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Use of serum and blood samples on filter paper to improve the surveillance of dengue in Pacific Island Countries

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ABSTRACT

Background: In Pacific Island Countries (PICs) the epidemiology of dengue is characterized by long-term transmission of a single dengue virus (DENV) serotype. The emergence of a new serotype in one island country often indicates major outbreaks with this serotype will follow in other PICs.
Objectives: Filter paper (FP) cards on which whole blood or serum from dengue suspected patients had been dried was evaluated as a method for transportation of this material by standard mail delivery throughout the Pacific.
Study design: Twenty-two FP-dried whole blood samples collected from patients in New Caledonia and Wallis & Futuna Islands, during DENV-1 and DENV-4 transmission, and 76 FP-dried sera collected from patients in Yap State, Majuro (Republic of Marshall Islands), Tonga and Fiji, before and during outbreaks of DENV-2 in Yap State and DENV-4 in Majuro, were tested for the presence of DENV RNA, by serotype specific RT-PCR, at the Institut Louis Malardé in French Polynesia.
Results: The serotype of DENV could be determined, by a variety of RT-PCR procedures, in the FP-dried samples after more than three weeks of transport at ambient temperatures. In most cases, the sequencing of the envelope gene to genotype the viruses also was possible.
Conclusions: The serotype and genotype of DENV can be determined from FP-dried serum or whole blood samples transported over thousands of kilometers at ambient, tropical, temperatures. This simple and low-cost approach to virus identification should be evaluated in isolated and resource poor settings for surveillance for a range of significant viral diseases.

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1. Background

Each year, dengue viruses (DENVs) infect more than 50 million people resulting in an estimated 500,000 hospitalizations and more than 12,500 deaths, mostly children.[1] The etiological agent of dengue fever is an RNA virus belonging to the *Flaviviridae* family and transmitted by *Aedes* mosquitoes, principally *Ae* (*Stegomyia*) *aegypti* but also *Ae albopictus* and endemic vectors like *Ae polynesiensis* in the Polynesian triangle.[2] There are four DENV serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) and infection with one serotype provides only short term cross-protective immunity against the other three. Each serotype may be divided into distinct phylogenetic clusters or genotypes based on the nucleotide sequences of the envelope (E) gene.[3] Infection with DENV may result in a wide spectrum of clinical manifestations ranging from a mild, undifferentiated fever through to hemorrhage and hypovolemic shock which, if untreated, may be fatal.[1]

Abbreviations: PICs, Pacific Island Countries; DENV, dengue virus; FP, filter paper; RT-PCR, reverse transcription polymerase chain reaction; WHO, World Health Organization; IPNC, Institut Pasteur de Nouvelle Calédonie; ILM, Institut Louis Malardé; NC, New Caledonia; WF, Wallis & Futuna Islands.

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The earliest reported dengue epidemics in the Pacific occurred during the second half of the nineteenth Century.[4] Epidemics due to each of the four DENV serotypes have occurred in the Pacific following the first virologically confirmed one, due to DENV-1, between 1943 and 1944.[5–7] The increase in both international and inter-island travel during the last 20–30 years has contributed to almost continuous dengue outbreaks in at least one PIC at any time, e.g. the DENV-1 outbreaks that occurred in many Pacific Island Countries (PICs) between 2000 and 2010.[8–14] After this decade of DENV-1 transmission, DENV-4 was introduced into the region. It spread to an increasing number of PICs after the second half of 2008 and caused several epidemics during the following years.[15–18] Concomitantly, DENV-2 was detected in Yap State, Federated States of Micronesia in 2011.[19]

