Application of Molecular Methods to the Diagnosis and Characterization of a Dengue Outbreak in Cuba

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ABSTRACT

After a 16-year period free of dengue in Cuba, the first clinical cases were detected in January 1997 in Santiago de Cuba district through active epidemiological surveillance with laboratory support. Reverse transcription-polymerase chain reaction/nested-polymerase chain reaction (RT-PCR/nPCR) procedures were carried out for rapid identification of dengue serotype 2 virus (DEN-2) as the etiologic agent. The application of the PCR fragment sequencing allowed us to know that the etiologic agent belongs to a Southeast Assian genotype related with dengue hemorrhagic fever epidemics. RT-PCR/nPCR was also used to detect DEN-2 virus in tissue samples of 10 fatal cases to discard the diagnosis of dengue in clinically suspected cases in other areas outside the focus, and to monitor the viral serotype present throughout the outbreak. These results demonstrate that molecular tools are useful for rapid confirmation of the clinical diagnosis of dengue and that they offer definitive data that help in the control, and recognition of the viral strain genotype and monitoring of the serotype throughout the outbreak.

Keywords: dengue, diagnosis, molecular characterization, polymerase chain reaction

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RESUMEN

Aplicación de métodos moleculares al diagnóstico y caracterización de una epidemia de dengue en Cuba. Después de un período de 16 años sin dengue en Cuba, los primeros casos clínicos fueron detectados en enero de 1997 en el municipio Santiago de Cuba mediante vigilancia epidemiológica activa con asistencia de laboratorio. Se realizó transcripción inversa-reacción en cadena de la polimerasa/reacción en cadena de la polimerasa anidada (TI-RCP/RCP anidada) para la identificación rápida del virus del dengue serotipo 2 (DEN-2) como agente etiológico. La secuenciación del fragmento de RCP permitió conocer que el agente etiológico pertenece a un genotipo del sudeste asiático relacionado con epidemias de fiebre hemorrágica de dengue. La TI-RCP/RCP anidada se empleó, además, para detectar el virus DEN-2 en muestras de tejidos de 10 casos mortales, descartar los casos clínicos de dengue en áreas fuera del foco y monitorear el serotipo viral presente durante el brote. Estos resultados demuestran que las herramientas moleculares son útiles para la confirmación rápida del diagnóstico clínico de dengue, y pueden ofrecer datos definitivos que ayuden al control y a la determinación del genotipo de la cepa viral y al monitoreo del serotipo durante todo el brote.

Palabras claves: caracterización molecular, dengue, diagnóstico, reacción en cadena de la polimerasa

*I*ntroduction

Dengue viruses (family Flaviviridae, genus *Flavivirus)* are plus-sense ssRNA viruses that cause dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in humans, and are serologically classified into four antigenically distinct serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). These viruses are maintained in a human-mosquito transmission cycle, primarily in tropical urban areas by *Aedes aegypti* mosquitoes and are currently endemic in most tropical areas of the world [1].

The genome (about 11 kb in length) consists of a single open reading frame that encodes a precursor polyprotein [2]. Proteolytic cleavages of the polyprotein result in the formation of the core (C), membrane (M) and envelope (E) proteins and the non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Studies of the molecular evolution and epidemiology of all four serotypes using genome sequence have demonstrated the occurrence of genotype groupings among these viruses [3–9].

Increased frequency of epidemic activity worldwide in the last 15 years has resulted in increased incidence and geographic expansion of the disease leading to predictions that DHF/DSS would become a major public health problem in America and Africa. Many countries in the American region had hyperendemic dengue transmission during the 1980s. Epidemics of DHF/DSS occurred for the first time outside Southeast Asia, with major outbreaks in the South Pacific in the 1970s and in the Americas in the 1980s [10].

Cuba had its first dengue epidemic of modern times in 1977 by DEN-1. A second epidemic in 1981, caused by DEN-2 virus, was unusually severe and widespread [11]. A virus isolated during the 1981 epidemic was classified in the same genotype as the prototype New Guinea 1944 strain [12]. After the epidemic ended in October 1981, a campaign to improve mosquito control and eradicate *Aedes aegypti* was immediately launched. Total eradication was not achieved but most of the 169 Cuban municipalities were free of the vector. The low *Aedes aegypti* premise indexes and the results of the passive surveillance system indicated no dengue transmission in Cuba between 1981 and the end of 1996. However, reinfestation occurred in

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some areas; the Santiago de Cuba district (located in Santiago de Cuba province, in the eastern part of the country) was reinfested in 1992 by *Aedes aegypti* harbored in imported tires. The convergence of several conditions associated with risk factors for the reemergence of dengue determined that in January 1997 the Institute of Tropical Medicine "Pedro Kourí" of the Cuban Ministry of Health established an active surveillance system in Santiago de Cuba district [13]. As a result of this system and the virological surveillance, dengue cases were detected on January 28, 1997, in one area of the district [14]. With the purpose of offering a detection, typing and molecular characterization system that would be rapid and accurate, all technologies available in our laboratory such as viral isolation, serological tests, PCR and nucleotide sequence were used. The present paper describes the application of molecular tools and the results obtained.

