

A New Tool for the Diagnosis and Molecular Surveillance of Dengue Infections in Clinical Samples

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Abstract

Dengue virus and dengue haemorrhagic fever are amongst the most important challenges in tropical diseases due to their expanding geographical distribution, increasing outbreak frequency, hyperendemicity and evolution of virulence.

Here, the use of a RT-nested PCR for both the diagnosis and genetic characterization of dengue infections in clinical samples is described.

Keywords: Dengue, dengue haemorrhagic fever, diagnosis, molecular epidemiology, surveillance, glycoprotein E gene, NS1 gene.

Introduction

Dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are considered to be the most important arthropod-borne viral diseases due to the high rates of morbidity and mortality caused by them. Over 2.5 billion people are at risk of the infection and more than 100 countries are home to endemic dengue transmission with an increasing incidence of DHF cases^[1-3], making dengue an archetypal "emerging" disease. International travel, urbanization, overpopulation, crowding, poverty and a weak public health

infrastructure in most endemic areas are the likely factors contributing to the surge in new cases^[4]. A major concern is the potential spread of dengue fever into the United States of America and Europe due to climate warming and the spread of its vector.

Travellers to areas where dengue is endemic are a potential source of the spread. Most infections manifest as a mild febrile illness during travel that coincides with the peak of viral shedding and risk for transmission. Imported dengue virus infections have been reported in several non-endemic countries; and dengue virus infection is one of the most frequent causes

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of febrile illness in tourists and people working in dengue-endemic areas^[5,6,7,8]. As early symptoms of DF mimic those of other diseases such as malaria or leptospirosis, rapid laboratory diagnosis is important for proper patient care. Dengue fever can be diagnosed by virus isolation, genome and antigen detection, or serological studies. Samples obtained for the detection of dengue virus by cell-culture require proper handling of the sample for viral viability. Serology, even for anti-dengue IgM antibodies, is feasible only after 5 days following the onset of the symptoms. Thus, molecular techniques fulfil an important role in the diagnosis of dengue infection during its early stages.

The characterization of circulating dengue virus serotypes is important in surveillance, since the introduction of a new variant to areas affected by pre-existing serotypes constitutes a risk factor for DHF/DSS^[9]. By defining intra-serotypic genetic variation, the global distribution and spread of virus strains can be mapped and followed up^[10-13], and the genetic differences associated with disease severity can be identified^[10,14-16]. Moreover, recent epidemiological analyses suggest that the more virulent genotypes are now displacing those of lower epidemiological impact^[17], resulting in dengue outbreaks^[18]. In this context, a methodology for real-time, worldwide surveillance is needed to track dengue strains and help anticipate changes in the epidemiology of the infection.

Here we report the amplification and analysis of a genomic interval spanning the E/NS1 junction of the dengue genome for the detection and typing of all four dengue virus serotypes in clinical specimens. This sensitive, specific and rapid alternative assay requires only a single acute phase serum sample.

Materials and methods

Virus isolates and clinical samples

Viral RNAs were provided by the National Collection of Pathogenic Viruses (Porton Down, Salisbury, UK): serotype 1 dengue virus (DEN-1; strain Hawaii), serotype 2 dengue virus (DEN-2; strain New Guinea C), serotype 3 dengue virus (DEN-3; strain H87), and serotype 4 dengue virus (DEN-4; strain H241); the RNAs from prototype strains of Japanese encephalitis (JEV), yellow fever (YFV), tick-borne encephalitis (TBEV), Murray Valley encephalitis (MVEV) and St. Louis encephalitis (SLEV) viruses were used to check the specificity of the dengue virus assay. Serial dilutions of this genome material were prepared to obtain the standards to assess the sensitivity of the assay.

Viremic human sera samples were obtained from patients with a clinical diagnosis of dengue infection (Sera 1794F02; 13VI02; 366VI03; 438VI03). These were travellers who presented at the Spanish Tropical Medicine Units with dengue-compatible symptomatology on their return from the Dominican Republic, India, Indonesia and Nicaragua, respectively, and suffered from classical DF as defined by the WHO criteria^[19].

