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Multiplex real-time RT-PCR for detecting chikungunya virus and dengue virus

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ABSTRACT

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Objective: To develop diagnostic test for detection chikungunya virus (CHIKV and Dengue virus (DENV) infection. **Methods:** We have performed a rapid, accurate laboratory confirmative method to simultaneously detect, quantify and differentiate CHIKV and DENV infection by single-step multiplex real-time RT-PCR. **Results:** The assay's sensitivity was 97.65%, specificity was 92.59% and accuracy was 95.82% when compared to conventional RT-PCR. Additionally, there was no cross-reaction between CHIKV, DENV, Japanese encephalitis virus, hepatitis C, hepatitis A or hepatitis E virus. **Conclusions:** This rapid and reliable assay provides a means for simultaneous early diagnosis of CHIKV and DENV in a single-step reaction.

1. Introduction

Chikungunya virus (CHIKV) belongs to the family *Togaviridae* and the genus *Alphavirus*[1]. It contains a single-stranded RNA genome of approximately 11.8 kb and comprises three different strains: the East, Central and South African strain, West African strain and Asian strain[2]. Dengue virus (DENV) is a member of the family *Flaviviridae*, genus *Flavivirus* with a single-stranded RNA genome of 11 kb which has been categorized into four serotypes, DENV1–4[3]. Both viruses are arthropod-borne viruses (arboviruses). Major mosquitoes vectors for the viruses are of the *Aedes* genus, including *Aedes aegypti* and *Aedes albopictus*. Worldwide an estimated 50 million are infected with DENV per year, culminating in about

25 000 deaths in severe cases[4]. The magnitude of problems caused by the spreading of CHIKV can be seen in the unprecedented spread of CHIKV on islands of the Indian Ocean in 2005[5] and the largest recorded outbreak with more than 1.38 million cases in 2006–2007[6,7]. Recent endemic areas are mainly urban areas in South, East and West Africa, the south-western Indian Ocean islands, India and Asia including Thailand. The report showed that the recent CHIKV outbreak started spreading in Thailand in 2008[8,9], and are of the ECSA strain confirmed by complete genome analysis[10,11].

CHIKV causes chikungunya fever (CHIKF) in both male and female individuals of any age. CHIKF is self-limiting with a very low death rate and usually presents as a rapid onset of fever, a characteristic rash, severe and incapacitating joint pain and other less common symptoms such as headache, fatigue, nausea, vomiting and conjunctivitis[12]. The clinical characteristics of dengue vary from asymptomatic to potentially fatal complications. Dengue fever (DF) may present as headache, fever, bone or joint pain while dengue hemorrhagic fever (DHF) may produce bleeding, plasma leakage, fever, and thrombocytopenia which can affect any age. A plasma leakage test is crucial as DHF usually presents plasma leakage while DF does not. DHF may lead to dengue shock syndrome and must be monitored

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closely^[13].

Discernment between DENV and CHIKV can be problematic because of their highly similar symptoms. For example, the Tanganyika (now Tanzania) epidemic of 1952–1953 was thought to be dengue infection while it was actually the first documented infection by CHIKV. A dengue-like epidemic on the Comoros Islands in 2005 was additionally a CHIKV outbreak^[14,15]. Furthermore, the two viruses not only show similar symptoms to each other but also to other fever such as measles, influenza infection and severe acute respiratory syndrome^[16]. The 2004 CHIKV epidemic in Kenya was originally thought to be malaria^[17]. Additionally, there have been several reports of co-circulation and co-infection of CHIKV and DENV in Delhi, Malaysia, the Maldives and India along with the increasing number of travelers and the spread of the vectors, *Aedes aegypti* and *Aedes albopictus*, for both viruses^[18–20].

Accurate and rapid diagnosis is critical, as this will facilitate treatment in case of atypical presentations and complications. Early distinction between dengue and other febrile illnesses could help identify patients with signs of DHF that could develop into life-threatening dengue shock syndrome. In addition, information on virus infections may contribute to the surveillance database and assist with molecular epidemiology research, as well as monitoring and aid in control of virus dissemination.

CHIKV and DENV infections can be confirmed by direct detection such as virus propagation, reverse transcriptase-polymerase chain reaction (RT-PCR) or real-time RT-PCR. Specific antibodies such as IgM or IgG (antibody capture ELISA) can be used to detect infection as well but they depend on the time of collection and availability. Real-time RT-PCR has several advantages such as high sensitivity and specificity, ability to be performed in multiplex and is quantifiable. The objective of this study was to develop multiplex real-time RT-PCR based on Taqman chemistry for detection of CHIKV and DENV in a single tube reaction. We evaluated the sensitivity and specificity by comparison with conventional RT-PCR.