Materials and Methods

Clinical samples and RNA extraction

All clinical samples used in this study are listed in Table 1 with the clinical picture (DF or DHF) and data of collection in relation to the onset of illness.

The serum samples and necropsy tissues were sent to our laboratory during the epidemic. The samples were transported to the laboratory at -20 ºC and were processed immediately upon arrival. Each necropsy specimen was processed as follows: 100 mg of tissue was homogenized in 1 mL of phosphate buffered saline (8 g NaCl, 0.2 g KCl, 0.14 g KH_2PO_4, 0.91 g Na_2HPO_4) (PBS) with 500 U of penicillin per milliliter. After low-speed centrifugation (1200 rpm, 10 min, at room temperature, Micro Centrifuge SIGMA 201m, BIOBLOCK SCIENTIFIC, France), 200 µL of supernatant were used for RNA extraction. Viral RNA was isolated from clinical samples using a methodology previously described [15].

RT-PCR/nested PCR

The detection and typing of dengue viruses were carried out according to Lanciotti *et al.* [16]. Briefly, 10 µL of target viral RNA were added to 90 mL of RT-PCR mixture which contained the following components: 50 mM KCL, 10 mM Tris (pH 8.5), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1 M dithiothreitol, 20 U RNAse inhibitor (RNAsin, Promega USA), 200 µM of each of the four deoxynucleotide triphosphates (Promega, USA), 50 pmol of each of the consensus primers designed to anneal to any of the four dengue virus types, 10 U of AMV-Reverse Transcriptase (Promega, USA), and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim GMbH, Germany). The reactions were developed in a MJ Research thermocycler (Watertown, Massachusetts, USA) programmed to incubate for 30 min at 42 ºC and then to proceed with 35 cycles of denaturation (94 ºC, 30 s), primer annealing (55 ºC, 1 min) and extension (72 ºC, 2 min). A second amplification reaction (nested PCR) was initiated with 1 µL of the first PCR product. The reaction mixture contained all the components described for the initial amplification reaction with the following exception: consensus primer was replaced with the dengue virus type-specific primers; dithiothreitol and AMV-RT were eliminated. The samples were subjected to 26 cycles of denaturation (94 ºC, 30 s), primer annealing (55 ºC, 1 min), and primer extension (72 ºC, 2 min). A 10-µL portion of the reaction product was electrophoresed on a 4% agarose gel (Sigma, USA) with ethidium bromide (10 mg/mL, Promega, USA) [17]. Because of the position of priming with each of the dengue virus type-specific primers, the size of the resulting DNA band was characteristic for each dengue virus type. Quantum Biotechnologies (Canada) provided all the primers. Positive and negative controls were included in all steps. The supernatant of cell cultures infected with strains of four serotypes (DEN-1: TVP1971, DEN-2: TVP965, DEN-3: H87 and DEN-4: H241) were used as positive control. Supernatant of non-infected cell culture and distilled water were included as negative control.

Table 1. Human samples used in this study.

Code	Source	Clinical pattern	Day of onset (month/day)	Sample collection (month/day)
6/97	Serum	DF	01/22	01/28
8/97	Serum	DF	01/21	01/29
11/97	Serum	DF	01/27	01/29
12/97	Serum	DF	01/27	01/29
13/97	Serum	DF	01/27	01/29
58/97	Serum	DF	Unknown	02/04
68/97	Serum	DF	Unknown	02/04
70/97	Serum	DF	Unknown	02/04
34/97	Liver, spleen	DHF	05/20	05/24
90/97	Liver, spleen, kidney, lung	DHF	05/31	06/04
91/97	Liver, spleen	DHF	06/01	06/07
125/97	Liver, spleen, kidney	DHF	06/05	06/08
170/97	Liver, spleen	DHF	06/05	06/11
171/97	Liver	DHF	06/06	06/08
184/97	Liver, spleen	DHF	06/07	06/13
199/97	Liver	DHF	06/25	06/27
205/97	Liver, spleen	DHF	06/23	06/30
210/97	Liver, spleen	DHF	06/27	07/02
216/97	Serum	DF-HM	07/04	07/09
218/97	Serum	DF-HM	07/11	07/13
220/97	Serum	DF-HM	07/04	07/10

DF, dengue fever; DHF, dengue hemorrhagic fever; DF-HM, dengue fever with hemorrhagic manifestation.