In contrast to the epidemiology of dengue in hyper-endemic continental regions of Asia and the Americas, where multiple DENV serotypes/genotypes co-circulate, there usually is long-term transmission of only a single DENV serotype in the PICs.[17] Introduction of a new DENV represents a high risk of serotype replacement and of major outbreaks due to the new serotype.[7,16,20] Most PICs have neither the human or financial resources to perform laboratory diagnosis of dengue and only two [Institut Pasteur de Nouvelle Calédonie (IPNC), New Caledonia, Institut Louis Malardé (ILM), French Polynesia] have the capacity to genotype DENV. Reference laboratories in Australia (WHO Arbovirus Reference Center, Queensland University of Technology; Queensland Health Forensic and Scientific Services), Hawaii (University of Hawaii), New Zealand (Institute of Environmental Science and Research) and Puerto Rico [U.S. Centers for Diseases Control and Prevention (CDC)] provide diagnostic and reference support as requested. However, because the shipment of frozen human sera is expensive and subject to restrictive regulations, routine laboratories in PICs send samples only occasionally to the reference centers and usually only to confirm the etiology of an outbreak.

2. Objectives

Because of the low risk to health they pose, FP-dried blood spots are exempted from dangerous goods requirements and regulations in a number of countries.[21,22] FP-cards therefore have the potential to facilitate many aspects of sample storage and shipment. The detection of DENV by reverse transcriptase-polymerase chain reaction (RT-PCR) in whole blood spiked with cultured DENV blotted on FP-cards had been described previously.[23] More recently, FP-dried whole blood spots have been employed for the molecular diagnosis of DENV.[24–26] We have evaluated whole blood/serum dried on FP-cards and transported by standard mail, at ambient temperatures, for use in DENV diagnosis and surveillance in a Pacific setting.

3. Study design

3.1. Sample collection

The laboratories at IPNC in New Caledonia and Sia hospital in Wallis & Futuna Islands sent FP-dried venous blood spots from confirmed or suspected dengue patients to ILM. At IPNC, DENV infections were confirmed by the detection of NS1 antigen (Platelia™ Dengue NS1 Ag ELISA or Dengue NS1 Ag Strip™, Bio-Rad Laboratories, France) or by the detection of anti-DENV IgM (Dengue IgM Capture ELISA, Panbio Diagnostics, Australia). Some of the sera containing NS1 were tested by RT-PCR followed by a serotype-specific hemi-nested PCR (classical RT-PCR) as previously described.[27] Following the LabNet meeting organized by the Secretariat of the Pacific Community in November 2010, representatives of laboratories in the 22 PICs were invited to participate in a pilot study to evaluate to evaluate blood/serum dried on FP-cards and transported at ambient temperatures as a source of DENV for serotyping and genotyping. Laboratories were provided with FP-cards (LDA[22] France) and were invited to send acute-phase sera collected from suspected dengue patients less than five days from onset of fever, to ILM. Routine diagnostic laboratories in the PICs employed a variety of assays to diagnose DENV infection: Dengue NS1 Ag Strip™, SD BIOLINE™ Dengue IgG-IgM, SD BIOLINE™ Dengue Duo NS1/IgM/IgG (Standard diagnostic Inc., South Korea). Within 48 h of blood collection, 240–600 μL of whole blood or serum were blotted on the pre-cut circles on the FP-card (20 μL/circle). The FP-card was stored for 2 h at room temperature, until dry, and then placed into a Ziploc plastic bag (Dominique Dutscher, France) and stored at 4 °C until shipment to ILM. Once received at ILM, the FP-cards were stored at 4 °C until processing.

