Original Article

Retrospective screening of acute undifferentiated fever serum samples with universal flavivirus primers

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Abstract

Introduction: Fever is a common symptom of many tropical diseases and in many cases the etiologic agent remains unidentified as a consequence of either the etiologic agent not being part of routine diagnostic screening or as a consequence of false negatives on standard diagnostic tests.
Methodology: This study screened a well characterized panel of 274 serum samples collected on day of admission from adult patients with acute undifferentiated fever admitted to a hospital in Nakhon Ratchasima, Thailand by RT-PCR using pan-flavivirus degenerate primers.
Results: Subsequent clinical diagnosis was achieved for 38 of the patients, and included 19 cases of dengue fever. RT-PCR screening identified seven positive samples (2.5%) which were revealed by sequence analysis to be dengue virus 1 (2 cases), dengue virus 2 (2 cases) and dengue virus 3 (3 cases). Only 5 out of 19 (26%) serum samples from patients subsequently diagnosed with dengue were positive, but 2 samples which clinically remained undiagnosed were shown to be positive for dengue virus. Sequence analysis suggested that the dengue virus 3 cases occurred as a result of importation of a strain of dengue from India or China. No other flaviviruses were identified.
Conclusions: No evidence was found of other flaviviruses besides dengue circulating in this population. Despite improved diagnostic tests, cases of dengue are still evading correct diagnosis.

Key words: fever; dengue; serum; flavivirus; phylogeny; RT-PCR.

Introduction

The 53 known species of viruses belonging to the genus *Flavivirus* (family *Flaviviridae*) are organized into three clusters, the mosquito borne cluster, the tick borne cluster and the no vector cluster based on their mode of known or suspected mode of transmission [1]. The known species of flaviviruses include a number of internationally important human pathogens, including *Yellow fever virus*, *Dengue virus* (DENV), *Japanese encephalitis virus* (JEV), *West Nile virus* and *Tick Borne Encephalitis virus* [1].

Thailand is known to be hyper-endemic for DENV [2], as well as to have JEV circulating in some regions of the country [3]. Zika virus (assigned to the Spondweni virus group) is believed to have been circulating in Southeast Asia for some 50 years [4] and a case of Zika fever was diagnosed in Cambodia in 2010 [5]. Tembusu virus (assigned to the Ntaya serocomplex) is a virus of uncertain human pathogenicity as while there are no reports of an association with human disease, there are reports of human seroconversion [6] and Tembusu virus has been detected in local mosquito populations [7].

There are an average of about 50,000 cases of dengue fever per year in Thailand [2] and while cases of Japanese encephalitis are rarer due to a successful vaccination program in Thailand, infection with JEV still leads to numerous hospitalizations each year [3]. There have been no reported local cases of infection with Tembusu virus or Zika virus, but two cases of international travelers who are believed to have contracted Zika fever during their stay in Thailand have been reported [8, 9]. It is possible therefore that cases of flavivirus infection by viruses other than DENV and JEV have not been reported in Thailand simply because these other viruses are not part of the normal spectrum of infections encountered, and specific diagnostic tests are not available for these viruses.

Acute undifferentiated fever is defined as acute fever associated with nonspecific symptoms and signs. Studies have shown that dengue infection, leptospirosis, and rickettsial infections such as scrub typhus and murine typhus are common causes of this syndrome in Southeast Asia including Thailand and that the cause of this syndrome remains unknown in 20-50 % of cases [10]. This study sought to screen serum samples from patients admitted to a tertiary hospital in Northeastern Thailand for acute undifferentiated fever for evidence of pathogenic flaviviruses, using degenerate primers capable of detecting all known flaviviruses with high sensitivity [11].

Methodology

Serum samples

The 274 serum samples collected on day of admission are part of a previously described prospective hospital based study on adult patients with acute undifferentiated fever undertaken at a hospital in Nakhon Ratchasima province in Northeastern Thailand [12]. Samples were collected between July 2011 and December 2012.

Samples were collected after study evaluation by the Ethical Review Subcommittee of the Public Health Ministry of Thailand and the Ethical Review Subcommittee of the Faculty of Medicine Siriraj Hospital, Mahidol University. Written inform consent was obtained from all study participants.