PCR fragment sequencing

RNA extraction and RT-PCR of the serum sample 13/97 was performed as previously described using primers D2/2452 5'-CCACATTTCAGTTCTTT-3' and D2/2578 5'-TTACTGAGCGGATTCCACAGATGCC-3'; the numbers indicate the map site at which the 5' end of the oligonucleotide hybridizes on the DEN-2 genome (Quantum Biotechnologies, Canada) to amplify E/NS1 genome segment (240 bp) [3]. Amplified DNA was purified after gel electrophoresis in low-melting point agarose (Sigma, USA) by phenol extraction and ethanol precipitation [17]. The DNA was resuspended in RNase-free water (treated with 0.01%, v/v diethyl pyrocarbonate) (Sigma, USA) [17] and 5 µL of this sample were used in dideoxy nucleotide sequencing reactions. The reactions were developed manually using the same primers and Termo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham-Pharmacia Biotech, Sweden) according to the manufacturer's instructions and [α-^{33}P]dATP (Amersham-Pharmacia Biotech, Sweden).

The nucleotide sequence obtained was compared with several strain nucleotide sequences analyzed in a

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previous study [12] (Figure). The sequence alignment and a dendogram were constructed using the CLUSTAL V program [18].

Results and Discussion

Early etiologic diagnosis of the outbreak

The convergence of several risk factors for the re-emergence of dengue in Santiago de Cuba district determined the establishment in January 1997 of an active surveillance system with laboratory confirmations for dengue disease, with the objective of detecting the possible reintroduction of dengue virus in Cuba [13].

When the first clinically suspicious cases were detected, the IPK Arbovirus laboratory began to receive samples taken from individuals during the beginning of symptoms with the aim of confirming the clinical diagnosis and to identify the agent. The first eight serum samples (Table 1, from 6/97 to 70/97) were assayed by ELISA/IgM [19], ELISA/IgG [20], viral isolation in C6/36 mosquito cell cultures [21] and the RNA extraction and RT-PCR method validated in our laboratory [22]. The results are shown in Table 2.

In less than 24 h we were able to detect the causative agent and type it as DEN-2. The rapid detection of IgM antibodies in some of the suspected cases and the presence of the DEN-2 virus detected by molecular methods allowed us to inform the national health authorities of the reemergence of dengue in Cuba after 16 years. These results were confirmed one week later by viral isolation in cell cultures and identification by indirect immunoflourescence assay (IFA) (Table 2). At least two samples (11/97 and 12/97) were positive for PCR and negative by means of cell culture viral isolation. Those same sera had levels of IgG antibodies that are common in patients with an infection to a second serotype (secondary infection). It is known that increased levels of specific antibodies and the formation of immune complexes directly interfere with cell culture viral isolation. In agreement with other studies, our results also showed that RT-PCR/nPCR was more useful than viral isolation when anti-dengue antibodies were detected. Previously, other authors carried out the same observation [23, 24]. However, it is worth highlighting the result obtained with sample 12/97 in which—despite that high level of antibodies—it turned out positive by PCR. Previous reports showed the high sensitivity of the PCR compared with viral isolation [25–27].

Preliminary genetic characterization of the agent

The epidemiological analysis at molecular level in dengue epidemics reveals that some strains of this virus are associated with benign epidemics, with the occurrence of few cases of DHF/DSS and an apparently inefficient virus transmission, while other are indeed involved in severe epidemics (epidemic strains) with a high incidence of DHF/DSS cases. The analysis of the nucleotide sequences of dengue viruses has been widely used to obtain information on the transmission dynamic of these viruses [3, 4, 8, 12]. Rico-Hesse proposes that the E/NS1 gene junction, which comprises less than 3% of the dengue genome, may provide sufficient information for estimating genetic relationships between DEN-1 and DEN-2 virus isolated from diverse geographic areas and hosts. This author reported five different genomic groups [3]. More recently, this same author described the circulation of various genotypes of DEN-2 in America [9].