3.2. Extraction of nucleic acids and detection of DENV by RT-PCR

For FP-dried blood samples, the viral RNA was extracted as previously described.[17] For FP-dried sera, the viral RNA was extracted using the Easymag extraction system (bioMérieux, France). Two rectangles (each containing 6 pre-cut circles and 120 μL of dried serum) were cut from the FP-card and incubated with 4.5 mL of lysis buffer at room temperature and mild agitation for 30 min. A volume of 3.5 mL of the final lysate then was collected and centrifuged for 5 min at 3200 × *g* to remove any remaining FP pieces. RNA was recovered from the 3 mL of supernatant remaining according to the manufacturer's instructions. Different RT-PCR protocols were performed to serotype the DENV. Real-time RT-PCR reactions were conducted in an iCycler iQ™ Real-Time PCR Detection System instrument using the iScript™ One-Step RT-PCR Kit for Probes (Bio-Rad Laboratories, France). Real-time multiplex and singleplex DENV RT-PCRs were performed using primers and probes designed previously.[28] The multiplex RT-PCR was modified as follows. Briefly, 5 μL of RNA template was added to a 20-μL mixture containing 1 μM of each DENV-1 and DENV-3 specific primer, 0.5 μM of each DENV-2 and DENV-4 specific primer and 180 nM of each probe. Reverse transcription was carried out at 50 °C for 10 min, followed by the initial denaturation step at 95 °C for 5 min and then 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The singleplex RT-PCR was performed as follows. The 25-μL reaction mixture contained 5 μL of RNA combined with 400 nM of each serotype-specific primer and 200 nM of probe. Reverse transcription was carried out at 50 °C for 10 min, followed by the initial denaturation step at 95 °C for 5 min and then 45 cycles at 95 °C for 15 s and 58 °C for 30 s. The classical DENV RT-PCR was performed using the One-Step RT-PCR® Kit and HotStarTaq® DNA Polymerase Kit (Qiagen, Germany), and the primers and protocol previously described.[27]

3.3. Nucleotide sequencing and genotyping of DENV

Amplification, sequencing and analysis of the E gene of the DENV strains were performed as previously described.[17] For DENV-4 strains, two oligonucleotides primers pairs were used D4E/777F-D4E/1766R and D4E/1639F-D4E/2509R. For DENV-2, the primers pair D2/618V (5′-ACC AGA AGA CAT AGA TTG TTG GTG C-3′) – D2/2578 (5′-TTA CTG AGC GGA TTC CAC AGA TGC C-3′) was used for the amplification of the E gene and five other primers [HINDII/D2+ (5′-GGGGTTTCAGGAGGAAGCTGGGTTGAC-3′),[29] ACCI/D2− (5′-CCCCATCCTCTGTCTACCATG-3′),[29] D2RS1812 (5′-TAGTTTGTCCATTCTCAGCC-3′), DEN-2F (5′-CAGGTTATGGCACTGTCACGA-3′)[28] and DEN2/2519 (5′-TTATATTGTTCTGTCCATGTG-3′)] were used for sequencing. Genetic analyses were carried out with the software MEGA version 5.[30] Nucleotide sequences were aligned using ClustalW and phylogenetic analyses were performed with Maximum

Likelihood based on the Kimura 2-parameter method,[31] with 1000 bootstrap replicates.[32] Previously published E gene sequences available on Genbank or provided by Li et al.[16] were included in the phylogenetic analyses.

4. Results

4.1. Detection of DENV by RT-PCR using FP-dried whole blood spots

ILM received 21 FP-dried blood samples from IPNC collected between February and March 2009, during an outbreak of DENV-1 and DENV-4 infections. The delivery by standard mail from New Caledonia to French Polynesia took 10–21 days. On arrival at ILM, the FP-cards were stored at 4 °C and processed within a month. Sixteen DENV-4, two DENV-1 and one DENV-2 were identified in these samples by multiplex RT-PCR (Table 1). No DENV RNA could be detected in the remaining two samples. DENV-4 was identified, by multiplex, singleplex and classical DENV RT-PCRs, in an additional sample from Sia Hospital in Wallis & Futuna Islands.

4.2. Detection of DENV by RT-PCR using FP-dried serum spots

ILM received 76 FP-dried sera sent by routine laboratories in Yap State, Tonga, Fiji and Majuro (Republic of Marshall Islands) between March 2011 and January 2012 (Table 2). The delivery by standard mail took three to four weeks. All FP-dried sera received at ILM were analyzed by multiplex RT-PCR. Once DENV was detected in one sample, all samples in that shipment were analyzed by three different DENV RT-PCR protocols. Among the 30 FP-dried sera collected in Yap State from mid-September to December 2011, DENV-2 was identified in 22 samples (including six detected by singleplex and classical RT-PCRs only). Among the seven samples collected in Majuro in October 2011, three were identified as DENV-4 (including one detected by singleplex and classical RT-PCRs only). The same sera also were sent, frozen, to the CDC laboratory in Puerto Rico, where DENV-4 was detected in an additional two samples.

