

A Diagnostic Polymerase Chain Reaction Assay for Zika Virus

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Zika virus (ZIKV) is a mosquito-borne flavivirus. Infection results in a dengue-like illness with fever, headache, malaise, and a maculopapular rash. Nearly all cases are mild and self-limiting but in 2007, a large outbreak of ZIKV was reported from the island of Yap (in Micronesia, northwest of Indonesia). Singapore is already endemic for dengue, and its impact on public health and economic burden is significant. Other dengue-like infections (e.g., Chikungunya virus) are present. Yet only 10% of reported dengue cases have laboratory confirmation. The identification and control of other dengue-like, mosquito-transmitted infections is thus important for the health of Singapore's population, as well as its economy. Given that ZIKV shares the same *Aedes* mosquito vector with both dengue and Chikungunya, it is possible that this virus is present in Singapore and causing some of the mild dengue-like illness. A specific and sensitive one-step, reverse transcription polymerase chain reaction (RT-PCR) with an internal control (IC) was designed and tested on 88 archived samples of dengue-negative, Chikungunya-negative sera from patients presenting to our hospital with a dengue-like illness, to determine the presence of ZIKV in Singapore. The assay was specific for detection of ZIKV and displayed a lower limit of detection (LoD) of 140 copies viral RNA/reaction when tested on synthetic RNA standards prepared using pooled negative patient plasma. Of the 88 samples tested, none were positive for ZIKV RNA, however, the vast majority of these were from patients admitted to hospital and further study may be warranted in community-based environments. **J. Med. Virol. 84:1501–1505, 2012.** © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

Arboviruses are recognized increasingly as a threat to global health. This group of pathogens is uniquely capable of emerging and distributing to new geographic regions as vectors spread and humans encroach on the environments of zoonotic reservoirs. Many have adapted to urban existence, and can cause explosive and disruptive outbreaks when new populations are exposed [Mackenzie and Williams, 2009; Weaver and Reisen, 2010]. Their propensity to emerge and establish in novel geographic regions brings new health threats and diagnostic challenges. Southeast Asia has been the center for emergence of several arboviruses. Chikungunya emerged from East Africa in 2004 causing an epidemic that affected millions and resulted in the virus becoming endemic throughout South and Southeast Asia including urban centers such as Singapore [Staples et al., 2009]. New serotypes of dengue continue to cause serious mortality and morbidity across the region. Japanese encephalitis virus has expanded its geographic range as far west as Pakistan and south to Papua New Guinea, Torres Strait and northern Queensland in Australia [Weaver and Reisen, 2010].

Zika virus (ZIKV) is a mosquito borne virus in the genus *Flavivirus*, family *Flaviviridae*. It was first isolated in 1947 from a febrile sentinel monkey in the

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Zika forest in Uganda during a project researching Yellow Fever virus [Dick et al., 1952]. ZIKV is endemic to East and West Africa, causing sporadic self-limiting febrile illness in humans [Macnamara, 1954; Fagbami, 1979; McCrae and Kirya, 1982]. The virus migrated across to Asia, with serological evidence of human infection in Malaysia in 1966 [Marchette et al., 1969]. Since then, evidence of ZIKV causing febrile illness in humans has been identified serologically in India, Indonesia, and Philippines [Rao, 1971; Olson et al., 1981]. The major vectors for ZIKV are mosquitoes from the *Aedes* genus including *A. africanus* among others in Africa, and *A. aegypti* in Malaysia [Marchette et al., 1969; Fagbami, 1979; McCrae and Kirya, 1982]. Non-human primates are thought to be sylvatic reservoirs, although other reservoir hosts have not been excluded [Hayes, 2009].

ZIKV remained relatively unrecognized until 2007 when it caused a large outbreak on Yap and surrounding islands in the Federated States of Micronesia [Lanciotti et al., 2008; Duffy et al., 2009; Hayes, 2009]. Initially this outbreak was misdiagnosed as dengue fever, however, serological and molecular diagnostic studies implicated ZIKV as the cause. Over 70% of Yap residents were affected. The most frequently reported syndrome was a febrile rash illness with arthralgia, myalgia, headache, or conjunctival injection. There were no documented cases of encephalitis or hemorrhagic disease. More recently, sexual transmission of ZIKV between a heterosexual couple was proposed, suggesting a novel transmission route for these adaptable viruses [Foy et al., 2011].

