EVALUATION OF TWO IgM RAPID IMMUNOCHROMATOGRAPHIC TESTS DURING CIRCULATION OF ASIAN LINEAGE CHIKUNGUNYA VIRUS

Herman Kosasih[1], Susana Widjaja[1], Edwin Surya[2], Sri H Hadiwijaya[1], Deni PR Butarbutar[1], Ungke A Jaya[1], Nurhayati[1], Bachti Alisjahbana[3] and Maya Williams[1]

[1]Viral Diseases Program, United States Naval Medical Research Unit 2, Jakarta;
[2]School of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta;
[3]Faculty of Medicine, Padjadjaran University, Bandung, Indonesia

Abstract. Chikungunya is an emerging viral disease, which is clinically difficult to distinguish from dengue. Current laboratory methods to diagnose chikungunya infection, such as virus isolation, RT-PCR and ELISA, are not readily available in many clinical settings. In order to provide a rapid and easy method for the diagnosis of chikungunya infection, rapid immunochromatographic tests to detect chikungunya IgM have recently become commercially available. The sensitivity and specificity of the *OnSite*® Chikungunya IgM Rapid Test-Cassette and the SD Bioline CHIK IgM rapid test were evaluated in comparison to a capture ELISA. The sensitivity of the *OnSite* test was 20.5% while its specificity was 100%. The sensitivity of the SD Bioline test was 50.8% while its specificity was 89.2%. The sensitivity of the SD Bioline test increased with increasing CHIK IgM titers and with days of onset in samples collected before day 21 of illness. Increasing the reading time from the manufacturer's suggested time of 10 to 20 minutes significantly increased the sensitivity of the SD Bioline test to 68.2%, but did not significantly change its specificity.

Keywords: chikungunya virus, CHIK IgM ELISA, CHICK RT PCR, rapid test

INTRODUCTION

Chikungunya virus (CHIKV) is an alphavirus belonging to the Togaviridae family. It is the causative agent of chikungunya fever, a disease that is transmitted to humans primarily through *Aedes aegypti* and *Aedes albopictus* mosquitoes. CHIKV is endemic in 23 countries (Powers and Logue, 2007) and has been reported to cause human epidemics in many areas of Africa, Asia, and a limited area of Europe. Recently, there has been a resurgence in the numbers of CHIKV outbreaks, with reports in the Republic of Congo in 2000, La Reunion in 2005, India, Sri Lanka, Malaysia, and Gabon in 2006, Italy in 2007, and Singapore and Thailand in 2008. Phylogenetic analysis has demonstrated that CHIKV likely originated in Africa with subsequent importation into southern Asia and is clustered into three major

Correspondence: Maya Williams, 3230 Lima Place, Washington DC 20521-3230, USA.
Tel: 51 1 614 4141 ext 210
E-mail: Maya.Williams@med.navy.mil

distinct lineages: Asian, East/Central/South African (ECSA) and West African (Powers *et al*, 2000).

Chikungunya fever can manifest as a debilitating illness characterized by incapacitating and sometimes prolonged joint pain and arthritis. Other symptoms of chikungunya fever (abrupt fever, headache, fatigue, and rash) are common among many arboviral infections including dengue (Robinson, 1955). Distinguishing between infection with dengue virus and CHIKV is difficult as in addition to sharing many clinical symptoms, dengue virus and CHIKV also share the same vector and geographical distribution (Deller and Russell 1968; Carey 1971). Because the prognosis and treatment for dengue and chikungunya fever are different, it is important to be able to differentiate between infection by these two viruses.

Current methods to identify CHIKV infection include viral and genome detection by culture and RT-PCR respectively, and the detection of CHIKV IgM/IgG antibodies by serology assays. Although these assays are very reliable (Pialoux *et al*, 2007; Staples *et al*, 2009), they require equipment and well-trained technicians that are not available in many diagnostic laboratories. To meet the need of a rapid point of care test that can accurately detect CHIKV infection, the *OnSite*® Chikungunya IgM Rapid Test-Cassette (CTK Biotech, San Diego, CA) and the SD Bioline CHIK IgM rapid test (Standard Diagnostic, Kyonggi-do, Korea) have recently become commercially available. The sensitivity of the SD Bioline rapid test in comparison to RT-PCR and ELISA has previously been reported to be 37% (Rianthavorn *et al*, 2010b). In a separate report, it was determined that the *OnSite* rapid test first detected CHIK IgM 3.75 to >7 days after fever onset (Yap *et al*, 2010). Both reports used samples from individuals collected during outbreaks of CHIKV belonging to the ECSA phylogroup (Rianthavorn *et al*, 2010a; Yap *et al*, 2010).

