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Phylogenetic study reveals co-circulation of Asian II and Cosmopolitan genotypes of Dengue virus serotype 2 in Nepal during 2013 ☆

Sneha Singh [a,1], Birendra P. Gupta [b,1], Anoop Manakkadan [a,2], Krishna Das Manandhar [b,*], Easwaran Sreekumar [a,*]

[a] Molecular Virology Laboratory, Rajiv Gandhi Centre for Biotechnology (RGCB), Thycaud P.O., Thiruvananthapuram 695014, Kerala, India
[b] Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal

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ABSTRACT

The re-emergence of dengue virus in Nepal and the recent widespread disease epidemics of unprecedented magnitude have raised a great public health concern. There are very few reports on Dengue virus (DENV) strains circulating in the country, especially at the molecular phylogenetics level. In this study, clinical samples from an outbreak in Nepal in 2013, which were positive for DENV serotype 2, were characterized by targeted genome sequencing. Envelope protein (E) coding region from fifteen samples were sequenced and compared with DENV-2 sequences of strains from different geographic regions obtained from the GenBank. Compared to the prototype New Guinea C strain, the samples had a total of eleven non-synonymous substitutions in the envelope protein coding region leading to amino acid change at positions 47, 52, 71, 126, 129, 149, 164, 390, 402, 454 and 462. However, none of these sites were found to be positively selected. A major observation was the presence of two distinct genotypes (Cosmopolitan Genotype IVa and Asian II) in the outbreak as seen by the phylogenetic analysis. It gives the first evidence of the introduction of Cosmopolitan Genotype IVa in Nepal. These strains replace the Genotype IVb strains prevalent earlier since 2004. Both genotypes had closer genetic relation to strains from other countries indicating possibility of exotic introduction. The Genotype IVa strain seems to be more adapted in C6/36 mosquito cells as indicated by its marginally increased replication rate than the Asian II strain in *in vitro* infection kinetics assays. The genotype replacement and co-circulation of two distinct genotypes may have significant consequences in dengue epidemiology and disease dynamics in Nepal in years to come.

1. Introduction

Dengue is a major re-emerging mosquito-borne disease in the tropical and subtropical countries. For the last few decades it remains on top with respect to disease burden and mortality (Bhatt et al., 2013). The disease manifests as a mild febrile episode or as severe and life threatening haemorraghic syndrome. Dengue virus belongs to the genus *Flavivirus*, family *Flaviviridae*. Its genome consists of approximately 11 kb positive-sense, single stranded RNA molecule without a poly-A tail (Chambers et al., 1990). Among the three structural proteins (Capsid, Membrane and Envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4 and NS5) encoded by the genome, the envelope (E) protein plays a critical role in virus attachment and fusion to the host cell membrane, and hence infectivity. Three domains (I, II and III) have been identified in the E protein and the domain III interacts with the cellular receptor and mediates endosome fusion (Modis et al., 2004). The presence of four classical serotypes among the Dengue viruses (DENV-1, 2, 3, and 4) that have no cross-protective immunity in infected patients dramatically increases the complexity of this disease.

DENV-2 has been attributed to cause a more severe disease even during primary infections (Rico-Hesse, 2003). At molecular genetic level, DENV-2 has been divided into 6 genotypes namely Cosmopolitan, Asian I, Asian II, American, American/Asian and Sylvatic. These genotypes have a 5% or more genetic divergence in their envelope as well as whole genome nucleotide sequences

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* Corresponding authors.
E-mail addresses: krishna.manandhar@gmail.com (K.D. Manandhar), esreekumar@rgcb.res.in (E. Sreekumar).

[1] These authors contributed equally.

[2] Present address: Department of Biotechnology, Cochin University of Science and Technology, Cochin-22, Kerala, India.

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(Weaver and Vasilakis, 2009). The Asian I genotype comprises of strains isolated from Thailand and Malaysia while Asian II genotype consists of strains circulating in China, Philippines, Sri Lanka, Taiwan and Vietnam. The strains circulating in China, Thailand, Vietnam, Brazil, Venezuela and the Caribbean belonged to American/Asian genotype while the isolates from Australia, Pacific Islands, South East Asia, Indian sub-continent, Middle East, Africa and Mexico fall in Cosmopolitan genotype (Twiddy et al., 2002; Mendez et al., 2012).

Nepal, a Himalayan country, is surrounded by India on three sides and China to the North. It is an endemic nation for many vector borne diseases, like malaria, kala-azar, Japanese encephalitis and lymphatic filariasis (Dumre et al., 2013). The first report of dengue fever came in 2004 from a Japanese traveler in Nepal and it was reported to be of serotype 2 (Takasaki et al., 2008) with >99% similarity with viral strains from India. It was followed by a native report in 2006 with detection of four dengue serotypes (Malla et al., 2008). A few intermittent cases were reported nationwide from 2007 to 2009 with 2, 8 and 16 cases per year, respectively (Dumre et al., 2013; Griffiths et al., 2013). An outbreak occurred in 2010 with 350 (30%) confirmed cases out of 1215 clinically reported cases. The first isolation was done during the 2010 epidemic and all the strains belonged to serotype 1, genotype V closer to the Asian sub-cluster (Pandey et al., 2013). The year 2013 witnessed a total shift in the circulating serotype and DENV-2 outbreak was reported in the Terai belt of the country (Birendra et al., 2014; accepted manuscript).