Selection and synthesis of oligonucleotide primers

A RT-nested PCR protocol was developed for the detection of the four serotypes of dengue virus in clinical samples. Dengue virus primers for amplification and/or sequencing (Table) were designed based on dengue virus sequences in the public sequence databases, using a computer-assisted analysis (MACAW version 32 software, 1995, NCBI, Maryland) to determine consensus sequences. The Table

shows the sequences and the respective primer positions in the prototype strains of the four dengue serotypes. To address the natural variability of dengue viruses, mixtures of degenerated primers were used to enable hybridization with all known serotypes.

RT-Nested PCR

Using purified dengue virus RNA as a template, relevant aspects of the RT-PCR and nested PCR assay (Mg^{2+} concentration, primers, RT temperature, number of cycles, annealing temperatures) were initially optimized to achieve the greatest sensitivity. A PTC-200 Peltier thermal cycler (MJ Research) was used throughout. 5 μ l of viral RNA solution were added to 45 μ l of a medium compatible with both the reverse transcription and PCR amplification steps (QIAGEN® OneStep RT-PCR kit). The RT-PCR mix contained 1 \times OneStep RT-PCR buffer, 400 mM of each dNTP, 20 pmol of each sense or antisense degenerated primer (S1871DEN1, 1871DEN2, 1871DEN3, 1871DEN4, AS2622DEN1, AS2622DEN2, AS2622DEN3, AS2622DEN4) and an optimized combination of Omniscript and Sensiscript reverse transcriptases and HotStar Taq DNA polymerase. The RT-PCR reactions were carried out using an initial reverse transcription step at 41 °C for 45 minutes followed by a denaturation and Hot Star Taq polymerase activation step (94 °C, 15 minutes) and 40 cycles of denaturation (94 °C, 30 seconds), primer annealing (55 °C, 1 minute), and primer extension (72 °C, 30 seconds). A final incubation was carried out at 72 °C for 5 minutes. A second amplification reaction (nested PCR) was seeded with 1 μ l of the initial amplification product. The reaction mixture contained 1 \times buffer B (60 mM Tris-HCl pH 8.5, 2 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 40 pmol of each sense and antisense primer (S2176DEN1,

S2176DEN2, S2176DEN3, S2176DEN4, AS2504DEN) and 2.5 U of DNA Taq Polymerase (Perkin-Elmer). The samples were subjected to a denaturation step (94 °C, 2 minutes) followed by 40 cycles of denaturation (94 °C, 30 seconds), primer annealing (57 °C, 4 minutes), and primer extension (72 °C, 30 seconds) and a further extension step at 72 °C for 5 minutes.

Dengue virus sequence database

A dengue sequence database was constructed by extracting sequences from the NCBI GenBank. Each sequence was identified by name, place, date and serotype. Previously described genotypes were taken from the references listed: DEN-1 strains genotypes were noted as described by Rico-Hesse for DEN-1, 3 and 4^[17] and by Twiddy et al. for dengue virus type 2^[20]. A manual search was employed for all the sequences in GenBank encompassing the targets of selected primers. Next, we used BUSSUB, a new tool developed at the Bioinformatics Unit of the Institute of Health Carlos III^[21]. This software simplifies and boosts the process of retrieving sequences contained between two given flanking regions, improving the final results of a search. Genetic characterization was performed on a total data set of 113 DEN-1, 191 DEN-2, 102 DEN-3 and 153 DEN-4 sequences.