In this context, the genomic classification of the causative agent of the outbreak becomes very important. The comparison of nucleotide sequences (240 bp) from the E/NS1 gene region of the dengue virus genome has shown to reflect evolutionary relationships and

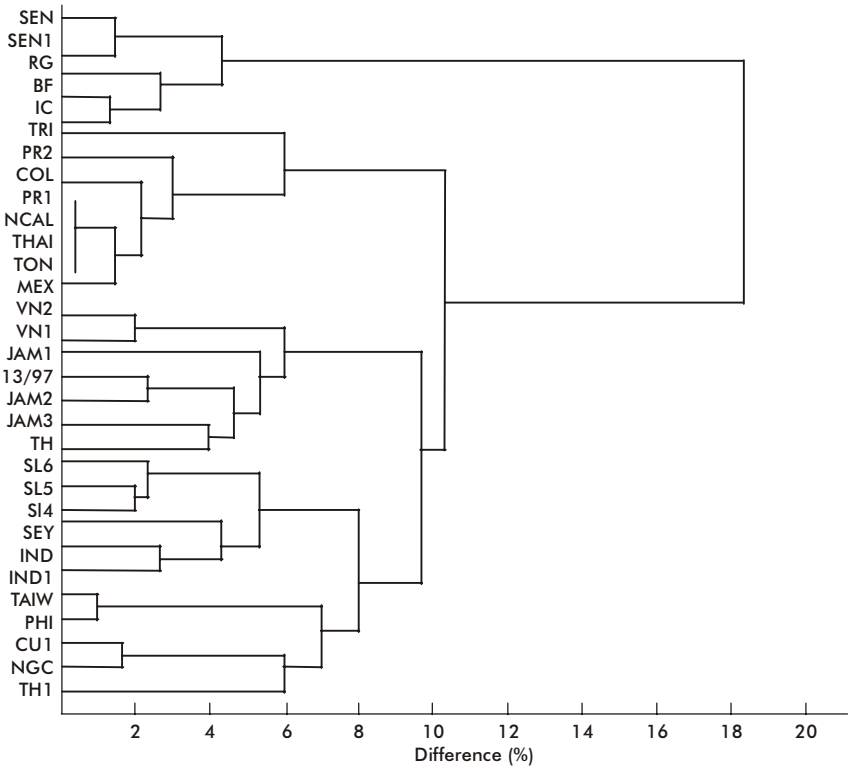

Figure. Dendogram showing the genetic relationships among DEN-2 viruses at the envelope protein/nonstructural protein-1 gene junction. SEN, HD10674 Senegal 1970; SEN1, ArD20761 Senegal 1974; PM33974 Republic of Guinea 1981; ArA2022 Burkina Faso 1980; DakA578 Ivory Coast 1980; Tri, 1751 Trinidad 1954; PR2, 1318 Puerto Rico 1981; COL, 330447 Colombia 1987; PR1, PR159 Puerto Rico 1969; NCAL, NC9163 New Caledonia 1972; THAI, 28741 Tahiti 1971; Ton, 1251 Tonga 1974; MEX, 044 Mexico 1985, VN2, 57S Vietnam 1987; VN1, 24H Vietnam 1987; JAM1, 8110827 Jamaica 1981, 13/97, Cuba 1997, JAM2, 1329 Jamaica 1982; JAM3, JAH Jamaica 1982; TH, 516 Thailand 1983; SL6, 975 Sri Lanka 1985; SL5, 1353 Sri Lanka 1982; SL4, 1334 Sri Lanka 1981; SEY, 8730 Seychelles 1977; IND, 8720 Indonesia 1973; IND1, 1223 Indonesia 1978; TAIW, PL046 Taiwan 1981; PHI, PhH 2172 Philippines 1983; CU1, A15 Cuba 1981; NGC, reference New Guinea 1944; TH1, 16681 Thailand 1964.

Table 2. DEN-2 virus identification on serum samples.

Code	RT-PCR/nPCR	ELISA/IgM	ELISA/IgG	Viral isolation in C6/36/IFA
6/97	-	+	1:5120	-
8/97	-	+	1:20480	-
11/97	+	-	1:80	-
12/97	+	+	1:20480	-
13/97	+	-	1:40	+
58/97	+	-	<1:20	+
68/97	+	-	1:20	+
70/97	+	-	1:20	+
216/97	+	ND	ND	+
218/97	+	ND	ND	+
220/97	+	ND	ND	+

+, positive; -, negative; ND, not determined.