4.3. Sequencing of the E gene and phylogenetic analyses

The sequences of the E genes of four DENV-4 strains detected in the FP-dried blood samples from New Caledonia and WF [NC09/060209-1528 (*Genbank:* JN832498), NC09/050209-1413 (JQ650086), NC09/170309-6652 (JQ650090), WF09/010409-0001 (JN832499)] were determined and aligned with those of 108 DENV-4 strains available on GenBank. The DENV-4 strains from New Caledonia and Wallis & Futuna Islands were most closely related to genotype IIa viruses and clustered in a distinct group of viruses collected in PICs from 2007 to 2010.[17] There was insufficient DENV RNA in the FP-dried sera collected in Majuro to permit RT-PCR of the full E gene for sequencing.

The sequence of the E gene of one DENV-2 strain detected in an FP-dried serum sample from Yap State [FM11/210911-18132 (JQ650046)] was determined and aligned with those of 84 DENV-2 strains available on Genbank and three DENV-2 strains collected during the previous sustained circulation of this serotype in PICs [PF98/150498-10598 (JQ650027), NC99/120599-2056 (JQ650040) and WF98/111298-5003 (JQ650044)]. The DENV-2 strain from Yap State was most closely related to Cosmopolitan genotype DENV-2 strains from South-East Asia, Africa, India and strains that circulated in the Pacific in the late 90s (Fig. 1). The most closely related strain to FM11/210911-18132 was one collected in China in 2010 (distance value of 0.8%).

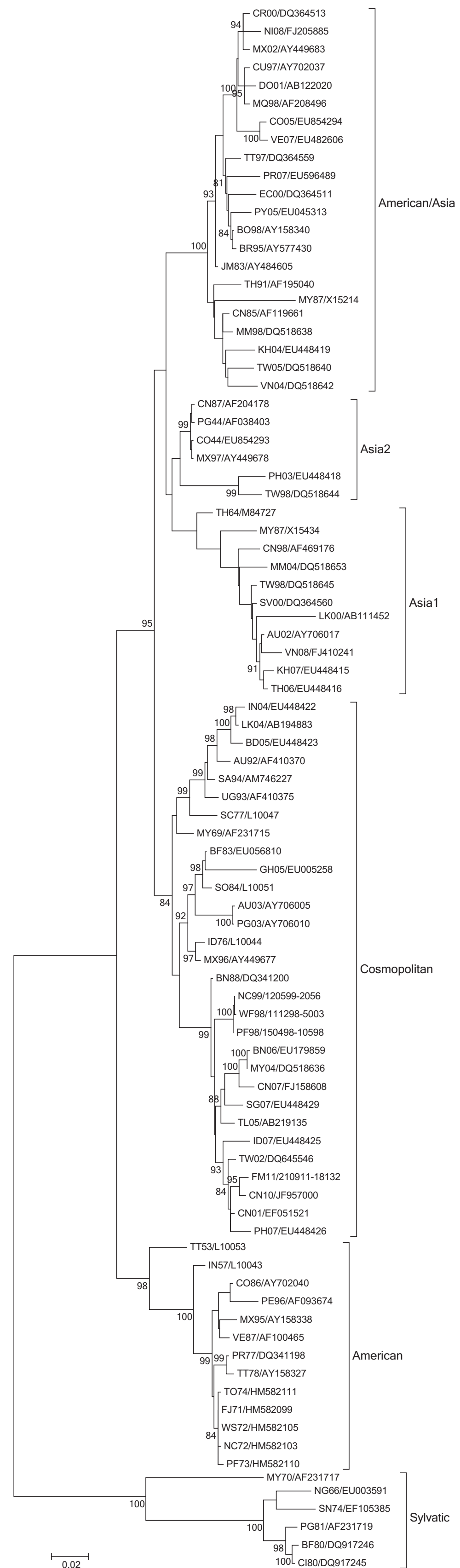

Fig. 1. Evolutionary relationships of DENV-2 E gene sequences. ML original tree derived from 88 DENV-2 E gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown for values over 80.