Singapore is endemic for dengue and Chikungunya. Of the 4,000–9,000 dengue cases notified annually in Singapore, only around 10% have laboratory confirmation [Lee et al., 2010]. Given the considerable overlap in clinical presentations and the presence of known mosquito vectors, it is not unreasonable to expect other flaviviruses including ZIKV may be responsible for milder cases clinically diagnosed as 'dengue' in Singapore. To investigate this, a diagnostic one-step reverse-transcription polymerase chain reaction (RT-PCR) assay for ZIKV was designed and tested against stored extracted RNA of patients with dengue-like illness presenting to the National University Hospital (NUH), Singapore.

MATERIALS AND METHODS

Genome Analysis, Primer Design, and Optimization

ZIKV has a ss(+)RNA genome consisting of 10,794 nucleotides encoding three structural genes and five nonstructural genes. It is most closely related to Spondweni virus, the only other member of the Spondweni clade within the mosquito borne flaviviruses. There appear to be three lineages of ZIKV based on NS5 homology—the East-African lineage, a West-African lineage, and the more distally related Yap lineage [Lanciotti et al., 2008]. Only two full genome sequences of ZIKV are published in GenBank, the prototype MR766, isolated from rhesus monkey 766 in Zika forest 1947, and ZIKA EC 2007, obtained from the Yap outbreak. The sequences have 89% sequence homology with 97% amino acid similarity.

The NS5 gene was chosen as target for the primers. Sequences of MR766 (GenBank accession number AY632535.2) and ZIKA EC 2007 (GenBank accession number EU545988.1) were aligned using Clustal W software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and a primer pair with complete homology to both sequences was designed (Table I). Internal control (IC) primers targeting glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), a human housekeeping gene, were selected to control for any potential sample inhibition of the PCR reaction. All primer sequences showed no cross-reactivity to any sequence other than their respective targets in the BLAST database (<http://blast.ncbi.nlm.nih.gov/>).

The primers pairs' sequences, concentration of primers, and cycling conditions used in a single reaction were carefully optimized to minimize possible formation of secondary structures and competitive inhibitions between primers. Optimal primer concentrations for each set of primer were carefully selected from a primer titration matrix experiment and the optimal annealing temperature was determined by temperature gradient.

Synthetic RNA Standards

No viable virus was isolated from the outbreak on Yap Island. An RNA transcript of MR766 was kindly donated by Division of Vector Borne Diseases, CDC (Fort Collins, CO) for use as positive control. The ZIKV

TABLE I. Primer Sequences and Characteristics

| Primers | Nucleotide position | Sequence (5'–3') | Sequence orientation | Amplicon size (bp) |
|---|---------------------|------------------------|----------------------|--------------------|
| ZIKVF9027 ^a | 9121–9141 | CCTTGGATTCTTGAACGAGGA | Forward | 192 |
| ZIKVR9197c ^a | 9312–9290 | AGAGCTTCATTCTCCAGATCAA | Reverse | |
| GAPDH112F22 (internal control) ^b | 112–133 | GTGAAGGTCGGAGTCAACGGAT | Forward | 264 |
| GAPDH357R19 (internal control) ^b | 375–332 | GCCAGCATCGCCCCACTTG | Reverse | |

^abased on Zika virus strain MR 766, complete genome (GenBank accession: AY632535.2).

^bbased on *Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA (GenBank accession: NM_002046).

target amplicon was cloned into a pDrive cloning vector (Qiagen, Valencia, CA) and transcribed, *in vitro*, into RNA transcripts using RiboMAX™ Large Scale RNA Production System -T7 (Promega, WI), according to manufacturers' instructions. Synthesized transcripts were subjected to quantification using NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE), at an absorption wavelength of 260 nm. Desired concentrations of RNA standards were diluted using pooled, negative RNA extracts to simulate the presence of matrix effect and *GAPDH* gene.