2. Material and methods

The research proposal has been approved by the ethics committee, Faculty of Medicine, Chulalongkorn University. All the study specimens were anonymized with coding numbers and permission granted by the directors of hospitals.

Table 1

Nucleotide sequence of primers and probe used in the assay.

Virus	Probe/primer	Sequence (5'–3')	Region, position*
CHIKV	ChikF10378–10398	GCATCAGCTAAGCTCCGGGTC	E1, 10378–10398
	ChikR10487–10508	CAATGTCTTCAGCCTGGACACC	E1, 10487–10508
	ChikP10443–10456	Cy5–ATGCCAAACGGCGACCATGCCGTCA--BBQ	E1, 10433–10456
DENV	DenF10520–10541	GACTAGYGGTTAGAGGAGACC	3'UTR, 10520–10541
	DenR10674–10694	GHRGAGACAGCAGGATCTCTG	3'UTR, 10674–10694
	DenP10610–10634	JOE–AAGGACTAGMGTTAGWGGAGACCC--BBQ	3'UTR, 10610–10634

*positions were approximated based on complete genome sequences.

2.1. Case selection and Clinical samples

RT-PCR confirmed CHIKV positive samples ($n=39$, accession number FJ882884–FJ882922)^[9] and positive DENV ($n=26$, accession number JF274206–JF274231), which were then confirmed by RT-PCR and NS1 antigen rapid test (SD BIOLINE Dengue Duo, Kyonggi-do, Korea) were included in this study^[21,22]. A total of 290 suspected cases were recruited in this study as well, comprised of acute serum samples collected within 7 days of the onset of illness, consistent with clinical diagnosis of dengue according to WHO criteria^[23] and of CHIKV samples from suspected infections in the endemic area. Moreover, 28 hepatitis C positive, 2 hepatitis A positive, 6 hepatitis E positive, 2 Japanese encephalitis virus (JEV) samples were included in this study to evaluate sensitivity and specificity of this method.

2.2. Nucleic acid extraction and reverse transcription

Viral RNA was extracted using a viral nucleic acid extraction kit (RBC Bioscience, Taipei, Taiwan) following the manufacturer's specifications. The extracted RNA was stored at -80°C until further use. RNA was transcribed into cDNA by using ImProm-IITM reverse transcriptase (Promega, Medison, WI). Briefly, the template was mixed with random primers diluted in RNase-free water. The mixture then was incubated at 70°C for 5 minutes and quickly chilled at 4°C for 5 minutes and then held on ice. Reverse Transcription Mix was added and annealing was performed at 25°C for 5 minutes, followed by extension at 42°C for 60 minutes. Reverse transcriptase was inactivated by holding the mixture at 70°C for 15 minutes.

2.3. Primer and probe design

Fifteen CHIKV and 11 DENV nucleotide sequences were retrieved from GenBank and aligned using CLUSTAL W (<http://www.ddbj.nig.ac.jp/searches-e.html>). The sequence accession numbers were FJ807891, EU703762, AF192898, GU301779, GU301780, GU301781, FJ807893, FJ445511, AM258994, FJ513655, EU244823, FJ445492, FJ807896, NC004162, and AF192907 for CHIKV and HQ166035, GQ398255, GQ398265, GQ398266, FJ744718, FJ744719, GQ398257, FJ744740, FJ744734, AY618993, AY618992 for DENV. Primer and probe sequences are shown in Table 1.

2.4. Plasmid preparation

The target regions between positions 10237 and 10544

of the CHIKV E1 region and between positions 10019 and 10695 of the DENV 3' UTR (positions were approximated from complete genome) were amplified, sequenced, cloned into pGEM®-T Easy vector (Promega, Madison, WI) and transformed into Competent JM109 *Escherichia coli*, which were subsequently spread onto LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. The white colonies (*Escherichia coli* with inserted region) were selected for further growth in LB broth. The plasmid was extracted using the HiYield™ Plasmid Mini Kit (RBC Bioscience, Taipei, Taiwan). The presence of the inserted region and its direction were confirmed by PCR and direct sequencing and submitted to GenBank (Accession number JF297648–JF297652).