Table 1
List of filter paper-dried blood samples sent from laboratories in New Caledonia (NC) and Wallis & Futuna Islands (WF) to ILM and results of the tests performed.

Location	Shipping	Reception	ID	Collection	Day of fever	Initial test results		RT-PCR performed at ILM		
						NS1	Classical RT-PCR	Classical RT-PCR	Real time multiplex	Real time singleplex
NC	02/10/2009	02/17/2009	NC1-0209	02/04/2009	4	Pos	–	–	DENV-1	–
			NC2-0209	02/04/2009	3	Pos	–	–	DENV-4	–
			NC3-0209	02/04/2009	5	Pos	–	Neg	Neg	Neg
			NC4-0209	02/05/2009	1	Pos	–	–	DENV-4	–
			NC5-0209	02/05/2009	5	Pos	DENV-2	–	DENV-2	–
			NC6-0209	02/05/2009	N/A	Pos	DENV-4	–	DENV-4	DENV-4
			NC7-0209	02/06/2009	1	Pos	–	–	DENV-4	–
			NC8-0209	02/06/2009	6	Pos	–	–	DENV-4	–
			NC9-0209	02/06/2009	5	Pos	–	Neg	Neg	Neg
			NC10-0209	02/06/2009	2	Pos	–	–	DENV-4	–
			NC11-0209	02/06/2009	7	Pos	–	–	DENV-4	–
			NC12-0209	02/06/2009	1	Pos	–	–	DENV-4	DENV-4
	02/17/2009	03/04/2009	NC13-0209	02/10/2009	4	Pos	–	–	DENV-4	–
			NC14-0209	02/10/2009	4	Pos	–	–	DENV-4	–
			NC15-0209	02/10/2009	3	Pos	–	–	DENV-4	–
			NC16-0209	02/10/2009	5	Pos	–	–	DENV-4	–
			NC17-0209	02/11/2009	3	Pos	–	–	DENV-4	–
			NC18-0209	02/10/2009	3	Pos	–	–	DENV-4	–
	03/07/2009	03/23/2009	NC1-0309	03/12/2009	2	Pos	–	–	DENV-1	–
			NC2-0309	03/12/2009	2	Pos	–	–	DENV-4	–
	03/24/2009	04/01/2009	NC3-0309	03/17/2009	2	Pos	–	–	DENV-4	–
WF	04/03/2009	04/21/2009	WF1-0409	04/01/2009	3	–	–	DENV-4	DENV-4	DENV-4

(–) not tested; (NA) not available.

Table 2
List of filter paper-dried sera sent from routine laboratories in PICs to ILM and results of the tests performed.

