COMPARISON OF PANBIO DENGUE IgM ELISA ASSAY WITH PENTAX DENGUE IgM PARTICLE AGGLUTINATION ASSAY TO EVALUATE FACTORS AFFECTING FALSE POSITIVE RESULTS

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Abstract. The objectives of this study were to conduct a further evaluation of performance characteristics (sensitivity/specificity, predictive values, cross-reactivity) of PanBio Dengue IgM (IgM-EIA test), particularly during non-epidemic periods in New Caledonia, and (ii) to evaluate an alternative test, Pentax Dengue IgM-Particle Agglutination (PA-IgM) test. A total of 1,808 samples were first tested with the IgM-EIA test and reactive specimens were then re-tested with IgM-PA test. Sensitivity and specificity were measured on a prospective mode from 2005 and 2006. Other etiologies were also investigated to confirm the non-specific reactive results. One hundred fifty-three samples were initially reactive with IgM-EIA test. Of these, 147 were classified as non specific and only 16 were reactive with the particle agglutination test (89.1% reduction of this interference). The specificity and positive predictive value of the ELISA test was 91.8% and 5.8%, respectively. The extrapolated specificity and positive predictive value for the particle agglutination test was 99.1% and 33.3%, respectively. Hepatitis A was identified as a major source of false positive, followed by rheumatoid factor and leptospirosis. Sensitivity of both tests was 100% on samples taken from the fifth day of the disease.

INTRODUCTION

Dengue is not believed to be endemic in the Pacific region and occurs as outbreaks of a limited duration, likely to be due to the importation of the virus from outside the region or from infection of neighboring islands (Kiedrzynski *et al*, 1998). Since mid-2000, circulation of dengue virus type 1 began in the Pacific and has continued until the present time. Starting in Palau, outbreaks have then been identified in French Polynesia, Cook Islands, Samoa, Vanuatu, Solomon Islands, Wallis and Futuna, Fiji and New Caledonia (Lepers, 2003). In New Caledonia, a major outbreak was reported in 2003, with 2,598

laboratory confirmed cases (http://www.institutpasteur.nc/IMG/pdf/Bilan_D2003.pdf). In 2004, a limited circulation of the virus occurred during the rainy season and probably ended in July, confirmed by absence of dengue PCR positive samples from local patients. In 2005 and 2006, only 11 imported cases were reported and it was not followed by any local circulation.

At the same time, another viral outbreak started in New Caledonia due to hepatitis A virus, probably reintroduced, as no laboratory confirmed cases were notified between January 2001 and November 2004. This virus caused a visible outbreak, involving mostly young people (http://www.dass.gouv.nc/static/sante/themes/hepatiteA.htm). This re-emergence of hepatitis A during a dengue inter-epidemic period was shortly identified as a probable cause of frequent false reactive samples in the dengue IgM ELISA assay uti-

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lized by the virology laboratory of the Pasteur Institute in New Caledonia (IPNC).

Thus, the purpose of this study was (i) to assess this interference and identify other situations leading to false positive dengue IgM results and (ii) to evaluate, in those reactive samples, the behavior of a newly released dengue IgM particle agglutination test of Pentax Corporation (Tokyo, Japan). This test is based on sensitized hydroxy-apatite (Ha-Ny) beads, which had already been successfully used by Pentax for the serological diagnosis of Japanese encephalitis (Yamamoto *et al*, 2000; Pandey *et al*, 2003).

MATERIALS AND METHOD

Patients

A total of 1,808 samples, taken from 1,698 patients, were tested for dengue IgM; 827 in 2005 and 981 in 2006. Additionally, PCR was performed on samples taken before the sixth day after onset of symptoms (Lanciotti *et al*, 1992). Of the total patients, 110 had both an early and a late sample. The test specimens were obtained by 2 mechanisms: (a) patients were recruited on a passive mode as part of a regular screening panel for presenting with an acute febrile syndrome and (b) active recruitment via sentinel sites, throughout the territory, by clinicians specially trained and educated for tracking dengue cases.