2.5. Thermal profile and optimization conditions

Separate reaction mixtures were carried out for each real-time RT-PCR with parameters adjusted for each virus target. The amplification reaction was performed in a Corbett Research Rotor-Gene 3000 in a 10 µL volume. The mixture contained 5 µL of 2× reaction mix, 0.2 µL of SuperScript™ III RT/Platinum® Taq Mix, 0.5 µM of ChikF10378–10398 and ChikR10487–10508, 0.2 µM of ChikP10443–10456, 0.25 µM of DenF10520–10541 DenR10674–10694 and 0.125 µM of DenP10610–10634, 1.25 mM of MgSO₄, 1.4 µL of distilled water and 1 µL of template. Initial melt was at 95°C for 2 minutes followed by 40 cycles of 95°C (15 seconds), 55°C (10 seconds), 60°C (10 seconds) and 72°C (20 seconds). Fluorescence signals were detected at the end of each 72°C step. Subsequently, both real-time RT-PCR were combined to perform in a multiplex format.

2.6. Sensitivity and specificity compared with RT-PCR

The clinical specimens were detected by conventional semi-nested RT-PCR using the primers previously defined by S. Chutinimitkul for DENV detection^[24] and Naresh Kumar C.V.M. for CHIKV detection^[25] and then compared to the real time RT-PCR for an evaluation of the sensitivity and specificity. Conventional RT-PCR serving as the reliable method because after RT-PCR, the result was confirmed

by sequencing the PCR products and BLAST with GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3. Result

3.1. Design of primers and probes

The specificity of primers and probes were tested against CHIKV and the four serotypes of DENV as shown in Figure 1A and Figure 1B, respectively. The amplification plot of the Cy5 signal showed that only CHIKV could be amplified in the assay as the signal of DENV in the Cy5 channel remained low at the base line. Likewise, the JOE signal appeared for DENV1–4 at 10–13 cycles and there was no cross-reactive signal of CHIKV. The control reaction performed without template did not produce amplification as evidenced by the low level signal (Figure 1A and Figure 1B) suggesting that we were able to detect CHIKV and four serotypes of DENV successfully.

3.2. Sensitivity and specificity

The result showed that our assay could detect all CHIKV and DENV confirmed positive samples and there was no signal for any of the other viruses tested.

3.3. Evaluation of assay using clinical specimens

In a total of 290 clinical samples, conventional RT-PCR was used to detect CHIKV and DENV. The results of conventional RT-PCR showed that 125 samples were positive for either CHIKV or DENV. The sensitivity, specificity and accuracy of real time RT-PCR were 97.65%, 92.59% and 95.82%, respectively when compare to conventional RT-PCR. Among 125 positive samples, 60 samples were positive for CHIKV and 65 for DENV. All positive samples tested by conventional RT-PCR were also positive for real-time RT-PCR. Out of 162 negative samples diagnosed by conventional RT-PCR, 12 tested positive by real-time RT-PCR. As a result, real-time RT-PCR was found to be more sensitive than conventional RT-PCR.

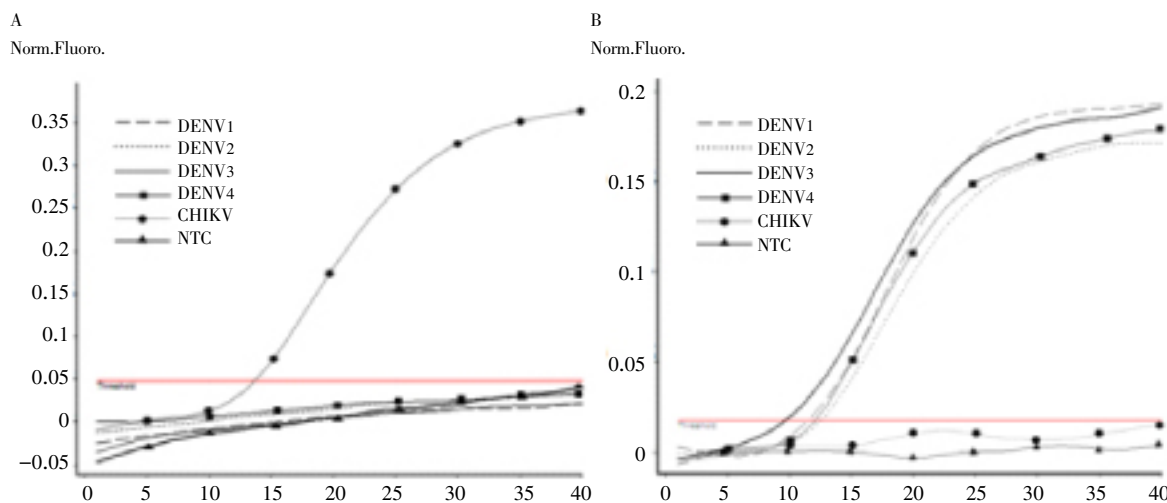


Figure 1. Amplification plot obtained for CHIKV and DENV1–4.