Analytical Evaluation

The limit of detection (LoD) within 95% confidence limits was determined using the *in-vitro* synthesized RNA transcripts. From 10^2 RNA copies/ μ l of RNA standard, one serial dilution series with semi logarithmic dilution steps was generated. All dilutions were aliquoted into three individual vials for one-time use to avoid repeated freeze and thaw cycles on the RNA dilution standards. A total of 24 replicates for each dilution step were carried out on 3 different days, with different reagent preparations by different operators. The 95% confidence limits were determined by polynomial probit analysis using SPSS version 19.0 (SPSS Inc., Chicago, IL). To ensure no cross reactivity with related viruses present in Singapore and with other common viruses which may have fever, rash, or arthralgia as presenting symptoms, specificity analysis confirmed negative results when tested with dengue virus 1–4, Japanese encephalitis virus, yellow fever virus, hepatitis C virus, Chikungunya virus, Ross River virus, cytomegalovirus, Epstein–Barr virus, varicella zoster virus, herpes simplex 1 virus, hepatitis B virus, parvovirus B19, adenovirus and human enterovirus subtypes including human enterovirus 71, echovirus 6, poliovirus Sabin type 1, 2, and 3, and coxsackieviruses A10 and B4. No samples of West Nile virus or rubella were available for testing.

Reverse RT-PCR

Amplification of ZIKV and IC were performed in a single-tube format using the conventional gel-based RT-PCR method. The RT-PCR was performed on the 96-Well GeneAmp® PCR thermal cycler (Applied Biosystems Inc, Foster City, CA), using the QIAGEN® OneStep RT-PCR (Qiagen, Valencia, CA). The 20 μ l of amplification reaction mix consisted of 5 μ l extracted RNA template, 0.4mmol/L dNTPs, 0.8 μ l enzyme mix, 1X buffer with final concentration of 3.0 mmol/L of $MgCl_2$, 0.5 μ mol/L of ZIKV forward primer and 0.8 μ mol/L of ZIKV reverse primer, 0.15 μ mol/L of IC forward primer and 0.15 μ mol/L of IC reverse primer. All primers were synthesized from Eurogentec AIT (Seraing, Belgium). The RT-PCR was initiated by reverse transcription (50°C, 30 min) and initial denaturation (95°C, 15 min), followed by 45 amplification cycles at 94°C for 15 sec, 57°C for 25 sec, and 72°C for

20 sec. Appropriate positive and non-template controls were included in every test run. Amplified products were visualized and analyzed using 2% agarose gel electrophoresis.

Sample Selection

Ethics approval (DSRB-E/10/182) was obtained to test archived extracted RNA for ZIKV from adult (i.e., aged 21 years or older), non-pregnant patients at NUH who had blood submitted for PCR testing for dengue and/or Chikungunya. Approval was not obtained to test samples from pediatric patients in this pilot study, but these represented relatively few samples as pediatric patients with suspected dengue fever were usually diagnosed on clinical grounds alone. Blood samples were less often drawn from pediatric patients due to the discomfort and stress that this may cause. Patients were included in the study if they were >21 years old, non-pregnant, had a documented history of fever and myalgia and/or arthralgia and/or rash, and there was a blood sample taken within 7 days of fever onset which was negative for dengue and (when tested) Chikungunya RNA on previous PCR testing. Eighty-eight unique patient samples taken between November 2008 and May 2011 met these criteria and were tested with the newly developed ZIKV assay.

Viral RNA Extraction

All blood samples collected in EDTA tubes were centrifuged at 3,000 rpm for 10 min. After centrifugation, 1 ml of blood plasma was collected for high speed centrifugation (13,200 rpm) for an hour at 4°C. After 1 hr, 800 μ l of plasma was carefully aliquoted away, leaving remaining 200 μ l for extraction with the Qiagen EZ1 Virus mini kit v2.0, using their respective proprietary Bio Robot EZ1 platform (Qiagen, Valencia, CA). For blood samples collected in plain tube, sera were used for extraction as described above. All blood samples were stored at 4°C and extracted within 2 days. All RNA extracts were previously tested for presence of dengue virus using a published real-time RT-PCR method [Johnson et al., 2005] and were negative for dengue. Tested RNA extracts were stored at –80°C upon initial testing without further thawing.

