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Simple, specific molecular typing of dengue virus isolates using one-step RT-PCR and restriction fragment length polymorphism

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A one-step RT-PCR and one-enzyme RFLP was used to detect and distinguish among flaviviruses, including the four serotypes of dengue and the St. Louis Encephalitis, West Nile and Yellow Fever viruses in cultured virus samples or acute-phase human serum. Using a previously described RT-PCR, but novel RFLP procedure, results are obtained in 24 h with basic PCR and electrophoresis equipment. There is 95% agreement between RT-PCR/RFLP results and those achieved by indirect immunofluorescence assays, and 100% agreement between RT-PCR/RFLP results and gene sequencing. This method is more rapid than tests of cytopathic effect based on virus isolation in tissue culture, and simpler than real-time PCR. It does not require specialized equipment, radioisotopes or computer analysis and is a method that can be applied widely in the developing world. It allows for prompt determination of whether a flavivirus is the cause of illness in a febrile patient, rapid identification of dengue serotypes in circulation, and improved patient management in cases where prior dengue exposure make dengue hemorrhagic fever or dengue shock syndrome a risk.

1. Introduction

The dengue viruses (DENV) are human pathogens with four serologically distinct types, DENV-1, -2, -3 and -4. They are transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes (Weaver and Reisen, 2010) and belong to the family *Flaviviridae* and genus *Flavivirus* (ICTV, 2005), comprising over 70 positive-sense RNA viruses, with 40 tick-borne or mosquito-borne pathogens among these (Kuno et al., 1998). Dengue causes up to 50 million cases each year worldwide and is the world's most important mosquito-borne viral disease (WHO, 2011).

Mild or moderate infections with dengue present with fever, headache, rash, muscle and joint pain, and lethargy (Nielsen, 2009). Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are more severe, complicated forms of dengue infection, manifesting in coagulopathy and increased vascular permeability, which results from a complex and only partially understood immunological process (Martina et al., 2009). Preexisting immunity to a related flavivirus, however, is known to be an important factor in the progression of DHF/DSS; 90% of DHF/DSS cases occur in patients with a secondary infection to a heterologous dengue serotype (Mathew and Rothman, 2008).

For this reason, in regions where more than one dengue serotype is known, simple and rapid methods of identifying the responsible serotype for a disease outbreak are important for the purposes of public health and vector control programs, risk mitigation for DHF/DSS, and diagnosis and management of individual patients. Current methods for serotype identification range from sophisticated techniques, which are sensitive but not widely applicable in diagnostic laboratories of the developing world (Nascimento et al., 2011); to viral culture techniques requiring more than 1 week for a result (Kuno et al., 1985); and a range of ELISA-based (Puttikhunt et al., 2011) and real-time (Hue et al., 2011), nested (Gomes et al.,

Abbreviations: DENV, dengue virus; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; JE, Japanese encephalitis virus; MVE, Murray Valley encephalitis virus; RT-PCR, reverse-transcriptase PCR; RFLP, restriction fragment length polymorphism; SLE, St. Louis encephalitis virus; WN, West Nile virus; YF, Yellow Fever virus.

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2007) and multiplex (Das et al., 2008; Saxena et al., 2008) PCR techniques.

A simple and rapid method is described for the identification of *Flavivirus* in the acute-phase sera of febrile patients, or from cultured virus samples, and the specific identification of dengue serotypes using a one-step, standard RT-PCR and RFLP. Other RT-PCR/RFLP techniques for the detection of dengue or *Flavivirus* have been described previously: Vorndam et al. (1994) described an RT-PCR/RFLP technique which identifies geographic subgroups of dengue, but which requires 2 days and amplification of the entire structural region of the flavivirus genome; Sudiro et al. (1997) described an RT-PCR method that is rapid and distinguishes dengue viruses from other flaviviruses in human sera, but does not identify definitively dengue serotype; and Gaunt and Gould (2005) described an RT-PCR/RFLP method which discriminates among 90% of known vector-borne flaviviruses with published full length E-gene sequences within 10 h, but have not yet published on the utility of this method with serum samples.

The RT-PCR primers used in this investigation, FLAVI-1 and FLAVI-2, amplify a region of the non-structural coding gene 5 (NS5) from the four most important American flaviviruses (dengue, Yellow Fever, West Nile and St. Louis encephalitis viruses), and the Japanese Encephalitis, Murray Valley Encephalitis, Kunjin and Usutu viruses (Ayers et al., 2006). The NS5 gene has already been established as a useful region of the genome for creating phylogenies and distinguishing among flaviviruses (Kuno et al., 1998), which allows confirmatory identification of the pathogen by sequencing if desired. The RT-PCR requires only one round of amplification after cDNA synthesis – no semi-nested PCR step is necessary – and production of an amplicon of 854–863 bp immediately allows determination of whether the fever-causing agent is a member of the *Flavivirus.* PCR products are then cut by *Hae*III and amplicons from DENV-1, -2, -3 and -4, WN and YF each yield different restriction patterns. Confirmatory identification of DENV-1 can be performed with an additional restriction digestion using *Msp*I.