Location	Shipping	Reception	ID	Age	Gender	Collection	Day of fever	Initial test results		RT-PCR performed at ILM		
								IgM/IgG	NS1	Classical RT-PCR	Real time multiplex	Real time singleplex
YAP	02/04/2011	03/03/2011	Y1-0311	20	M	11/11/2010	3	–	–	–	Neg	–
			Y2-0311	14	F	12/22/2010	3	Pos/Neg	–	–	Neg	–
			Y3-0311	51	M	01/10/2011	5	Neg/Pos	–	–	Neg	–
			Y4-0311	8	M	01/11/2011	4	Neg/Neg	–	–	Neg	–
	03/22/2011	04/20/2011	Y1-0411	1	M	03/04/2011	4	Neg/Neg	–	–	Neg	–
			Y2-0411	14	M	03/05/2011	3	Pos/Pos	–	–	Neg	–
			Y3-0411	33	M	03/07/2011	3	Neg/Neg	–	–	Neg	–
			Y4-0411	20	F	03/11/2011	3	Neg/Neg	–	–	Neg	–
			Y5-0411	33	M	03/14/2011	3	–	–	–	Neg	–
TONGA	03/25/2011	04/11/2011	T1-0411	27	F	NA	1	–	–	–	Neg	–
			T2-0411	21	M	NA	4	–	–	–	Neg	–
			T3-0411	10	F	NA	2	–	–	–	Neg	–
			T4-0411	16	F	NA	5	–	–	–	Neg	–
			T5-0411	12	F	NA	5	–	–	–	Neg	–
FIJI	04/27/2011	05/09/2011	F1-0511	31	F	NA	NA	–	Pos	Neg	Neg	–
			F2-0511	33	F	04/11/2011	NA	–	Pos	Neg	Neg	–
TONGA	N/A	05/25/2011	T1-0511	59	M	NA	2	–	–	–	Neg	–
			T2-0511	14	F	NA	1	–	–	–	Neg	–
			T3-0511	1	M	NA	3	–	–	–	Neg	–
			T4-0511	12	NA	NA	5	–	–	–	Neg	–
			T5-0511	77	F	NA	3	–	–	–	Neg	–
			T6-0511	36	F	NA	2	–	–	–	Neg	–
			T7-0511	64	F	NA	3	Neg/Neg	–	–	Neg	–
YAP	06/14/2011	07/05/2011	Y1-0711	20	F	03/28/2011	2	–	–	–	Neg	Neg
			Y2-0711	72	F	04/01/2011	2	Neg/Neg	–	–	Neg	Neg
			Y3-0711	20	M	04/08/2011	2	Neg/Neg	–	–	Neg	Neg
			Y4-0711	11	F	05/06/2011	3	Neg/Neg	–	–	Neg	Neg
			Y5-0711	4	F	05/20/2011	3	Neg/Neg	–	–	Neg	Neg
			Y6-0711	60	F	05/20/2011	5	Neg/Neg	–	–	Neg	Neg
			Y7-0711	63	M	05/22/2011	3	Neg/Pos	–	–	Neg	Neg
			Y8-0711	21	M	05/30/2011	6	Neg/Pos	–	–	Neg	Neg
			Y9-0711	22	M	06/13/2011	1	Neg/Neg	–	–	Neg	Neg
	09/06/2011	09/27/2011	Y1-0911	54	F	06/15/2011	3	Neg/Pos	–	–	Neg	–
			Y2-0911	56	M	07/21/2011	3	Neg/Neg	–	–	Neg	–
			Y3-0911	20	F	07/26/2011	5	Neg/Neg	–	–	Neg	–
			Y4-0911	18	F	08/06/2011	3	Neg/Pos	–	–	Neg	–
			Y5-0911	12	M	09/03/2011	2	Neg/Neg	–	–	Neg	–
			Y6-0911	62	F	09/03/2011	2	Pos/Pos	–	–	Neg	–
			Y7-0911	17	M	09/05/2011	5	Neg/Neg	–	–	Neg	–
	10/27/2011	11/16/2011	Y1-1111	15	F	09/11/2011	3	Neg/Neg	–	DENV-2	DENV-2	DENV-2
			Y2-1111	14	M	09/21/2011	6	Neg/Neg	–	DENV-2	DENV-2	DENV-2
			Y3-1111	8	M	09/21/2011	3	Neg/Neg	–	DENV-2	DENV-2	DENV-2
			Y4-1111	4	M	09/30/2011	2	Neg/Neg	–	DENV-2	Neg	DENV-2
			Y5-1111	6	M	09/30/2011	2	Neg/Neg	–	DENV-2	DENV-2	DENV-2
			Y6-1111	13	M	10/05/2011	2	Pos/Pos	–	DENV-2	DENV-2	DENV-2
			Y7-1111	11	M	10/05/2011	5	Pos/Neg	–	DENV-2	DENV-2	DENV-2
			Y8-1111	8	F	10/05/2011	5	Pos/Neg	–	DENV-2	Neg	DENV-2
			Y9-1111	31	M	10/07/2011	NA	Neg/Neg	–	Neg	Neg	Neg
			Y10-1111	10	F	10/08/2011	3	Neg/Neg	–	DENV-2	DENV-2	DENV-2
			Y11-1111	13	M	10/08/2011	4	Neg/Neg	–	DENV-2	Neg	DENV-2
			Y12-1111	9	F	10/08/2011	3	Neg/Neg	–	DENV-2	DENV-2	DENV-2
			Y13-1111	15	M	10/08/2011	2	–	–	DENV-2	DENV-2	DENV-2
MAJURO	N/A	11/21/2011	M1-1111[a]	11	M	10/19/2011	5	–	–	Neg	Neg	Neg
			M2-1111[a]	10	F	10/19/2011	4	–	–	DENV-4	Neg	DENV-4
			M3-1111	7	F	10/19/2011	NA	–	–	Neg	Neg	Neg
			M4-1111[a]	24	M	10/20/2011	5	–	–	Neg	Neg	Neg
			M5-1111[a]	30	M	10/21/2011	1	–	–	DENV-4	DENV-4	DENV-4
			M6-1111[a]	12	M	10/22/2011	4	–	–	DENV-4	DENV-4	DENV-4
			M7-1111	34	F	10/27/2011	5	Neg/Neg	–	Neg	Neg	Neg
YAP	12/28/2011	01/19/2012	Y1-0112	16	M	12/02/2011	4	Neg/Neg	Pos	DENV-2	Neg	DENV-2
			Y2-0112	16	M	12/02/2011	1	Neg/Neg	Neg	DENV-2	Neg	DENV-2
			Y3-0112	51	F	12/02/2011	2	Neg/Neg	Neg	Neg	Neg	Neg
			Y4-0112	23	M	12/02/2011	1	Neg/Neg	Pos	DENV-2	DENV-2	DENV-2
			Y5-0112	4	M	12/02/2011	3	Neg/Neg	Neg	Neg	Neg	Neg
			Y6-0112	5	F	12/02/2011	3	Neg/Neg	Pos	DENV-2	DENV-2	DENV-2
			Y7-0112	17	F	12/02/2011	1	Neg/Neg	Pos	DENV-2	DENV-2	DENV-2
			Y8-0112	7	M	12/02/2011	2	Neg/Neg	Neg	Neg	Neg	Neg
			Y9-0112	23	F	12/02/2011	2	Neg/Neg	Neg	Neg	Neg	Neg