A: in Cy5 channel labeled for CHIKV probe; B: in JOE channel labeled for DENV probe.

3.4. Limit of detection and standard curves

We used template of known concentration to estimate the limit of detection for this method. The results showed that

the minimum concentration was 10 copies/ μ L for both CHIKV and DENV. Each concentration was plotted against Ct values as shown in Figure 2A and Figure 2B. The standard curves also showed a high correlation coefficient, $R^2 = 0.994$ and 0.999 for CHIKV and DENV, respectively.

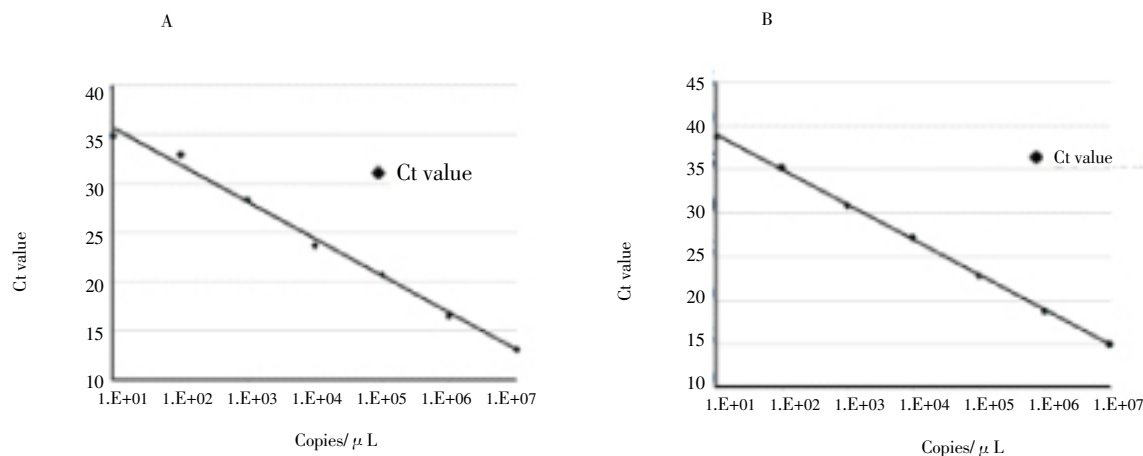


Figure 2. Standard curve with serially diluted pGEM plasmid.
A: CHIKV; B: DENV1.

4. Discussion

CHIKV and DENV diagnosis may be approached via several methods such as conventional RT-PCR, commercial rapid test, ELISA, HI test, virus culture and antibody screening (IgM, IgG). Screening for antibody of virus is commonly used but IgM of CHIKV may cross-react with other alphaviruses such as O'nyong nyong virus^[26–28] and the antibody of DENV may cross react with other members of the *Flaviviridae* family such as JEV^[29], resulting in false positives. Virus culture has shown reliable results but is laborious and time consuming to perform. To our knowledge, there is no method available to detect both viruses simultaneously and thus we have developed a method to detect CHIKV and DENV in a single tube.

The challenge of evaluating the assay is the difficulty in defining a gold standard for detecting the viral genome. Conventional RT-PCR has been traditionally used as the gold standard, but from our results real-time RT-PCR yields higher sensitivity than this method. Applying this assay, we were able to detect viral DNA at titers too low for amplification by normal RT-PCR ($n=12$). Additionally, real-time RT-PCR also has an advantage over conventional RT-PCR in terms of expediency. There are no post-amplification processes such as gel electrophoresis, gel visualization and sometimes confirmation by direct sequencing. Compared to semi-nested RT-PCR for DENV amplification, a two-step procedure, real-time RT-PCR requires less time to arrive at a diagnosis. By using multicolor probes, we were able to design oligonucleotide sequences of specific probes labeled with different colors^[30]. Primers and probes were designed so that they could be used under similar amplification conditions but provide immediate viral identity feedback. Additionally, regional mutations were taken into account during the design process. During the largest outbreak of CHIKV on islands in the Indian Ocean, a signature mutation, E1-A226V, was found in the E1 region. Hence, we designed a set of primers and probe that do not target on this spot so