A confirmed diagnosis was subsequently available for 38 of the patients whose samples were included in this study, of which 19 were confirmed as dengue fever, 9 as scrub typhus and 10 as leptospirosis [12]. Dengue serology (IgG and IgM) and NS1 ELISA were undertaken in house at Siriraj hospital, Bangkok, Thailand according to established protocols [13, 14].

RNA extraction and amplification

Total RNA was extracted from 150 µl of patient plasma (n = 274) with TRI LS Reagent (Molecular Research Center, Inc., Cincinnati, USA) in accordance with the manufacturer's suggested protocol. RT-PCR was carried out using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, USA) with 6 µl RNA, essentially as described by Maher-Sturgess and colleagues using primers Flav100F and Flav200R [11]. Dengue viral RNA from DENV 2 (strain 16681) and water were included as positive and negative controls, respectively. PCR products were separated on 1.0% agarose gels by electrophoresis and products visualized by ethidium bromide staining.
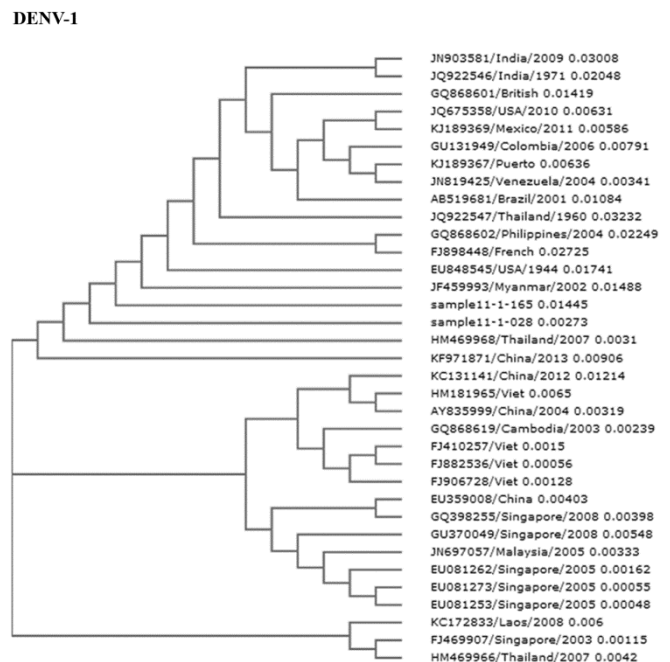
DNA sequencing

Positive samples were re-amplified, the products excised from the gel and purified with Gel/PCR DNA fragments extraction kit (Geneaid Biotech, New Taipei City, Taiwan) before undergoing commercial sequence analysis (1st BASE, Singapore). Sequence data was compared using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenic trees were constructed using the Clustal Omega program [15]. All sequences were deposited with the GenBank database (Accession numbers KM488324, KM488325, KM488326, KM488327, KM488328, KM488329 and KM488330).

Results

A total of 274 serum samples taken on day of admission from adult patients with acute undifferentiated fever collected at a hospital in Northeastern Thailand were screened with universal degenerate primers capable of detecting all known flaviviruses [11]. A total of 7 samples (2.5%) gave positive PCR products which were subsequently re-amplified and sent for sequence analysis. Sequence analysis confirmed that all samples were positive for DENV, and that three of the samples were positive for