Sequence analysis of amplified products

Original sequence data were first analysed by the CHROMAS software (version 1.3, McCarthy 1996; School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Brisbane, Queensland, Australia); forward and reverse sequence data of each sample were aligned using the programme EDITSEQ (DNASTAR

Inc. Software, Madison, Wisconsin, USA). The consensus sequence was compared and aligned to other samples or DNA database sequences using the programme CLUSTAL X, version 1.83^[22]. Programmes from the MEGA package^[23] were used to produce phylogenetic trees using NJ as the method to reconstruct the phylogeny and Kimura-2p as nucleotide substitution calculation method. The statistical significance of a particular tree topology was evaluated by bootstrap re-sampling of the sequences 1,000 times. Published sequences used in the comparisons were obtained from the GenBank databases. Pair-wise comparisons of the dengue virus database were done by global alignment using the Needleman Wunsch^[24] algorithm using the implementation from EMBOSS, the European Molecular Biology Open Software Suite^[25]. Z-

Scores were calculated to test the significance of each pair-wise alignment by Monte Carlo simulation on the shuffled sequences. Statistical analysis was conducted with the SPSS statistical package (SPSS Software, Chicago, IL).

Results

Design of the primers

The E/NS1 region of the genome was chosen for the development of a RT-nested PCR. The primers selected specifically amplify the four dengue viruses with no cross reactivity to other members of the flavivirus family. A mix of degenerate primers representing each serotype was used to ensure coverage for the highly variable dengue serotypes (Table).

Table. Primers used in RT-nested PCR assays and sequencing

Primer*	Sequence ^o	Genome position [†]	PCR
S1871DEN1 S1871DEN2 S1871DEN3 S1871DEN4	5'-TGGCTGAGACCCARCATGGNAC-3' 5'-TAGCAGAAACACARCATGGNAC-3' 5'-TCTCCGAAACGCARCATGGNAC-3' 5'-TGGCAGAAACACARCAYGGNAC-3'	1869 to 1890 1871 to 1889 1863 to 1884 1873 to 1894	RT-PCR
AS2622DEN1 AS2622DEN2 AS2622DEN3 AS2622DEN4	5'-CAATTCATTTGATATTTGYTTCCAC-3' 5'-CAATTCTGGTGTTATTTGYTTCCAC-3' 5'-CAGTTCATTRGCTATTTGYTTCCAC-3' 5'-TAGCTCGTTGTTATTTGYTTCCAC-3'	2620 to 2644 2622 to 2646 2614 to 2638 2624 to 2648	RT-PCR
S2176DEN1 S2176DEN2 S2176DEN3 S2176DEN4	5'-ATCCTGGGAGACACYGCNTGGG-3' 5'-ATTTTRGGTGACACAGCNTGGG-3' 5'-ATCTTGGGAGACACAGCNTGGG-3' 5'-ATTCTAGGTGAAACAGCNTGGG-3'	2174 to 2195 2176 to 2197 2168 to 2189 2178 to 2199	Nested
AS2504DEN	5'-TGRAAYTTRTAYTYGTCTGTCC-3'	2506 to 2527 DEN-1 2504 to 2525 DEN-2 2496 to 2517 DEN-3 2506 to 2527 DEN-4	Nested

*Primers names beginning with "S" indicate a genome (plus)-sense orientation; names beginning with "AS" indicate a complementary sense orientation.

[†]The genome positions are given according to each dengue virus serotype prototype strain (DEN-1; strain Mochizuki, DEN-2; strain Jamaica N-109, DEN-3; strain H87, DEN-4; strain 814669)