that we are able to detect CHIKV regardless of E1-226 wild-type or mutant. For DENV detection, our method is able to detect all four serotypes of DENV. Although detecting serotypes of DENV may be advantageous, the clinical treatments for all serotypes are identical and it may not be effective to use different probes to detect different serotypes. Evaluation of detection at minimum concentration revealed that this method can detect concentrations as low as 10 copies/ μ L. When testing the specificity of our assay, we found no cross reactivity between CHIKV, DENV, hepatitis C virus, hepatitis A virus, hepatitis E virus and JEV. Dual infection of CHIKV and DENV confirmed by conventional RT-PCR produced identical results with multiplex real-time RT-PCR, suggesting that this method remains useful for detecting two viruses in a single step reaction.

In conclusion, our set of primers and probes allows for the rapid and early detection of CHIKV and DENV infection in a single step reaction. This method presents a useful tool for studying seroprevalence and quantitative determination of viral infection and may shed light on the correlation between clinical severity and immune response. Detection of CHIKV and DENV presents a challenge, as these viruses may only be traceable during the acute phase, 3–7 days post infection, making other techniques crucial in confirming infections. Awareness of virus dissemination will help the people to take preventive measures and the governments to develop vector control policies.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

- [1] Strauss JH, Strauss EG. The alphaviruses: gene expression, replication and evolution. *Microbiol Rev* 1994; **58**: 491–562.
- [2] Khan AH, Morita K, del Carmen Parquet M, Hasebe F, Mathenge EGM, Igarashi A. Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. *J Gen Virol* 2002; **83**: 3075–3084.
- [3] Rice CM. Flaviviridae: the viruses and their replication. In: Fields BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, et al. *Fields virology*. 3rd ed. Philadelphia: Lippincott–Raven; 1996.
- [4] World Health Organization. Dengue and dengue haemorrhagic fever. Fact sheet N° 117. March 2009. [Online] Available from <http://www.who.int/mediacentre/factsheets/fs117/en/2009> [Accessed on January 24, 2011].
- [5] Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med* 2006; **3**: 1058–1070.
- [6] Lahariya C, Pradhan SK. Emergence of chikungunya virus in Indian subcontinent after 32 years: a review. *J Vector Borne Dis* 2006; **43**: 151–160.
- [7] World Health Organization. Chikungunya fever, chikungunya in South–East Asia. [Online] Available from http://www.searo.who.int/EN/Section10/Section2246_13975.htm 2009 [Accessed on January 24, 2011].
- [8] Theamboonlers A, Rianthavorn P, Praianantathavorn K, Wuttirattanakowit N, Poovorawan Y. Clinical and molecular characterization of chikungunya virus in south Thailand. *Jpn J Infect Dis* 2009; **62**(4): 303–305.
- [9] Rianthavorn P, Prianantathavorn K, Wuttirattanakowit N, Theamboonlers A, Poovorawan Y. An outbreak of chikungunya in southern Thailand from 2008 to 2009 caused by African strains with A226V mutation. *Int J Infect Dis* 2010; **145**: 161–165.
- [10] Pongsiri P, Auksornkitti V, Theamboonlers A, Luplertlop N, Rianthavorn P, Poovorawan Y. Entire genome characterization of Chikungunya virus from the 2008–2009 outbreaks in Thailand. *Trop Biomed* 2010; **27**(2): 167–176.
- [11] Auksornkitti V, Pongsiri P, Theamboonlers A, Rianthavorn P, Poovorawan Y, Manujum K, et al. Whole–genome characterisation of Chikungunya virus from *Aedes albopictus* collected in Thailand. *Ann Trop Med Parasitol* 2010; **104**(3): 265–269.