Figure 1. Phylogenic tree of DENV 1 showing the sequences of the two isolates identified in this study (samples 11-1-165 and 11-1-028) and comparative DENV 1 isolate sequences selected from the GenBank database.
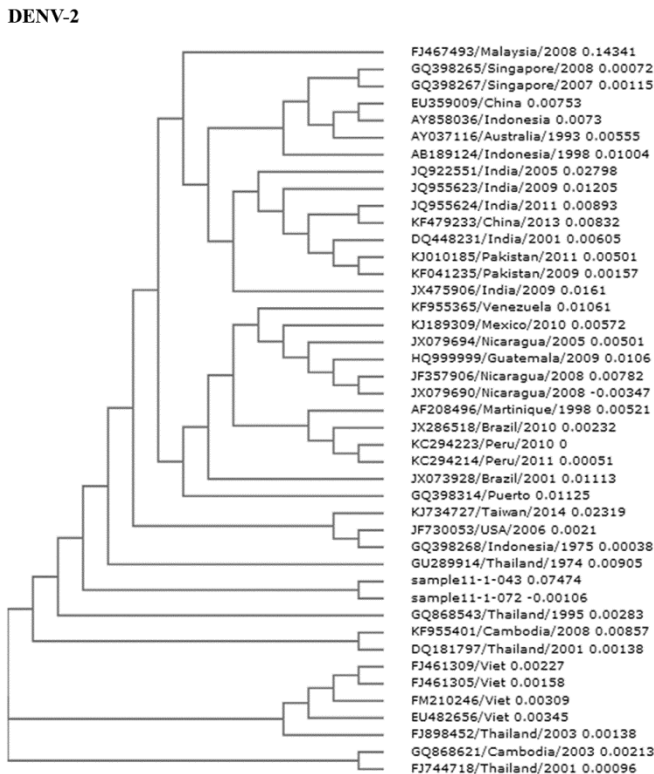
DENV-1

JN903581/India/2009 0.03008
JQ922546/India/1971 0.02048
GQ868601/British 0.01419
JQ675358/USA/2010 0.00631
KJ189369/Mexico/2011 0.00586
GU131949/Colombia/2006 0.00791
KJ189367/Puerto 0.00636
JN819425/Venezuela/2004 0.00341
AB519681/Brazil/2001 0.01084
JQ922547/Thailand/1960 0.03232
GQ868602/Philippines/2004 0.02249
FJ898448/French 0.02725
EU848545/USA/1944 0.01741
JF459993/Myanmar/2002 0.01488
sample11-1-165 0.01445
sample11-1-028 0.00273
HM469968/Thailand/2007 0.0031
KF971871/China/2013 0.00906
KC131141/China/2012 0.01214
HM181965/Viet 0.0065
AY835999/China/2004 0.00319
GQ868619/Cambodia/2003 0.00239
FJ410257/Viet 0.0015
FJ882536/Viet 0.00056
FJ906728/Viet 0.00128
EU359008/China 0.00403
GQ398255/Singapore/2008 0.00398
GU370049/Singapore/2008 0.00548
JN697057/Malaysia/2005 0.00333
EU081262/Singapore/2005 0.00162
EU081273/Singapore/2005 0.00055
EU081253/Singapore/2005 0.00048
KC172833/Laos/2008 0.006
FJ469907/Singapore/2003 0.00115
HM469966/Thailand/2007 0.0042

DENV 3, two samples were positive for DENV 1 and two were positive for DENV 2. No other flavivirus was detected. A phylogenic analysis showed that while the DENV 1 and DENV 2 viruses were grouped with Southeast Asian isolate sequences (Figures 1 and 2) the DENV 3 isolates were most closely related to sequences from China (2013 and 2009) and India (2008) as shown in Figure 3.

Discussion

Of the 274 serum samples screened with pan-flavivirus primers, only 7 positive samples were detected, and all were subsequently shown to be DENV, and three different serotypes (DENV 1, DENV 2 and DENV 3) were detected. As the samples were all collected between July 2011 and December 2012, it can be assumed that these three viruses were circulating simultaneously in Thailand, consistent with the known hyper-endemic nature of DENV in Thailand [16]. While no case of DENV 4 was detected, this might be a reflection of the total number of DENV positive samples, and it cannot be concluded that DENV 4 was not circulating during the study period.

The patients who provided 19 of the samples were subsequently shown to have dengue fever by serologic and/or NS1 screening [12]. Only 5 of these patients were amongst the PCR positive samples, showing a relatively low sensitivity for virus detection by RT-PCR in serum samples. While optimized RT-PCR methodologies specific for DENV have reported detection rates of over 75% [17], the objective of this study was to survey for different flaviviruses, and so the lower detection rate with a non-optimized protocol for DENV was not surprising. Two of the samples shown to be positive by RT-PCR in this study were not diagnosed as dengue fever patients by serologic and/or NS1 testing [12] suggesting that some cases of dengue fever in Thailand are still missed during clinical screening, possibly as a consequence of . As the samples studied were "day of admission samples" from adults, there may be some variation in the timing of the individual samples in relation to disease onset. The PCR methodology used here would be more sensitive for samples from very early after fever onset [18], while both NS1 and serological testing would be more sensitive for samples taken later after fever onset [19]. This would possibly account for the samples detected by PCR, but not by serology or NS1 testing (samples collected early after fever onset) and samples

Figure 2. Phylogenic tree of DENV 2 showing the sequences of the two isolates identified in this study (samples 11-1-043 and 11-1-072) and comparative DENV 2 isolate sequences selected from the GenBank database.