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geographic origins of dengue virus strains [3]. To this end, isolation number 13/97 with first passage in C6/36 cells was used. The comparison of this nucleotide sequence with the nucleotide sequence of strains from different geographic sources available in our data bank revealed a 97.8% nucleotide identity in the zone studied with the Jamaica 1982 strain (Figure). This genotype has been disseminated throughout the Caribbean and South America. Strains belonging to this genotype have been associated with DHF/DSS outbreaks worldwide [9]. This virus was possibly introduced in Cuba from one of the other Caribbean countries or from South America via human transmission due to an increased number of visitors from other countries in the last decade, since Santiago de Cuba has become a tourism center in this region.

The genetic characterization of the viral agent of this epidemic allowed us to warn the public health authorities about the potential emergence of severe cases of the disease during this outbreak, which were unfortunately suffered several months later.

Other important diagnostic strategies

Once the diagnosis and the molecular characterization were defined, the RT-PCR/nPCR was reserved for its strategic use in specific circumstances: i) the emergence of clinically suspicious cases in areas distant from the initial focus, which thanks to the fast results of this technique, allowed us to define in few hours a change in the strategy for vector control; ii) the definition of the cases "imported" from the focus in the Santiago de Cuba district, which helped us know rapidly that the transmission never occurred in other regions of the country; iii) the definition of cases with an atypical clinical picture, particularly in fatal cases. Other older techniques that can be applied to these samples are cell culture, viral isolation and immune histochemistry, but they provide conclusive information more slowly (2–7 days) [11].

One or more samples from different organs of 10 of the 12 fatal cases were analyzed by RT-PCR/nPCR (Table 3). The method of viral RNA extraction described for serum samples [15] was used in the viral RNA extraction of tissue samples seemingly showing good results. As it can be seen in Table 3, RT-PCR/nPCR detected DEN-2 virus in 17 tissue samples, 12 of which were also positive for viral isolation and the same serotype was identified by IFA. This allowed us to make the etiologic diagnosis in 9 out of the 10 fatal cases analyzed here, 3 of which were diagnosed only by PCR. One of the cases (199/97) was only confirmed by the presence of IgM antibodies in the serum sample but it could not demonstrate the presence of the virus or the viral genome in the tissues analyzed. In the literature, the rates of isolation of dengue virus from tissue of fatal cases are low, possibly owing to the presence of virus-neutralizing activity and the insensitivity of the viral isolation systems [28].

The positive results of DEN-2 by RT-PCR/nPCR in the samples 216/97, 218/97 and 220/97 (Table 2) obtained from children with clinical diagnosis of dengue fever with hemorrhagic manifestations also reaffirms the usefulness of this quick method. Early diagnosis of dengue fever contributes to the appropriate management of the disease and its potentially severe complications [29].

Table 3. DEN-2 virus identification on tissue samples from fatal cases.

Cases	Clinica samples	RT-PCR/nPCR	Viral isolation in C6/36/IFA
34/97	Liver	+	-
	Spleen	+	-
90/97	Kidney	+	+
	Lung	+	+
	Liver	+	+
	Spleen	+	+
91/97	Liver	+	+
	Spleen	+	+
125/97	Liver	+	+
	Kidney	-	-
	Spleen	-	-
170/97	Spleen	+	-
	Liver	+	-
171/97	Liver	+	ND
184/97	Liver	+	-
	Spleen	+	-
199/97*	Liver	-	-
	Spleen	-	-
205/97	Liver	+	+
	Spleen	+	+
210/97	Liver	+	+
	Spleen	-	-

+, positive; -, negative; ND, not determined; *confirmed by positive ELISA/IgM.

Serotype monitoring throughout the outbreak

The RT-PCR/nPCR results allowed us to monitor during the outbreak period (January–July) that serotype 2 virus was always identified as the causative agent. This is undoubtedly important in our country because the introduction of other serotypes such as DEN-3 or DEN-4 could have had a very different repercussion in the scope and seriousness of the outbreak due to the fact that they did not have circulation previously, which means that a great percentage of the population is susceptible [30]. When we analyze all the results shown here it is obvious the value of the molecular methods for the fast diagnosis, follow-up and genetic characterization of the dengue outbreak, even in a laboratory with an important set of diagnostic methods and a highly trained personnel. This experience shows that RT-PCR/nPCR is a very useful tool for small laboratories with restricted resources for validating the clinical diagnosis, as well as for providing definitive data to help in the control of the disease through the rapid diagnosis of cases outside of the dengue outbreak. On the other hand, the studies of molecular characterization of the dengue strains offer valuable information about the evolutionary relationship and the geographical origin of the strains, as well as about the association of the genotype with severe forms of the disease (DHF/DSS) [9].

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