Because the sensitivity of a CHIKV diagnostic assay may vary depending upon the viral phylogroup in circulation in the region, it is important that these rapid tests are also evaluated using samples collected from individuals who reside in areas where other lineages of CHIKV are known to circulate. To this end, we conducted a retrospective study on stored samples from CHIK and non-CHIK cases in Indonesia collected when CHIKV of the Asian phylogroup was known to be circulating (unpublished results). These samples were used in order to evaluate the sensitivity and specificity of the SD Bioline CHIK IgM and *OnSite*® Chikungunya IgM rapid tests in comparison to a capture IgM ELISA.

MATERIALS AND METHODS

Sample collection

A retrospective study was conducted on de-identified stored specimens from volunteers presenting with a febrile illness in Jakarta or Bandung, Indonesia. The collection included 132 samples from individuals with confirmed recent CHIK cases and 74 samples from individuals with confirmed dengue or a non-dengue, non-CHIK febrile illness. A confirmed recent CHIKV infection is defined by at least one of the following criteria: IgM seroconversion, a four-fold or greater increase in IgM antibody between acute and convalescent samples, detection of CHIKV nucleic acid by RT-PCR.

CHIK IgM and IgG ELISA

These tests were performed as previously described (Porter *et al*, 2004). The antigen was prepared from Vero E6 cell

cultures infected with CHIKV 23574, an Asian lineage virus. Uninfected Vero E6 cell cultures were used as negative controls. For detection of CHIK IgM, 96-well microtiter plates (Immulon 2, Dynex Technologies, Chantilly, VA) were coated with anti-human IgM antibodies (Kirkegard and Perry, Gaithersburg, MD). Excess antibodies were washed with 0.1% Tween Phosphate-buffered saline (PBS). Serum was diluted 1:100 in serum dilution buffer (PBS, 0.1% Tween-20, and 5% Bacto skim milk), and incubated at 37ºC for one hour. Plates were then washed and clarified cell culture supernatant, diluted 1:6, was added. Normal human serum (diluted 1:50) was added to each lysate preparation. Anti-CHIK hyperimmune mouse ascitic fluid (diluted 1:1,000) and horseradish peroxidase-conjugated anti-mouse IgG (Kirkegard and Perry, Gaithersburg, MD) was used to detect IgM specific to CHIKV. ABTS substrate was allowed to react for one hour and absorbance was determined at 415 nm. For the detection of CHIK specific IgG antibodies, a 96-well microtiter plate was coated directly with cell lysate antigen diluted in PBS. After washing, test serum (diluted 1:100) was added, and incubated for one hour at 37ºC. Horseradish peroxidase conjugated mouse anti-human IgG Fc (Kirkegard and Perry, Gaithersburg, MD), and ABTS were used to detect bound antibody. The adjusted optical density value (OD) at 415 nm for each sample was determined by subtracting the OD obtained with the control antigen from the OD obtained using the CHIK antigen. A sample was considered positive if its OD value exceeded the mean plus three standard deviations of the normal control sera. The endpoint ELISA IgM titers were determined by testing ELISA-positive samples at serial four-fold dilutions starting from 1:100. The highest dilution showing a positive result is considered the endpoint titer.

CHIK RT-PCR

CHIK RT-PCR was performed as previously described (Porter *et al*, 2004). Viral RNA was extracted using a QIAamp Viral RNA Isolation Kit (QIAGEN, Hilden, Germany). RNA was then used in a nested RT-PCR assay using JM1 (5′ GCAGAC GCAGAGAGGGCCAG 3′; bp 1,201 to 1,220) and JM2 (5′ CGTGCTGCAAGG TAGTTCTC 3′; bp 1,440 to 1,421) primers. A second nested PCR was performed using the product from the first reaction and primers JM3 (5′ GCTATTTGTAAGAAC GTCAG 3′; bp 1,221 to 1,240) and JM4 (5′ TACCGTGCTGCGGTCGGGAA 3′; bp 1,420-1,401). Amplified PCR products were resolved by electrophoresis on a 2% agarose gel and visualized using ethidium bromide.