Table 2 (*Continued*)

Location	Shipping	Reception	ID	Age	Gender	Collection	Day of fever	Initial test results		RT-PCR performed at ILM		
								IgM/IgG	NS1	Classical RT-PCR	Real time multiplex	Real time singleplex
			Y10-0112	9	F	12/02/2011	2	Neg/Neg	Pos	DENV-2	DENV-2	DENV-2
			Y11-0112	1	M	12/03/2011	1	Neg/Neg	Pos	DENV-2	DENV-2	DENV-2
			Y12-0112	10	M	12/03/2011	1	Pos/Pos	Neg	DENV-2	DENV-2	DENV-2
			Y13-0112	5	F	12/03/2011	5	Neg/Neg	Pos	NA	NA	NA
			Y14-0112	55	F	12/03/2011	1	Pos/Pos	Neg	DENV-2	Neg	DENV-2
			Y15-0112	28	F	12/03/2011	2	Neg/Neg	Neg	Neg	Neg	Neg
			Y16-0112	8	F	12/03/2011	1	Neg/Neg	Pos	DENV-2	DENV-2	DENV-2
			Y17-0112	5	F	12/03/2011	2	–	Neg	Neg	Neg	Neg

(–) not tested; (NA) not available.
[a] Sample positive for DENV-4 at the CDC lab.