- [12] Robinson MC. An epidemic of a dengue–like fever in the southern province of Tanganyika. *Cent Afr J Med* 1956; **2**(11): 394–396.
- [13] Halstead SB. Epidemiology of dengue and dengue hemorrhagic fever. In: Gubler DJ, Kuno G. *Dengue and dengue hemorrhagic fever*. Wallingford, United Kingdom: CAB International; 1997, p. 23–44.
- [14] Ross RW. The Newala epidemic III; the virus: isolation, pathogenic properties and relationship to the epidemic. *J Hyg* 1956; **54**(2): 177–191.
- [15] Sergon K, Yahaya AA, Brown J, Bedja SA, Mlindasse M, Agata N, et al. Seroprevalence of Chikungunya virus infection on Grande Comore Island, union of the Comoros, 2005. *Am J Trop Med Hyg* 2007; **76**(6): 1189–1193.
- [16] Wilder–Smith A, Earnest A, Paton NI. Use of simple laboratory features to distinguish the early stage of severe acute respiratory syndrome from dengue fever. *Clin Infect Dis* 2004; **39**(12): 1818–1823.
- [17] Sergon K, Njuguna C, Kalani R, Ofula V, Onyango C, Konongoi LS, et al. Seroprevalence of Chikungunya virus (CHIKV) infection on Lamu Island, Kenya, October 2004. *Am J Trop Med Hyg* 2008; **78**(2): 333–337.
- [18] Chahar HS, Bharaj P, Dar L, Guleria R, Kabra SK, Broor S. Co–infections with Chikungunya virus and dengue virus in Delhi, India. *Emerg Infect Dis* 2009; **15**(7): 1077–1080.
- [19] Nayar SK, Noridah O, Paranthaman V, Ranjit K, Norizah I, Chem YK, et al. Co–infection of dengue virus and chikungunya virus in two patients with acute febrile illness. *Med J Malaysia* 2007; **62**(4): 335–336.
- [20] Yoosuf AA, Shiham I, Mohamed AJ, Ali G, Luna JM, Pandav R, et al. First report of chikungunya from the Maldives. *Trans R Soc Trop Med Hyg* 2008; **103**(2): 192–196.
- [21] World Health Organization. *Guideline for treatment of dengue fever/dengue hemorrhagic fever in small hospitals, regional office for south–east Asia*, New Delhi. Geneva: WHO; 1999.
- [22] Chutinimitkul S, Payungporn S, Theamboonlers A, Poovorawan Y. Dengue typing assay based on real–time PCR using SYBR Green I. *J Virol Methods* 2005; **129**: 8–15.
- [23] M Naresh Kumar CV, Anthony Johnson AM, R Sai Gopal DV. Molecular characterization of chikungunya virus from Andhra Pradesh, India & phylogenetic relationship with Central African isolates. *Indian J Med Res* 2007; **126**(6): 534–540.
- [24] Rianthavorn P, Wuttirattanakowit N, Prianantathavorn K, Limpaphayom N, Theamboonlers A, Poovorawan Y. Evaluation of a rapid assay for detection of IgM antibodies to chikungunya. *Southeast Asian J Trop Med Public Health* 2010; **41**(1): 92–96.
- [25] Tontulawat P, Pongsiri P, Thongmee C, Theamboonlers A, Kamolvarin N, Poovorawan Y. Evaluation of the rapid test for NS1 and anti–Dengue IgM, semi–nested PCR and IgM ELISA for detection of Dengue virus. *Southeast Asian J Trop Med Public Health* 2011; **42**(3): 194–202.
- [26] Demanou M, Antonio–Nkondjio C, Ngapana E, Rousset D, Paupy C, Manuguerra JC, et al. Chikungunya outbreak in a rural area of Western Cameroon in 2006: A retrospective serological and entomological survey. *BMC Res Notes* 2010; **5**(3): 128.
- [27] Taraphdar D, Sarkar A, Chatterjee S. Mass scale screening of common arboviral infections by an affordable, cost effective RT–PCR method. *Asian Pac J Trop Biomed* 2012; **2**(2): 97–101.
- [28] Arya SC, Agarwal N. Rapid point–of–care diagnosis of chikungunya virus infection. *Asian Pac J Trop Biomed* 2011; **1**(3): 230–231.
- [29] A–Nuegoonpipat A, Panthuyosri N, Anantapreecha S, Chanama S, Sa–Ngasang A, Sawanpanyalert P, et al. Cross–reactive IgM responses in patients with dengue or Japanese encephalitis. *J Clin Virol* 2008; **42**(1): 75–77.
- [30] Carl TW, Mark GH, Cameron NG, Kojo SJ Elenitoba–Johnson. Real–time multiplex PCR assays. *Methods* 2001; **25**: 430–442.