^oDegenerate positions: N:A/C/g/T, R:A/g, Y:T/C

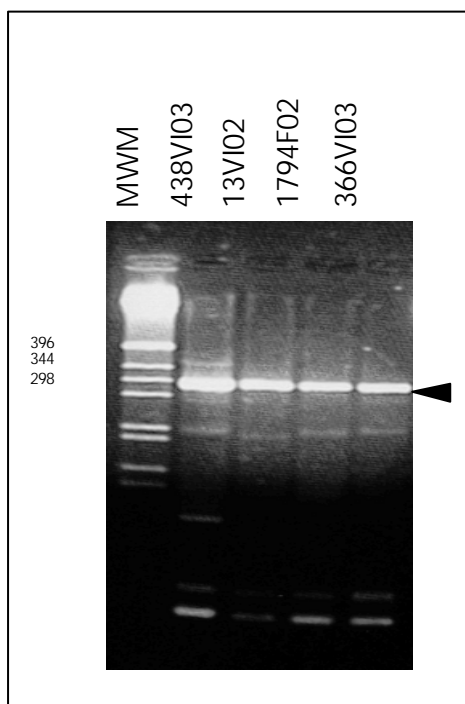
Dengue virus RT-n PCR specificity

The specificity of the RT-n PCR was determined by analysing serial dilutions of RNA from related flavivirus (JEV, MVEV, SLEV, TBEV, WNV, YFV) and no amplification was obtained (data not shown).

The amplification was successful with both commercial RNA and serum samples for all dengue virus serotypes as shown (Figure 1), yielding a distinct DNA product of the expected size (328-pb) in agarose gels.

Figure 1. Amplification products obtained through PCR analysis of sera from subjects with acute dengue virus infection

[Arrow indicates 328 bp E/NS1 products. 1% agarose gel. MWM: Molecular weight markers; 438VI03 (DEN-1); 13VI02 (DEN-2); 1794F02 (DEN-3); 366VI03 (DEN-4)]



One hundred and sixty-four serum samples from cases of febrile illness associated with travel were tested with the assay. Thirty-seven cases were diagnosed as of classical dengue fever by the WHO criteria^[19]. Sixteen of these cases were found positive by using our E/NS1 assay. Convalescent sera were available for 13 of these cases; all were later confirmed to be seroconvert. All serum samples found positive by RT-n PCR were collected in the first week after the onset of the symptoms.