Dengue IgM Capture ELISA (IgM-EIA)

IgM-EIA (PanBio, Brisbane, Australia) was performed according to the manufacturer's instructions. This kit uses conventional ELISA immunocapture microplates and its performance has already been assessed (Sang *et al*, 1998; Groen *et al*, 2000). Patient serum was diluted 1:100 in the diluent provided and added to the assay plate, which contained anti-human IgM antibodies coated on the surface of the wells, and incubated at 37°C for 60 minutes. Concurrently, peroxidase-labeled anti-dengue monoclonal antibody conjugate

was added to vials containing lyophilized dengue virus types 1 to 4, which resuspended the antigen and allowed formation of antigen-antibody complex. After washing the plate to remove residual serum, the antigen-antibody complexes were transferred from the antigen vials to the assay plate. After a further 60-minute incubation at 37°C, the assay plate was washed and tetramethylbenzidine substrate added. After 10 minutes, the reaction was stopped by addition of 1 M phosphoric acid and absorbance was read at 450 nm. The kit includes negative, positive and cut-off controls and results are based on a sample/cut-off optical density ratio. The test results were interpreted as follows: positive for sera giving a sample/cut-off ratio of ≥1.1, equivocal (in an established grey zone) for a ratio between 0.8-1.1, and negative for ratios <0.8.

Dengue IgM particle agglutination test (IgM PA)

Patient specimen was diluted 1:100 with a diluent provided (Hapalyse Dengue-M PA Kit, Pentax Corporation, Tokyo, Japan) and added to the assay plate coated with anti-human IgM antibodies and incubated for 30 minutes. If present, specific anti-dengue human IgM antibodies are captured by the anti-human antibodies coated on the assay plate. Unreacted antibodies were washed off and Ha-Ny beads, coated with dengue virus antigen and dyed red for test detection purpose, were added and the solution was incubated for one hour. If IgM antibodies against the dengue virus are present, the captured human dengue and anti-human IgM antibodies bind to the Ha-Ny beads, causing agglutination, which occurs as a diffuse pattern. In the absence of IgM antibodies against the dengue virus, there are no human dengue and anti-human antibody complexes. The Ha-Ny beads, having no complex to bind, do not agglutinate and instead precipitate as a button at the bottom of the microplate well. Because the Ha-Ny beads are dyed, the agglutination and precipitation pattern are readily visible within the well. A dif-

fuse pattern of agglutination or a red ring in the lower part of the well is interpreted as positive. The absence of a diffuse agglutination and the presence of a red precipitate button at the bottom of the microplate well are interpreted as negative. The assay took less than 2 hours. The washing step was performed manually with a wash bottle. In the present study, according to the manufacturer's instructions, screening dilution for the qualitative protocol was 1/100 and positive samples were then quantified by up to a 1/800 dilution, although the manual indicates a quantification up to a 1/12,800 dilution for positive samples.

Detection of hepatitis A virus IgM

An automated enzyme linked immuno fluorescent assay (ELIFA) test was used (Vidas HAV IgM, bioMérieux SA, Marcy-L'Etoile, France), following the manufacturer's instructions.

RESULTS

Dengue prevalence

During the 2005-2006 period, only 10 patients (12 samples) out of 1,698 (1,808 samples) were identified as true imported dengue cases, infected by type -1, -3 or -4 viruses, leading to a dengue prevalence of 0.59% in this study population. All 10 patients were identified and diagnosed after they returned from a country where dengue was prevalent; 2 were positive by PCR but negative for IgM-EIA; 5 were positive for both PCR and IgM-EIA; 3 were negative by PCR but positive for IgM-EIA, but were unequivocally considered true cases in the presence of a strong epidemiological context.

Sensitivity assessment

Among the 12 samples from dengue patients, 9 were positive or equivocal for the IgM-EIA test, 8 of those were positive with the IgM-PA test (Table 1). The discrepant serum (case no. 7, early sample) was only exhibiting an equivocal signal by ELISA (86% of the cut-off value). The sensitivity of the IgM-EIA kit is 75% when considering equivocal results as positive or 58% when only taking in account the sera with a sample/cut-off ratio greater than 1.

Table 1

Dengue IgM levels obtained by 2 different testing kits in 12 samples from 10 confirmed dengue imported cases in New Caledonia, 2005-2006.

Case no.	Year	Imported from	DbO[a] (Day)	Dengue PCR	IgM-EIA[b]	IgM-PA[c]
1	2005	The Philippines	2	Positive DEN-3	0.24	<1/100
2	2005	Indonesia	4	Positive DEN-4	0.29	<1/100
3	2006	Indonesia	7	Positive DEN-1	6.41	1/400
4 early	2006	French Polynesia	2	Positive DEN-1	0.21	<1/100
4 late	2006	French Polynesia	20	Not tested	5.51	1/400
5	2006	French Polynesia	15	Negative	6.45	1/400
6	2006	French Polynesia	37	Negative	0.95	1/400
7 early	2006	French Polynesia	4	Positive DEN-1	0.86	<1/100
7 late	2006	French Polynesia	10	Not tested	2.08	1/400
8	2006	India	9	Positive DEN-3	2.32	1/800
9	2006	French Polynesia	5	Positive DEN-1	2.01	1/800
10	2006	French Polynesia	5	Negative	7.36	1/800

[a]Delay between onset of symptoms and sample date.
[b]IgM-EIA (PanBio Capture ELISA Test). Sample/cut-off ratio: positive >1.1; equivocal 0.8-1.1; negative <0.8.
[c]IgM-PA (Pentax IgM Particle Agglutination Test). Serum highest dilution giving a visible agglutination pattern, cut-off value: 1/100.