2. Materials and methods

2.1. Collection of samples

Virus isolates from 364 febrile patients, collected 1994–2004 were obtained from the archives of the Virology Department of the Gorgas Memorial Institute for Health Studies (GMI). These isolates had tested positive previously for dengue infection by indirect immunofluorescence assays (IFA; Section 2.2). An additional 37 acute-phase sera were collected from patients in 1999, 2005 and 2007–2010. Approval of the local ethics committee was deemed unnecessary as samples were collected for diagnostic purposes. Informed patient consent was obtained upon the patient's agreement for sample collection. Six of the acute-phase patients could specify the number of days of fever they had experienced prior to the serum collection date. Five serum samples from healthy volunteers were cultured and subject to PCR-RFLP as negative controls.

2.2. Isolation of virus and characterization of serotypes by indirect immunofluorescence

Between 1994 and 2004, virus was isolated and cultured from sera obtained from febrile patients within the first days of symptoms, just prior to the end of the fever period. Samples were stored at 4 °C during transport to the laboratory where individual sera were diluted 1/3 in PBS with gelatin plus 2% penicillin/streptomycin and 25 μg/mL amphotericin B. A sample volume of 100 μL of serum, diluted to 1 mL, was used to inoculate C6/36 *A. albopictus* mosquito cell lines which were at 90% confluency after 2 days of plating. Inoculated cells were incubated at 33 °C. Cytopathic effect was observed within 12–14 days. Supernatants from each sample demonstrating cytopathic effects were extracted in two aliquots to cryogenic vials for storage at −70 °C, and these were used for subsequent extraction of viral RNA for RT-PCR (Section 2.3.1).

A sample of the culture was taken for immunofluorescence using standard methods (2003; PAHO, 2002). FITC-conjugated MABs, used at 1/10 dilution, were a kind gift from the Center for Disease Control. They were obtained from mouse ascites fluid and were specific for dengue virus types DENV-1, DENV-2, DENV-3 or DENV-4.

2.3. Preparation of viral RNA

2.3.1. Extraction of RNA from viral isolates

A 250 μL aliquot of each viral isolate was extracted using TRIzol® LS Reagent (Invitrogen, Carlsbad, California, USA), following manufacturer's instructions and the modified method of Chomczynski and Sacchi (1987). Extracted RNA was rehydrated in 60 μL molecular-grade H_2O and stored at −70 °C.

2.3.2. Extraction of RNA from acute-phase sera

Acute-phase sera were centrifuged for 2 h at 14,000 × *g* for sample concentration and 140 μL of the precipitate was used in extracting viral RNA using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, California, USA), according to manufacturer's recommendations.

2.4. RT-PCR/RFLP

2.4.1. RT-PCR for Flavivirus

The protocol of Ayers et al. (2006) was used, with primers FLAVI-1 (5′-AATGTACGCTGATGACACAGCTGGCTGGGACAC-3′) and FLAVI-2 (5′-TCCAGACCTTCAGCATGTCTTCTGTTGTCATCCA-3′) to a 854–863 bp region of the non-structural 5 gene (NS5), highly conserved among the flaviviruses. One-Step RT-PCR (Qiagen) was performed with 16 μl water, 10 μl 10× Buffer, 10 μl Q-Solution, 2 μl dNTPs (10 mM each dNTP), 2 μl Enzyme Mix, 1 μl each (25 pmol) of primers FLAVI-1 and FLAVI-2, and 8 μl of extracted RNA in a 2470 Thermal Applied Biosystem thermocycler (Carlsbad, California, USA). Genetic material for the YFV control sample was obtained from vaccine isolate 17D RKI with 100% identity to Genbank sequence JN628279.1. SLE material, 99% identical to strain GML 903797, Genbank sequence EF158060.1, was obtained from a clinical sample and WNV RNA was obtained from the CAREC Caribbean Epidemiology Center and showed 99% identity at the nucleotide level to strain 68856, represented by Genbank sequence EU249803.1. The programmed cycling was 30 min at 50 °C, 15 min at 94 °C and then 40 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by a final 10 min extension step at 72 °C. Electrophoresis was performed with 1 μl of the amplification product and 2.0% agarose gels, 0.5× TBE and ethidium bromide. Amplicons were analyzed on a UV transilluminator.

2.4.2. Restriction enzyme incubation

For restriction enzyme analysis, 3 μl of the unpurified amplification product was added to 14.3 μl water, 2 μl Buffer C (10×), 0.2 μl BSA (10 mg/ml) and 0.5 μl *Hae*III (10 U/μl) (Promega, Madison, Wisconsin, USA). The reaction was incubated at 37 °C for 2 h, after which restriction products were subject to 2% agarose gel electrophoresis. Distinctive restriction patterns were obtained at this stage for YF, WN and SLE viruses, and DENV-2, DENV-3 and DENV-4. If no digestion was observed for the amplicon of DENV-1, the sample was subject to another reaction using the reagents and incubation