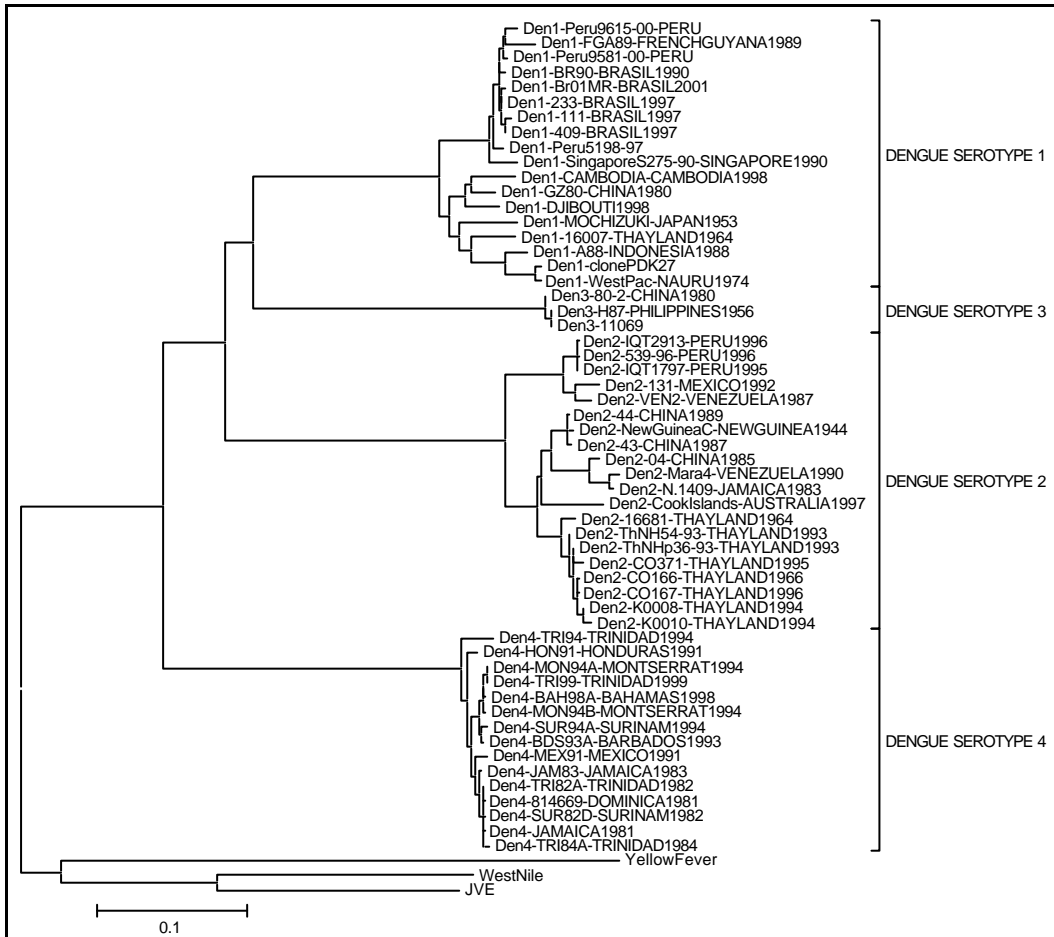
Sequence analysis results

The phylogenetic trees obtained by the analysis of the representative strains of the four serotypes and unknown sample sequences allowed rapid differentiation of the corresponding serotype (Figure 2).

Pair-wise sequence analysis using Needleman Wunsch global alignment was carried out on the 220bp sequence where a higher amount of sequences were available for comparison. As expected, comparisons between serotypes showed a low sequence similarity and could be easily grouped. An all-against-all sequence comparison was done within each serotype to evaluate the possibility of using sequence similarity to classify genotypes. Significant sequence similarity was observed when comparing sequences within the same genotype. This was evaluated by an analysis of variance between groups (ANOVA), comparing the scores of sequence comparisons within genotypes to comparisons between genotypes. Each group was significant to the $P < 0.001$ level. Genotypes with only one member sequence were excluded from the analysis.

Figure 2. **Phylogenetic tree constructed with the E/NS1 fragment which identifies the four dengue serotypes**

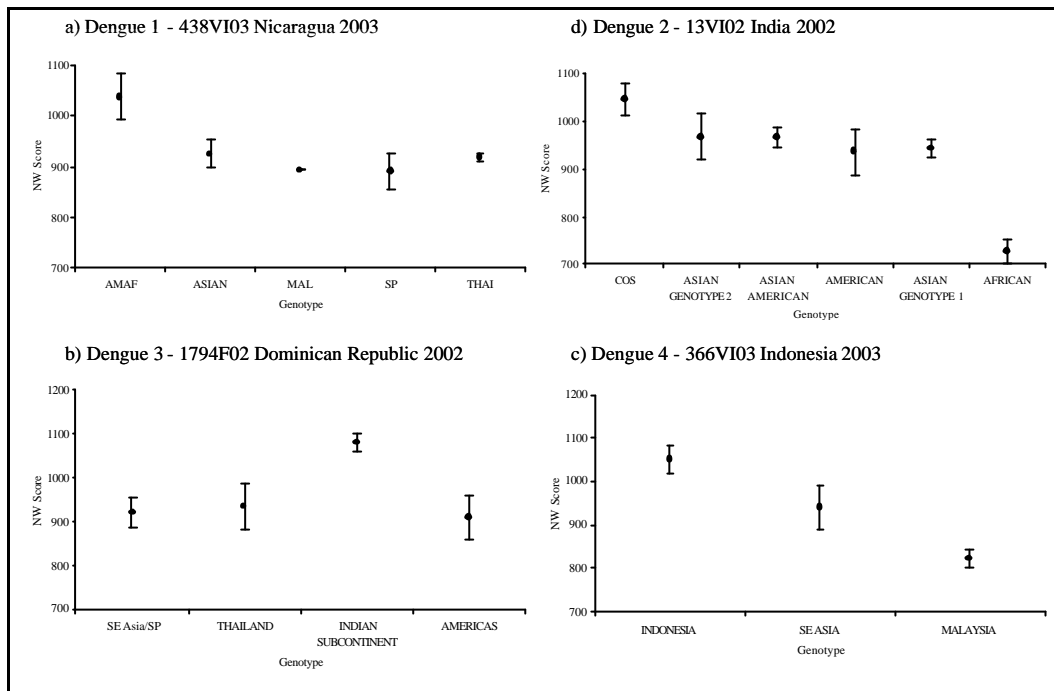
[Phylogenetic analysis was performed using the Kimura-two parameter model as a model of nucleotide substitution and using the neighbor joining method to reconstruct the phylogenetic tree (MEGA version 2.1 software package)]



