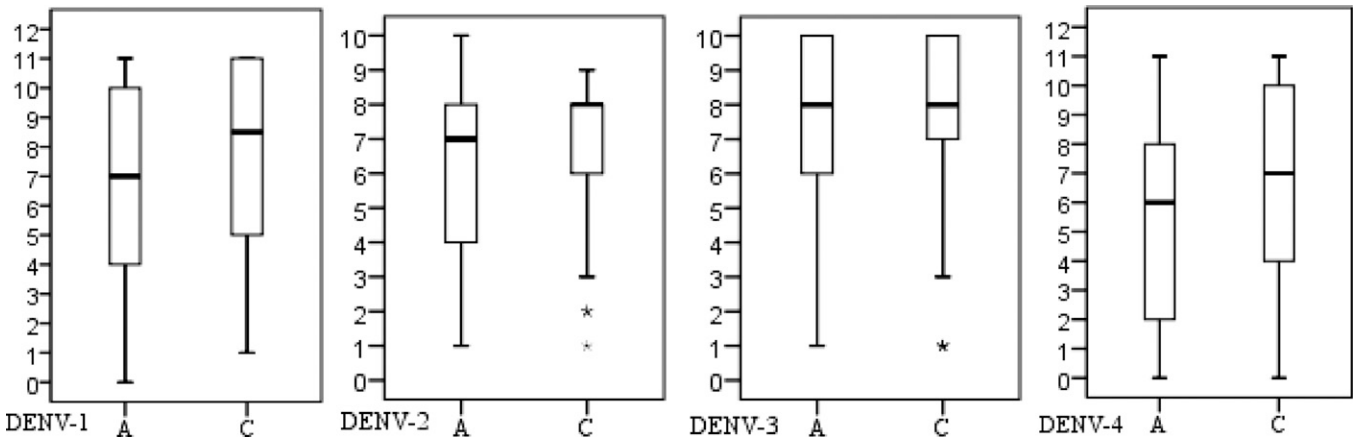

FIGURE 1. The enzyme-linked immunospot microneutralization test (ELISPOT-MNT$_{50}$) titers to each dengue virus serotype (DENV 1–4) in acute and convalescents samples from 26 cases of dengue infection. Box and whisker plot represent the range, median; and 25.0–75.0% interquartile ranges are shown for acute (A) and convalescent (C) samples for each dengue serotype. Antibodies titers are represented as an integer value along the *y* axis. 0 = lower than 1:20; 1 = 1:21–1:40; 2 = 1:41–1:80; 3 = 1:81–1:160; 4 = 1:161–1:320; 5 = 1:321–1:640; 6 = 1:641–1:1,280; 7 = 1:1,281–1:2,560; 8 = 1:2,561–1:6,400; 9 = 1:6,401–1:10,240; 10 = 1:10,241–1:40,960; 11 = 1:40,961–1:163,840 and 12 = higher than1:163,841.

TABLE 1
Overall sensibility and specificity of the tests

Test ($N = 41$)	Panbio® IgM Capture ELISA	Panbio® IgG Capture ELISA	RT-PCR	Combination of the three tests	SD BIOLINE IgM	SD BIOLINE IgG	NS1 antigen	BIOLINE Dengue Duo NS1 + IgM/IgG
Sensibility % (95CI)	26.9 (8.0–45.9)	34.6 (14.4–54.8)	30.8 (11.1–50.4)	53.9 (32.8–74.9)	30.8 (11.1–50.4)	26.9 (7.9–45.9)	11.5 (0.0–25.7)	30.8 (11.1–50.4)
Specificity % (95CI)	86.7 (66.1–100.0)	60.0 (31.9–88.1)	93.3 (77.4–100.0)	80.0 (56.4–100.0)	73.3 (47.6–99.1)	66.7 (39.5–93.9)	100.0 (96.7–100.0)	73.3 (47.6–99.1)
PPV % (95CI)	77.8 (45.1–100.0)	60.0 (31.9–88.1)	88.9 (62.8–100.0)	82.4 (61.3–100.0)	66.7 (35.8–97.5)	58.3 (26.3–90.4)	100.0 (83.3–100.0)	66.7 (35.8—97.5)
NPV % (95CI)	40.6 (22.1–59.2)	34.6 (14.4–54.8)	43.8 (25.0–62.5)	50.0 (27.9–72.1)	37.9 (18.6–57.3)	34.5 (15.5–53.5)	39.5 (22.6–56.3)	37.9 (18.6–57.3)

95CI = 95% confidence interval; ELISA = enzyme-linked immunosorbent assay; NPV = negative predictive value; PPV = positive predictive value; RT-PCR = real-time polymerase chain reaction.