Rapid tests

The tests were performed according to the manufacturer's procedures using serum or plasma. Results were determined independently by two individuals at 10, 15, and 20 minutes. When discrepancies between two experimenters occurred, specimens were considered positive.

Data analysis

The rapid test results were analyzed for sensitivity, specificity, and overall agreement in comparison to a CHIK IgM capture ELISA. Statistical analysis was calculated using Stata 9 (Stata Corp, College Station, TX).

RESULTS

A total of 206 samples collected from febrile volunteers in Bandung or Jakarta, Indonesia were used to evaluate the performance of the *OnSite* and SD Bioline CHIK IgM rapid tests. When determined

Table 1
Performance of *OnSite* and SD Bioline rapid tests to detect CHIK IgM.

	Percentage		
	OnSite	SD Bioline	*p*-value
Sensitivity	20.5% (27/132)	50.8% (67/132)	0.000
Specificity	100% (74/74)	89.2% (66/74)	0.004
Overall agreement	49.0% (101/206)	64.6% (133/206)	0.002

by IgM capture ELISA, 117 samples were CHIK IgM positive and CHIK PCR negative, 17 samples were CHIK IgM positive and CHIK PCR positive, and 74 samples were CHIK IgM negative and CHIK PCR negative. The rapid tests were performed according to the manufacturer's instructions and evaluated independently by two investigators (results were considered positive when at least one of the investigators read a test as positive). The sensitivity of the *OnSite* test was 20.5%, its specificity was 100%, and overall agreement with ELISA results was 49%. The sensitivity of the SD Bioline rapid test was 50.8%, specificity 89.2%, and overall agreement with ELISA results 64.6% (Table 1).

Effects of illness day and IgM titer on rapid test sensitivity

The sensitivity of the rapid tests could be dependent on the distribution of the CHIK IgM titers in the samples used for analysis; therefore, the sensitivities of the rapid tests were stratified according to IgM titer. The sensitivity of the SD Bioline rapid test increased with increasing IgM titers reaching a peak sensitivity of 75% in samples with CHIK IgM titers ≥ 102,400. The sensitivity of *OnSite* test increased with increasing IgM titers up to a titer of 6,400 but was not higher than 26% even in the highest titer samples (Table 2). During the course of disease, the sensitivity of the rapid tests may vary as antibody titers rise and fall. In order to determine the sensitivity of the rapid tests according to day post-illness onset, results also were stratified by day post-illness onset. Sera were grouped into 5 categories: 1-5, 6-10, 11-15, 16-20 and ≥ 21 days post-illness onset. The geometric mean titer (GMT) of CHIK IgM increased with days post-illness onset, peaking in the 16-20 days group before decreasing in samples collected ≥ 21 days after illness onset (Table 3), and the sensitivity of both rapid tests was also highest in the 16-20 days post-illness onset group.

Table 2
Sensitivity of *OnSite* and SD Bioline rapid tests according to IgM titer.

IgM titer	*OnSite*	SD Bioline
100	0.0% (0/3)	33.3% (1/3)
400	6.3% (1/16)	18.8% (3/16)
1,600	17.6% (6/34)	44.1% (15/34)
6,400	25.7% (9/35)	48.6% (17/35)
25,600	25.0% (6/24)	66.7% (16/24)
102,400	25.0% (5/20)	75.0% (15/20)

Effect of determination time on rapid test sensitivity

The recommended determination time for the *OnSite* test is 15 minutes

Table 3
Sensitivity and corresponding geometric mean titers (GMT) according to days post-illness onset.

Days post-illness onset	*OnSite*	SD Bioline	IgM GMT
1 - 5	22.7% (5/22)	40.9% (9/22)	2,297
6 - 10	28.6% (4/14)	50.0% (7/14)	3,045
11 - 15	17.9% (10/56)	46.4% (26/56)	9,108
16 - 20	23.1% (6/26)	65.4% (17/26)	10,907
≥ 21	14.3% (2/14)	57.1% (8/14)	10,500

Table 4
Performance of SD Bioline rapid test according to test time.