5. Discussion

This study has demonstrated the utility of FP-dried serum/blood samples as a means of surveillance for dengue in a resource poor setting and where samples have to be transported over thousands of kilometers, at ambient temperatures, to reference centers for analysis. Knowledge of the serotype and genotype of DENV is important because the appearance of a "new" DENV serotype, which differs from that currently circulating, often is associated with major outbreaks of dengue due to the new serotype.[7,16,20] As more DENV genotype data becomes available, Pacific Island Communities may gain a clearer idea of from where most DENV introductions are coming and be able to undertake risk mitigation activities.

Previous studies had demonstrated that FP-dried blood samples were suitable for the detection of DENV by RT-PCR[23–25] and blood samples on FP have been used recently for prospective virological surveillance for dengue in the confines of the French West Indies.[26] The present study has confirmed these observations in a regional Pacific setting and shown that serum initially collected for routine viral serology can be substituted for whole blood. Moreover, using an appropriate extraction protocol it was demonstrated that FP-dried serum or blood samples can provide DENV RNA in a quantity and a quality that allows sequencing of the full E gene and DENV genotype identification.

Since FP-cards have been employed, traditionally, as a substrate for whole blood, the study began by testing for DENV in FP-dried blood samples collected from dengue patients in New Caledonia and Wallis & Futuna Islands. The results of the tests performed at ILM (Table 1) were consistent with epidemiological data provided by IPNC who reported transient DENV-1 and DENV-4 co-circulation and occasional importations of other serotypes into New Caledonia in 2009 (the sample that contained DENV-2 had been reported as from a case imported from India).

Since laboratories in PICs perform initial dengue diagnostic tests on patients' serum, it appeared more convenient for laboratory technicians to blot serum, rather than whole blood, on FP-cards. The use of FP-dried sera for the molecular diagnosis of infections had been described previously for other viruses.[32–35] In this study, FP-dried sera were used successfully as sources of viral RNA for DENV serotyping (Table 2). The singleplex and classical RT-PCRs detected DENV in seven more samples (total 25) than the real-time multiplex RT-PCR. Although less sensitive, the multiplex RT-PCR was the less expensive one-step/one-tube assay for DENV serotype identification. While DENV-4 was detected in five frozen sera sent to CDC Puerto Rico but in only three of the corresponding FP-dried samples sent to ILM for testing, the FP-dried sampling strategy appears robust enough to identify the serotype and genotype of DENV involved in an outbreak in a Pacific setting. Since 13 of 18 FP-dried serum samples which contained detectable DENV RNA were collected from patients whose serum also contained NS1 antigen, the efficiency of DENV serotyping and genotyping could be improved by diagnostic laboratories referring only sera which were known to contain NS1 antigen.

The sequences of the E genes of DENV-4 from New Caledonia and Wallis & Futuna Islands determined from RNA recovered from FP-dried serum confirmed previous reports that DENV-4 strains circulating in PICs from 2007 to 2010 resulted from a single introduction from South-East Asia, probably Indonesia.[16,17] The origins of DENV-4 strains collected in Majuro in 2011 are yet to be determined. The nucleotide sequence of the DENV-2 recovered from an FP-dried serum sample from Yap State suggested that it also was introduced from Asia, supporting previous claims of the Asian/South-East Asian origins of DENVs that emerged in the Pacific during the last decade.[8,12,16,17]

This study has found that sera collected for routine diagnostic purposes, dried onto FP and transported over extended distances and times at ambient temperatures, is suitable for DENV surveillance in a resource poor setting. This system should be evaluated for use in surveillance for an extended range of viral disease of humans in these settings.

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Competing interests

None declared.

Ethical approval

This study was a non-interventional study in which all acts were practiced and products used in a usual manner, the *in vitro* diagnostic medical devices (DM-DIV) were used without any additional or unusual procedure of diagnostic or monitoring. The information provided by routine laboratories to ILM was the following: laboratory ID number, age, gender, date of sample collection, number of days from fever onset, and result of the initial IgM/IgG or NS1 antigen detection tests if performed. According to the French law (Code de la Santé Publique, art. L 1121-1.1) such protocol does not require

approval of an ethics committee and is exempted from informed consent application.[36]

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