DENV-2

Figure 3. Phylogenic tree of DENV 3 showing the sequences of the three isolates identified in this study (samples 11-1-669, 11-1-688 and 11-1-030) and comparative DENV 3 isolate sequences selected from the GenBank database.
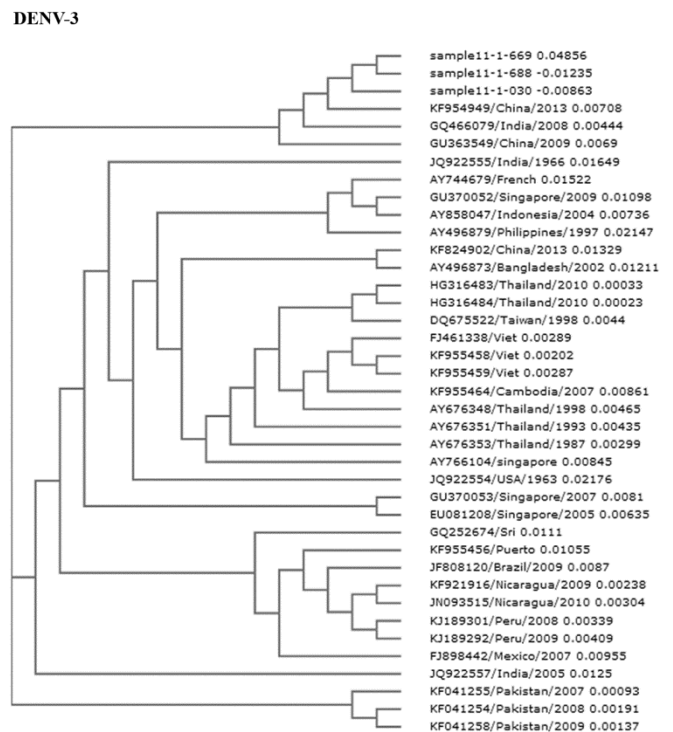
DENV-3

positive by serologic/NS1 but negative by PCR (samples collected later in the course of the fever).

While DENV 1 and DENV 2 samples clustered with representative Southeast Asian isolates, the DENV 3 sequences showed highest homology to sequences from China and India suggesting that this DEN 3 strain was imported to Thailand before becoming locally established. The importation of DENV lineages to Thailand from different countries has been observed previously [16].

In this study, no other flaviviruses were detected, suggesting that there is no major circulation of unknown flaviviruses in northeastern Thailand. JEV is known to circulate in Thailand, and it has been estimated that 15% of hospitalized encephalitis cases in Thailand occur as a consequence of JEV infection [3]. However, clinically, symptomatic Japanese encephalitis is distinctive [20] and it is unlikely that a patient with JE would meet the inclusion criteria of acute undifferentiated fever. It should be noted that all of the samples screened were from adult patients with acute undifferentiated fever, and screening of a similar cohort of pediatric patients could produce different results.

The tourists who were diagnosed with Zika fever upon their return to Canada [8] and Germany [9] both visited Phuket, one of the southern provinces of Thailand as a common part of their holiday [8,9]. Phuket, Thailand's largest island, is located on the Andaman Sea side of Thailand and is thus geographically quite separate from Cambodia, where Zika virus infection has been reported [5]. This suggests that while no evidence was found of Zika infection in this cohort from the Northeast of Thailand, other populations, particularly those of Southern and Southeastern Thailand (adjacent to Cambodia) may show evidence of Zika transmission.

Overall, the results support the supposition that DENV is the major circulating flavivirus in Thailand, and potential emerging flaviviruses such as Zika virus are not currently impacting on the population of Northeastern Thailand.

Acknowledgements

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