Table 2
Dengue IgM results obtained using the Hapalyse Dengue-M PA Kit in 147 non-dengue cases exhibiting a non-specific reactivity with the Dengue IgM Capture assay in New Caledonia, 2005-2006.

Diagnosis	N	Panbio dengue IgM capture (Sample/cut-off ratio)					Pentax hapalyse dengue –M PA Kit (titer)				
		0.8-1	1 - 2	2 - 3	>3	Total	1/100	1/200	1/400	1/800	Total
Unknown[a]	91	27	51	9	4	91	4	6	2	0	12
Hepatitis A	37	7	14	11	5	37	1	0	0	0	1
Autoimmune disease	8	2	0	0	6	8	1	0	0	0	1
Hepatitis B	1	0	0	0	1	1	0	1	0	0	1
HIV	3	1	2	0	0	3	0	0	0	0	0
Leptospirosis	5	1	2	1	1	5	0	0	0	0	0
Malaria	1	0	0	1	0	1	0	1	0	0	1
Septicemia	1	0	0	0	1	1	0	0	0	0	0
Total non-dengue samples	147	38	69	22	18	147	6	8	2	0	16

[a]Cases finally classified as non-dengue patients in the absence of any epidemiological argument and secondary cases.

The IgM-PA kit has a sensitivity of 66%. Sensitivities calculated only with results from samples taken 5 days after onset or later were 100% for both assays.

Specificity and predictive values assessment

Among the 1,808 samples tested in 2005 and 2006 by the IgM-EIA method, 156 exhibited a result interpreted as positive or equivocal. Of these, 9 (6%) were linked to a true dengue imported case, as presented in the previous section. The other 147 (94%) reactive samples were finally considered non-dengue specific. Of the 147 samples, a differential diagnosis was found for 56 (38.1%), including auto immune and acute infectious diseases (Table 2). The presence of hepatitis A antibody in 37 (25%) of these samples could be considered as a contributing factor of cross reactivity, resulting in false positive. The remaining 91 cases reported as "unknown" in Table 2 were classified as non-dengue cases in the absence of any epidemiological findings or secondary cases. The specificity of the IgM-EIA assay, determined for this 2 year period, was 91.8%, the negative predictive value

(NPV) was 99.8% and the positive predictive value (PPV) was 5.7%.

All 147 IgM-EIA positive samples considered non-dengue specific by clinical data were re-tested using the IgM-PA test. Of the 147, only 16 were positive by the IgM-PA test (Table 3). This represented a significant drop (89.1%) in the occurrence of this non-specific interference. In the 37 hepatitis A IgM positive samples, only one had a positive result with the IgM-PA test at the threshold dilution of 1/100.

As none of the 1,649 IgM-EIA and PCR negative samples were tested using the IgM-PA test, no true values for specificity, NPV or PPV were calculated. However, assuming none of the IgM-EIA samples would have given a positive result with the IgM-PA assay, those characteristics would have been 99.1% specificity, 99.8% NPV and 33.3% PPV.

DISCUSSION

Dengue exists under two major epidemiological patterns. It can be endemic, generally

Table 3

Comparison of Hapalyse Dengue-M PA Kit and Dengue IgM Capture assay.

	Sample tested	IgM PA PENTAX	IgM EIA PANBIO
True positive	12	8	9
False negative	0	4	3
False positive	0	16	147
True negative	1,796	1,780[a]	1,649
Total	1,808	1,808	1,808
Sensitivity		66.7%	75.0%
Specificity		99.1%[a]	91.8%
Positive predictive value		33.3%[a]	5.8%
Negative predictive value		99.8%[a]	99.8%

[a]As the 1,649 samples classified as true negatives using the IgM-EIA test were not tested by the PA kit, those figure are extrapolated assuming no false positive among them using the PA kit.