RESULTS

The 95% confidence limits of the LoD for ZIKV calculated from the three serial dilution series PCR tested on three consecutive days, using polynomial probit analysis was 140 RNA copies/PCR. No cross reactivity was observed for analytical specificity testing which included other closely related flaviviruses such as dengue virus, yellow fever virus, and Japanese encephalitis virus.

Of the 88 patients included for study: 61.4% were male (median age was 38 years), 96.6% were inpatients

admitted for investigation of febrile illness, and 3.4% were investigated as outpatients either in the dengue outpatient management clinic or in other specialist outpatient clinics. All had a febrile illness with an onset within 7 days of blood collection. The median time between fever onset and blood collection was 4 days. 84.1% had documented myalgia and in 44.3 and 30.7% had documented arthralgia and rash, respectively. None of the patient samples tested positive for ZIKV when tested on this assay.

DISCUSSION

The aim was to develop a sensitive and specific one-step RT-PCR assay for screening patients who present with clinical symptoms and signs compatible with ZIKV infection. Inclusion of a GAPDH IC allowed detection of PCR inhibitors within the patient samples and avoided false negative results without an unacceptable loss of sensitivity. Fortunately, no ZIKV was detected in these selected patient samples, suggesting that ZIKV is not a widespread infection in Singapore—if it is present at all.

Only two other assays for detection of ZIKV have been published previously. One, using degenerate primers, was designed from ZIKV samples isolated from mosquitoes and humans in West Africa [Faye et al., 2008]. The other was a real-time PCR assay constructed for investigation of the Yap outbreak [Lanciotti et al., 2008]. Both assays target the envelope gene. In this assay, primers targeting the NS5 region were chosen as this region is usually highly conserved among flaviviruses and may allow detection of circulating variants whereas envelope genes could be more subject to variation driven by antigenic pressure. This was important as the sequence of other ZIKV variants which have been reported in South East Asia is unknown. Having 100% homology with the archetypal strain, MR766, and with the most recently identified strain ZIKA EC 2007 ensured a well-conserved region was targeted. The assay is also capable of being converted to a real-time format using fluorescent probes, as appropriate probes were designed and tested to ensure specificity and sensitivity of the assay was not altered (data not shown). This allows the rapid application of the assay within a fast workflow, should an outbreak of Zika fever occur in this region.

The samples were largely taken from patients where the diagnosis was thought to be critical enough that a laboratory test was required. In fact, most patients presenting with dengue-like illness at this hospital are mainly diagnosed and managed purely on clinical grounds for dengue or Chikungunya, as outpatients without having blood taken and sent for PCR testing. This sampling bias represents a limitation of the study in that if most ZIKV cases in this population present as a relatively mild clinical illness, no blood sample would have been taken for dengue or Chikungunya testing and would therefore not be available for screening with this ZIKV assay.

Furthermore, most of the published reports are in patients younger than 18 years of age, and these patients were excluded from this study as ethics approval for testing of these samples was not granted. However, it is unlikely that the exclusion of these samples would affect the performance or the sensitivity of the assay. A strength of the study is that samples over an 18-month period were tested and thus should cover any seasonality as increased case numbers toward the end of the rainy season has been previously postulated [Olson et al., 1981].

Given these limitations, the presence of ZIKV cannot be excluded in Singapore, but we were not able to detect it in a sample of febrile patients hospitalized in NUH during 2008–2011. Duration of viremia in Zika fever is not known, but expected to be short as for other flaviviruses [Lanciotti et al., 2008]. Prospective collection of blood from patients presenting to community medical clinics within the first 3–4 days of fever is likely to be a more suitable sample group and could be an area for future research.

Although no cases of ZIKV were identified in this Singaporean patient cohort through retrospective testing, knowing that flaviviruses are capable of sudden and sometimes explosive shifts in their distribution and range of clinical manifestations, periodic surveillance with an in-house assay such as this, in areas supportive of the mosquito vector, on hospitalized and non-hospitalized patients with compatible symptoms and signs, or even on the mosquito vectors themselves, may provide a greater understanding of the epidemiology of this virus in South East Asia.

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