61.3–100.0%), the negative predictive value was 50.0% (95% CI = 27.9–72.1%), the LR+ was 2.69 (95%CI = 0.9–7.9), and the LR− was 0.6 (95%CI = 0.4–0.9). SD Bioline Dengue Duo rapid test correctly identified eight of the 26 acute cases determined using ELISPOT-MNT. The rapid test identified 11 cases as negative and three cases as positives DENV infections among the 15 non-recent infections determined using ELISPOT-MNT. The overall sensitivity and specificity of SD Bioline Dengue Duo rapid test were 30.8% (95% CI = 11.1–50.4%) and 73.3% (95%CI = 56.4–100.0%), respectively. The positive predictive value was 66.7% (95%CI = 35.8–97.5%), the negative predictive value was 37.9% (95%CI = 18.6–57.3%), the LR+ was 1.2 (95%CI = 0.4–3.2), and the LR− was 0.9 (95%CI = 0.6–1.4). In addition, the sensitivity of the combination of RT-PCR plus Panbio® IgM/IgG ELISAs was significant higher than the SD Bioline Dengue Duo rapid test ($P < 0.05$).

The agreement between the ELISPOT-MNT and the combination of RT-PCR plus Panbio® IgM/IgG ELISAs was considered to be fair by a Cohen's κ coefficient test (κ = 0.30), whereas it was considered to be poor for SD Bioline Dengue Duo rapid test (κ = 0.034). The McNemar's association test demonstrated a significant disagreement between each of the diagnostic strategies used and ELISPOT-MNT ($P < 0.05$), suggesting that both strategies do not give the same results as that of the ELISPOT-MNT.

The performances of the individual test were also assessed (see Table 1). As expected, individual performances were lower than the performances of the strategies combining several tests.

In addition, we evaluated the performance of the combination of the direct detection tests (RT-PCR plus NS1 antigen) versus ELISPOT-MNT. We found that the sensitivity and specificity were 34.6% (95%CI = 14.4–54.8%) and 93.3% (95%CI = 77.4–100.0%), respectively. Positive predictive value was 90.0% (95%CI = 66.4–100.0%) and the negative predictive value was 45.2% (95%CI = 47.5–79.4%). The agreement between the ELISPOT-MNT and the combination of RT-PCR plus NS1 antigen was considered to be fair by a Cohen's κ coefficient test (κ = 0.228). Finally, virus isolation was possible in 95.1% (39/41) of the samples, and DENV-1 was recovered in three of them.

DISCUSSION

We evaluated the performance of commercially available assays for the diagnosis of acute DENV infection in 41 schoolchildren from an epidemiological study in Colombia, a country where DENV is endemic. Two strategies for acute DENV diagnostic were used: Panbio® IgM and IgG ELISAs plus RT-PCR and SD Bioline Dengue Duo rapid test, using ELISPOT-MNT as comparative standard method.

ELISPOT-MNT evidenced acute dengue infection in 26/41 cases. Panbio® IgM and IgG ELISAs plus RT-PCR only evidenced 14 cases of dengue infections and SD Bioline Dengue Duo rapid test only detected eight positive cases. This led to low sensitivity of both strategies when compared with ELISPOT-MNT (53.9% for the combination of Panbio® ELISAs and RT-PCR and 30.8% for the SD Bioline duo test) and to a significant disagreement between both strategies and ELISPOT-MNT. Moreover, all positive cases were

classified as secondary dengue infections with the ELISPOT-MNT assay whereas Panbio® and SD Dengue Duo rapid tests only classified 23.1% and 26.9% of the positive cases as secondary dengue infections, respectively.

Interestingly, all our observations indicate previous exposures to DENV in all the patients, despite the young age of the tested population. Indeed, all the patients had detectable neutralizing antibodies titers in the acute sample as well as heterotypic antibody responses. In addition, all acute dengue infections were non-severe and undifferentiated of other febrile illnesses. It is well known that infection with any DENV serotype results in a transient increase of neutralization titers to all the other serotypes.[14] Different studies suggest that preexisting neutralizing antibodies play an important role in protecting patients from clinical manifestations after repeated DENV infections, specifically when intervals of 2–3 years occur between the infections.[19,20] In a recent study, samples collected from a Sri Lankan pediatric dengue cohort (799 children) were used to investigate antibody responses in children with clinically unapparent and clinically apparent DENV infections.[19] It was reported that children with repeated unapparent infections had a greater number of broadly preexisting neutralizing antibodies against DENV serotypes than children with apparent infections.[19]