in continental territories, such as in Southeast Asia, where seasonal peaks primarily conditioned by the climate are described, on a background of permanent viral circulation. Conversely, it can be epidemic, with periods of outbreaks separated by inter-epidemic periods of variable duration during which no viral transmission occurs. An extreme case of the latter situation is often seen in Pacific island regions, which are protected by their insularity but become vulnerable in the absence of a strong herd immunity in the population and by permanently existing conditions of transmission due to the endemic presence of one or more vectors (primarily mosquito species *Aedes aegypti, Ae. polynesiensis* and *Ae. albopictus)*. In such places, dengue surveillance activities during inter-epidemic periods are crucial and the role of laboratories in the rapid confirmation of reintroduced cases is fundamental. Although early virological tests (viral isolation or PCR detection) are mostly recommended in this situation, they are usually not available locally due to a lack of resources to support such testing (*eg* funding, equipment, technical personnel). For this reason, a suitable serology test to detect IgM antibodies becomes a desirable alternative laboratory confirmation method to aid in di-

agnosis and management of dengue disease. It is important that the selected serology test has optimal performances in terms of sensitivity, specificity and negative and positive predictive values. Whereas false negatives can limit viral detection resulting in decreased diagnostic and disease management, false positives can lead to over management of dengue disease resulting in the implementation of needless and expensive response measures in states with limited resources.

This study showed comparable sensitivities of the two IgM tests, in agreement with previous assessments available for the IgM-EIA providing values around 88% (Sang *et al*, 1998; Groen *et al*, 2000). As with the study by Sang *et al* (1998), weaker performances were observed in samples collected at early onset of symptoms (36%, in early samples taken at the time of the first medical contact in the evaluation by Sang *et al*, 1998). Both tests detected 100% of true positives among samples taken five days or later after onset of symptoms, confirming the value of IgM testing in the later or convalescent phase of the disease.

This evaluation also demonstrated a lower specificity for the IgM-EIA test than in the studies of Sang *et al* (1998) and Groen *et al* (2000) (92% versus 96%), but consistent with the

assessment by Vajpayee *et al* (2001). Consequently, the IgM-EIA test, when used within a population with a very low prevalence, presents a positive predictive value of 5.8%, which must be regarded as unacceptable. This characteristic was already mentioned for this test in a German study (Wichmann *et al*, 2006), dealing with febrile travellers returning from dengue endemic areas. However, as the dengue prevalence in this study population is 3%, five times higher than among the patients tested in New Caledonia in 2005-2006, the estimated PPV is 50%. The authors recommended that results should be confirmed with a more specific technique.

Among the documented false positives identified in this study, the causative agents most frequently involved were hepatitis A virus (37 cases), autoimmune diseases (8 cases) and leptospirosis (5 cases). Autoimmune diseases are conditions where such interference have already been described with the IgM-EIA test (Takasaki *et al*, 2002) or other rapid dengue immunochromatographic assays (Jelinek *et al*, 2000; Berlioz-Arthaud *et al*, 2005). In those studies, the presence of a circulating rheumatoid factor accounts for 10% of the false positives in microplate format tests and up to 26% using strip tests. The major cross reaction among our patients was acute hepatitis A that has so far not been described and should be taken into consideration in countries where this virus is endemic or among travellers returning from those places.

When run in parallel with IgM-EIA test, IgM-PA test, based on a different principle, demonstrated significantly less cross-reactive results particularly in the presence of hepatitis A antibodies. In a previous regional study conducted in western Pacific, IgM-PA test demonstrated a higher specificity than the rapid immunochromatographic tests (95.2% *vs* 83.3) (Berlioz-Arthaud *et al*, 2005). The current study indicates a superior specificity and positive predictive value of IgM-PA test compared to IgM-EIA test, although further extensive studies need to be performed to confirm this superiority.

As recommended by Wichmann *et al* (2006), we now have introduced IgM-PA test to our current serology testing algorithm for confirmation of all IgM-EIA positive samples and consider as positive only those reacting with both assays. The risk of missing a true case appears negligible and the reduction of false positives has led to significantly less useless spraying around residences of suspect patients. This has an obvious economical impact and is likely to slow down the emergence of insecticide resistant populations of mosquitoes, a real concern in insular situations, where such mutated vectors could rapidly replace wild populations.

An accurate confirmation of the first dengue case of an outbreak is particularly important in the Pacific insular context where resources are often limited and the herd immunity of the population low. The very satisfactory positive predictive value of the IgM-PA test, assessed in this study in an inter-epidemic period, makes it a reliable tool for use in disease diagnosis, management, and control. However, additional detailed studies need to be conducted for further evaluation of these test methods as to the best approach to take in finalizing a clear dengue disease testing algorithm for the Pacific island region.

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