	10 minutes[a]	15 minutes	20 minutes
Sensitivity	50.8% (67/132)[b]	62.9% (83/132)	68.2% (90/132)[b]
Specificity	89.2% (66/74)	85.1% (63/74)	85.1% (63/74)
Overall agreement	64.6% (133/206)[b]	70.9% (146/206)	74.3% (153/206)[b]

[a]Manufacturer recommended time; [b]$p<0.05$

while that of the SD Bioline rapid test is 10 minutes. In order to determine the optimal time required for each test, both tests were conducted by two investigators for 10, 15 and 20 minutes. Increasing the testing time from 15 to 20 minutes did not significantly change the calculated sensitivity or specificity of the *OnSite* test (data not shown); however, increasing the time from 10 to 20 minutes resulted in a significant increase in the sensitivity of the SD Bioline rapid test from 50.8% to 68.2% while not significantly changing its specificity (Table 4).

DISCUSSION

A simple, reliable, and rapid diagnostic kit would be a valuable tool to overcome the difficulties in establishing a diagnosis of CHIKV infection, especially in remote areas. The sensitivity of the SD Bioline rapid test was high and could be improved without significantly affecting its specificity by increasing the test time to 20 minutes. The sensitivity of the *OnSite* test was poor at all test times but demonstrated 100% specificity. When comparing the rapid test results to those from the capture IgM ELISA, one must consider the possibility of cross-reaction with antibodies against other alphaviruses. To date, chikungunya virus is the only alphavirus found in Indonesia (Tesh *et al,* 1975), so it is unlikely that the positive results in this study are due to cross-reaction with other anti-alphavirus antibodies. As with all serological tests, interpretation of the CHIK IgM rapid test results must be done carefully. In the majority of cases, CHIK IgM antibodies reach a detectable level starting between day 4 and day 7 of illness

(Pialoux *et al*, 2007) resulting in false negative results when early acute sera are tested. Follow-up serum, collected in the second week of illness, should be tested when initial results are negative. Furthermore, CHIK IgM antibodies can remain at detectable levels for 1-2 years (Grivard *et al*, 2007). Thus, especially in endemic areas, a positive CHIK IgM rapid test result may not always signify a recent CHIKV infection. Most symptomatic CHIKV infections are primary infections and CHIK IgG does not appear until convalescence (Pialoux *et al*, 2007). Thus, in endemic areas, the addition of CHIK IgG detection to the rapid tests would be useful in order to distinguish whether a positive CHIK IgM result is due to a recent infection or is residual IgM from a previous CHIKV infection.

The sensitivities of the two rapid tests evaluated in this study differ from previous reports. A previous evaluation of the *OnSite* test reported 90.3% sensitivity and 100% specificity when compared to MAC-ELISA (CTK Biotech). This differs dramatically from the 20% sensitivity determined in our study. The differences between these values could be due to genetic differences in the viruses circulating in the regions where the samples were collected. The samples used in our study were collected in an area where CHIKV of the Asian phylogroup is known to circulate (unpublished data), while information about the genotype of the samples used in the previous study was not provided.

A recent study in Thailand reported 37% sensitivity for the SD Bioline IgM rapid test when the sensitivity was calculated in comparison to the detection of CHIK infection by IgM ELISA or PCR (Rianthavorn *et al*, 2010b). The sensitivity of the reported test is low in samples collected before day 10 of illness (26%), but increases in samples collected more than ten days after illness onset (89%). In comparing only samples collected after day 10 of illness, the sensitivity was reported to be 89% while the sensitivity calculated in our study was 68%. The samples used in the report of Rianthavorn *et al* (2010a) were collected during a CHIKV outbreak caused by a virus of the ECSA phylogroup. One possibility for the difference in the reported sensitivitiy of the SD Bioline rapid test is the difference in the phylogroups of viruses circulating when the samples were collected. This would not be surprising as it has previously been shown that serological assays have varying sensitivities depending on chikungunya viruses of the ECSA phylogroup containing an A or V at position 226 (Yap *et al*, 2010). Unfortunately, requests for information concerning the phylogroup of the antigens used in the rapid tests was not available. As the sensitivity of the rapid tests may vary according to the predominant phylogroup in the region, further studies are necessary in order to directly compare rapid test performance using samples collected from regions where different phylogroups circulate.

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