A large number of secondary infections are usually reported from the country and may be attributable to the circulation of multiple serotypes in the region (Malla et al., 2008; Dumre et al., 2013). There is lack of reliable dengue surveillance data from the country and inadequate response in accordance with international guidelines (Griffiths et al., 2013) that may hamper dengue control measures in the region. Adding to these is the lack of information on nucleotide sequences of DENV strains from the country (Malla et al., 2008; Pandey et al., 2013), which may also affect the epidemiological interventions.

In the present study, DENV strains from an outbreak in Nepal in 2013 were characterized at the molecular level by sequencing of the complete envelope protein coding region and subsequent phylogenetic analysis. Strains representing different genotypes were studied by *in vitro* infection kinetics analysis in mosquito cells to understand the differences in infection competence and adaptation. The results shed light on the genotypes of dengue virus currently circulating in the region and add country specific information to the DENV sequence database.

2. Materials and methods

2.1. Ethical approval and patient samples

Ethical approval for the study was obtained from the Nepal Health Research Council (NHRC) Ethical Review board (2071-04-15). A total of 52 venous blood samples from patients clinically diagnosed as dengue were obtained from the 2013 dengue outbreak in Nepal, as described earlier (Birendra et al., 2014; accepted manuscript).

2.2. Cell lines

The *Aedes albopictus* mosquito cell line C6/36 was maintained in culture at 28 °C in L-15 medium (Invitrogen, New York) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAN Biotech, Germany). Baby hamster kidney cells (BHK-21) were cultured in DMEM (Invitrogen, New York) supplemented with 10% FBS (PAN Biotech, Germany) and was maintained at 37 °C in a 5% CO_2 incubator (Sanyo, Japan).

2.3. Reverse transcriptase PCR and envelope (E) protein coding region sequencing

Viral RNA was extracted from 150 µl of patient serum using viral RNA isolation kit (Macherey Nagel, Germany) in accordance with the manufacturer's instructions. Initial dengue viral RNA detection in samples was done by a single-step RT-PCR using a set of generic primers (D1F-DencomR2) (Supplementary Table 1) that amplifies the Core-Pre-membrane (C-PrM) region of any of the four viral serotypes, as described previously (Anoop et al., 2010, 2012). Subsequently, serotype identification was done using a second step, multiplex, semi-nested PCR of this primary amplification by serotype-specific primers described previously (Lanciotti et al., 1992), with minor modifications in the primers (D1F-NTS1/NTS2/NTS3/nDen4) (Supplementary Table 1) (Anoop et al., 2012). Amplification of a 1616 bp region spanning the complete E gene was performed using a two-step RT-PCR with the primers D2Seq3F and D2Seq7R (Supplementary Table 1). Briefly, it consisted of a reverse transcription of 5 µl of the isolated RNA using AMV reverse transcriptase system (Fermentas) at 45 °C for 1 h, and denaturation of the enzyme at 98 °C for 5 min. This was followed by a PCR reaction for 35 cycles with cycling conditions of 98 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 4 min. The amplified region was subjected to bi-directional sequencing using overlapping primers (Supplementary Table 1) and the Big Dye Terminator cycle sequencing kit in an ABI3730 Genetic Analyzer automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

2.4. Virus isolation in C6/36 cells and in vitro infection kinetics analysis

Briefly, the serum samples (1:10 diluted in phosphate buffered saline, PBS; pH 7.4) was allowed to adsorb on to the C6/36 cells for 2 h at 33 °C with slow shaking followed by a gentle wash with 1 × PBS and addition of L-15 medium supplemented with 2% FBS and further incubation with shaking. The virus containing supernatant was harvested on the third day post-infection and stored in −80 °C as aliquots.

For infection kinetics analysis, the C6/36 cells were infected at an MOI of 0.1. The supernatant was collected at 0 h, 6 h, 12 h and then every 24 h till five days post infection. The amount of virus in the supernatant was titrated by plaque assay on BHK-21 cells.

2.5. Infection of BHK-21 cells, immunofluorescence and plaque assay

The BHK-21 cells were infected with DENV-2 isolates by adsorption of the virus for 2 h at 37 °C followed by two washes with 1 × PBS. The cells were further grown in DMEM supplemented with 2% FBS for three days. Cells were fixed with 4% paraformaldehyde 72 h post-infection followed by washes with 1 × PBS and permeabilization with 0.5% Triton X 100. The presence of dengue virus antigen was detected by in-house rabbit polyclonal anti-dengue envelope protein antibody as primary antibody and anti-rabbit Alexa Fluor 488 secondary antibody. Fixed cells were incubated with primary antibody at 4 °C overnight, followed by washes and incubation with secondary antibody at 37 °C for one hour. The cells were washed and counter stained with DAPI stain and visualized under an inverted fluorescent microscope (Nikon Eclipse Ti).

For plaque assay and virus titration, 90% confluent BHK-21 cells were infected by adsorption of the virus for 2 h at 37 °C followed by two washes with 1 × PBS (Sigma–Aldrich) and overlaying with 1 × DMEM (Invitrogen, New York) containing a final concentration