The fact that all patients included in our study were classified as acute dengue secondary infections (using the ELISPOT-MNT method) may be a contributing factor to the poor performances of the two dengue diagnostics strategies. Indeed, the presence of immune complexes of anti-NS1 IgG antibodies, which occur most frequently in secondary DENV infections[21] may interfere with the detection of NS1 antigen, which may raise a concern for the accuracy of the SD Bioline Dengue Duo assay. In this study, we found that the sensitivity of the SD Bioline Dengue Duo assay using NS1 antigen detection only was as low as 11.5%. This is in agreement with the low sensitivity of NS1 detection observed during a dengue outbreak in 2010 in Santos, Brazil, where most of the infections were secondary infections and DENV-2 was the infecting serotype.[22] Sequencing analysis of the *NS1* gene of these viruses did not reveal any mutation that could altered its reactivity with this diagnostic test. Low sensitivity (11.0%) was also reported when samples were drawn 4–5 days after the onset of symptoms.[22] In other studies where NS1 antigen detection with SD Bioline Dengue Duo was compared with RT-PCR, the SD Bioline assay displayed better performances in specimens from patients with primary infections and when DENV-1 or DENV-3 were the infecting serotypes.[21,23] Guzman and others[8] also reported lower sensitivity of NS1 antigen detection in American countries (Nicaragua and Venezuela) compared with Asian countries.[8,24]

The occurrence of secondary infections also seems to have an impact on the accuracy of IgM detection. Indeed, higher sensitivity of the anti-IgM SD Bioline Dengue Duo was evidenced in primary DENV infections compared with secondary infections.[21] This is in accordance with our results that evidenced poor performances of both Panbio® IgM Capture ELISA and IgM SD Bioline Dengue Duo rapid test (sensitivity of 26.9% and 30.8%, respectively). It has been documented that IgM antibody production is lower and transitory in secondary infections and that some of the patients with secondary infections may even have no detectable IgM antibodies.[10,25]

Despite the high neutralizing antibody titers detected by ELISPOT-MNT in all cases, both tests Panbio® IgG Capture ELISA and IgG SD Bioline Dengue Duo displayed low performances at detecting IgG antibodies in patients with acute DENV secondary infections (sensitivity of 34.6% and 26.9%, respectively). This is consistent with a published study comparing the performances of Panbio® IgG Capture ELISA with IgG antibody Capture ELISA developed by AFRIMS (Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand) on samples from Thailand and Sri Lanka, which reported a sensitivity of 39.8% for Panbio® ELISA.[26]

In our study, specificity and sensitivity of these tests were not determined regarding each DENV serotype, since it was possible to amplify the genes of only 30.8% of the positive cases by ELISPOT-MNT even though the tests was done within 7 days after the onset of symptoms. In addition, it was only possible to isolate DENV-1 from three samples.

Despite a rigorous approach concerning sample processing (standardized conditions of cell and viral cultures, simultaneous processing of acute and convalescent samples in triplicates), our results were obtained on a small sample size of the population, which prompted us to interpret the data with caution. Additional studies with increased amount of well-characterized paired samples may be considered, as well as studies on adult population. However, it has to be stressed that many reports that granted acceptable performances to commercially available dengue assays used ELISAs as comparators instead of gold standard methods such as PRNT or HI.[8,21,23,26] A recent study showed that ELISPOT-MNT had a sensitivity of 95.6% and specificity of 88.2%, with a good correlation ($R^2 = 0.7$) with PRNT.[16] Because of its very high sensitivity, the use of ELISPOT-MNT as the comparative standard method in our study leads inevitably to lower statistics performances of the assays that are compared with it, which can explain the relatively low performances of both diagnostic strategies.

As a conclusion, in our pediatric population of secondary infected patients, combination of commercially available "easy-to-use" tests did not give satisfactory results in detecting acute dengue infection when compared with the ELISPOT-MNT assay, which is unsuitable for routine diagnostic tests. In population where dengue infection is endemic, the predominance of secondary infections combined with the wide range of other febrile illnesses with similar clinical characteristics to DENV infections increase the difficulty of making accurate dengue fever diagnostic. However, an early and precise dengue diagnostic is important for patient management, to avoid dengue severity and mortality and, ultimately, to control epidemics.[8,21,26] Our results agree with others who reported that the combination of two or more diagnostic strategies is needed to improve the diagnostic accuracy of DENV infection.[8,21–23,26] Combining direct DENV detection and antibody-based tests seems to be a particularly promising strategy. However, as seen in this study and evidenced by the low LR+ and LR− values, combinations of current assays do not guarantee the accuracy of the diagnosis when compared with standards such as PRNT or ELISPOT-MNT. Several studies evidenced that the diagnostic performances of different dengue diagnosis kits vary depending on geographical and temporal factors.[8,22,24,27] Thus, validation of those kits in one country should take into account 1) the epidemiological settings (endemic, epidemic, and hyperepidemic periods),

2) the genetic background and immunological status of studied population, the different phases of the dengue disease, and 3) the DENV serotypes and genotypes that are circulating in the area.

While other studies are needed to further document our findings, it is important that national public health services and clinicians are aware of the current limitations of using commercial assays for dengue diagnostic in endemic regions. Accordingly, clinicians should not rule out a dengue infection based solely on a negative result from any of these tests, especially in countries with high dengue prevalence. Facing a patient with febrile syndrome and highly suspected dengue infection, clinician should go ahead with adapted supportive care and rely on clinical observations for diagnostics even in case of a negative laboratory result.

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