Sequences that had no known genotype were classified with respect to the group to which they were most similar. To verify the utility of this method, a phylogenetic tree was built in parallel with the unknown and characterized sequences. Bootstrap values in the 220bp region were too low to

generate a full taxonomy tree, but did fully differentiate the genotypes (data not shown). Even with this simple method, all unknowns were classified correctly into their genotypic group (Figure 3 illustrates one example result for each serotype, compared to known sequences).

Figure 3. Pair-wise analysis of four dengue strains detected by PCR amplification of 328 bp E/NS1 products from patient sera. Samples are (a) 438VI03, DEN-1 AMERICAN-AFRICAN genotype, (b) 13VI02, DEN-2 COSMOPOLITAN genotype, (c) 1794F02 DEN-3 INDIAN genotype, and (d) 366VI03, INDONESIA DEN-4 genotype



Discussion

The efficient worldwide control of dengue virus requires the definition of sources of epidemic viruses and the precise identification of virus genotypes. A key objective of DF and DHF surveillance programmes is early detection of outbreaks to permit the implementation of control measures. DHF outbreaks can be anticipated by monitoring the emergence of new genotypes in a region. The need for surveillance is warranted, since air travellers can quickly move viruses from an endemic area to a receptive area. Dengue virus surveillance should be implemented in endemic and non-endemic areas to aid

governments and healthcare workers in planning for potential outbreak situations. The advent of a simple and accurate method for diagnosis and surveillance could improve the establishment of these programmes in developing countries affected by the disease, and in non-endemic areas where dengue is a travel-acquired infection.

The RT-nested PCR described here allows rapid direct diagnosis of acute dengue infection in laboratories without BSL-3 (bio-safety level 3) facilities.

Pair-wise comparison to classify sequences has been used for enteroviruses and potyvirus^[26-28]. Multiple alignment and

rigorous phylogenetic methods are preferable to establish exact lineages of sequence strains and discover recombination events. Pair-wise comparisons can substitute if only a high level of taxonomic classification is desired. Our method allows classification of dengue genotypes using the sequence of the 220bp region amplified by the PCR assay. The advantage of pair-wise comparison for classification is its speed, simplicity and availability. The database and classification scheme provides a repository for sequences, complementing efforts in tracking dengue genotype distribution. A website could be deployed wherein clinical laboratories post their sequences, location and circumstances of isolation. This would allow rapid centralized analysis detailing the genotype, date and location of the most similar sequence isolate in the database. New genotypes could be rapidly identified by failure to relate them to a described group.

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Acknowledgements

This investigation received financial support from the Instituto de Salud Carlos III (ISCIII) through research project grants (MPY 1194/02 and C03/04). G. Palacios and WI Lipkin were supported by the Ellison Medical Foundation and the National Institutes of Health (AI 51292 and U54 AI57158-Lipkin). C. Domingo was contracted by an agreement between the Public Health Division of the Spanish Ministry of Health (DGSP-MS) and the Instituto de Salud Carlos III (ISCIII) for the development of the Haemorrhagic Viral Fevers Surveillance and Control Programme. The authors thank Dr J. Gascón, Dr R. López-Vélez and Dr S. Puente and the many scientists who contributed dengue-infected patient samples for this work. The authors are grateful to Dr J.E. Mejía for assisting in manuscript preparation.

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