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► **To cite this version:**

Maïté Aubry, Jérôme Finke, Anita Teissier, Claudine Roche, Julien Broult, et al.. Silent Circulation of Ross River Virus in French Polynesia. *International Journal of Infectious Diseases*, 2015, 37, pp.19-24. 10.1016/j.ijid.2015.06.005 . hal-01452897

**HAL Id: hal-01452897**

**<https://hal.univ-reunion.fr/hal-01452897v1>**

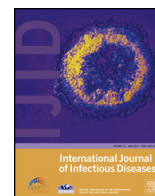
Submitted on 27 Jun 2018

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## Silent Circulation of Ross River Virus in French Polynesia



Maite Aubry<sup>a,\*</sup>, Jérôme Finke<sup>b</sup>, Anita Teissier<sup>a</sup>, Claudine Roche<sup>a</sup>, Julien Brout<sup>c</sup>,  
Sylvie Paulous<sup>d</sup>, Philippe Desprès<sup>d,e</sup>, Van-Mai Cao-Lormeau<sup>a</sup>, Didier Musso<sup>a</sup>

<sup>a</sup> Unit of Emerging Infectious Diseases, Institut Louis Malardé, PO BOX 30, 98713 Papeete, Tahiti, French Polynesia

<sup>b</sup> Hochschule Emden/Leer, Constantiaplatz 4, D-26723 Emden, Germany

<sup>c</sup> Centre de Transfusion Sanguine de la Polynésie française, Hôpital du Taaone, PO BOX 4530, 98713 Papeete, Tahiti, French Polynesia

<sup>d</sup> Département Infections and Epidemiology, Institut Pasteur, 75724 Paris, France

<sup>e</sup> UMR PIMIT (12T) Université de La Réunion, INSERM U1187, CNRS 9192, IRD 249, GIP-CYROI, 97491 Sainte-Clotilde, France

### ARTICLE INFO

#### Article history:

Received 6 May 2015

Received in revised form 6 June 2015

Accepted 9 June 2015

**Corresponding Editor:** Eskild Petersen, Aarhus, Denmark

#### Keywords:

Ross River

French Polynesia

Seroprevalence

Blood donors

### SUMMARY

**Objectives:** Ross River is an emerging mosquito-borne disease in the Western Pacific. Ross River virus (RRV) circulation has been sporadically reported in some Pacific Island Countries and Territories but never in French Polynesia. To determine if RRV has circulated locally among the French Polynesian population, we conducted a seroprevalence study on blood donors.

**Methods:** Sera of 593 blood donors were collected from July 2011 to October 2013 and tested by ELISA for the presence of RRV-specific Immunoglobulin G (IgG) antibodies.

**Results:** A total of 204 (34.40%) blood donors were found seropositive for RRV. Among the 132 blood donors that were born in French Polynesia and had never travelled abroad, 56 (42.42%) had RRV-specific IgGs.

**Discussion:** Our results support the existence of autochthonous RRV transmission and suggest that this pathogen has silently circulated in French Polynesia. These findings raise the question of possible undetected circulation of RRV in other Pacific Island Countries and Territories.

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### 1. Introduction

French Polynesia (FP) is a French overseas territory of about 270,000 inhabitants in the Southeast Pacific, and one of the 22 Pacific Islands Countries and Territories (PICTs). Arthropod-borne virus (arbovirus) infections are major public health concern in the PICTs, with dengue virus (DENV), a *Flavivirus* of the *Flaviviridae* family, as the most common circulating and widespread arbovirus.<sup>1</sup> In FP, DENV outbreaks have been recorded since the 40s.<sup>1–4</sup> DENV used to be the only arbovirus isolated until October 2013 when FP experienced the largest outbreak of Zika virus (ZIKV) ever reported,<sup>5,6</sup> followed by an outbreak of chikungunya virus (CHIKV) that started in October 2014.<sup>7</sup> These two viruses, belonging respectively to *Flavivirus* and *Alphavirus*

genera, have also been recently involved in outbreaks in other PICTs.<sup>6,8,9</sup> Other arboviruses have been reported in PICTs, but not in FP, such as Japanese encephalitis virus (JEV)<sup>10</sup> and Ross River virus (RRV).<sup>11</sup>

RRV is an arbovirus belonging to the *Alphavirus* genus of the *Togaviridae* family.<sup>12</sup> It was first isolated from *Aedes vigilax* mosquitoes in Australia in 1959<sup>13</sup> and further from more than 30 mosquito species.<sup>12</sup> The most common clinical manifestations of RRV infections are fever, arthralgia and rash. Asymptomatic infections range from 55 to 75%.<sup>12,14–16</sup> The first reports of unusual epidemics of “pain, skin eruption and general manifestations” thought to have been due to RRV occurred in 1928 in New South Wales, Australia.<sup>17,18</sup> A large RRV outbreak occurred in the Pacific in 1979–1980 with over 500,000 cases reported,<sup>16</sup> and affected several PICTs including Fiji, Cook Islands, American Samoa, New Caledonia, Wallis & Futuna, and Vanuatu.<sup>14,19–23</sup> In FP, due to the neighbouring RRV epidemics, patients presenting with a “dengue like syndrome” were routinely tested for RRV in addition to DENV between April and August 1980 then sporadically until February 1984. Although no RRV infection was detected during this period

\* Corresponding author: Maite Aubry, Institut Louis Malardé, PO BOX 30, 98713 Papeete, Tahiti, French Polynesia.  
Tel./fax: +689 40 416 460/(689) 40 431 590.  
E-mail address: [maubry@ilm.pf](mailto:maubry@ilm.pf) (M. Aubry).

(unpublished data), circulation of RRV in FP cannot be excluded. After the 1979–1980 outbreak, RRV apparently disappeared from the PICTs. However, cases of RRV infections were subsequently reported from travelers returning from Fiji: 2 Canadian travelers, respectively in 2003 and 2004,<sup>24</sup> and 5 New Zealand travelers, respectively in 1997, 2000, 2001, 2003 and 2009.<sup>16</sup> Evidence of RRV infections were also found among residents of Papua New Guinea.<sup>25</sup>

To date, few data are available about the circulation, incidence and prevalence of RRV in the Pacific. To our knowledge, 13 serosurveys have been conducted for RRV in the region.<sup>12,14,20</sup> Among the studies performed in Australia,<sup>12</sup> seropositivity ranged from 8% for sera collected in North and Southeast Queensland, Darwin and Northern Territory area in 1974–1975<sup>26</sup> to up to 55% for sera collected from aboriginal communities in North Queensland and Northern Territory in 1966–1971.<sup>27</sup> In a serosurvey conducted by Tesh et al. in several PICTs,<sup>28</sup> evidence of RRV infections was found among populations sampled in Papua New Guinea between 1960 and 1969 and in Solomon Islands between 1960 and 1966, with rates of RRV seropositivity among localities ranging from 10% to 64% and 1% to 17%, respectively. In contrast, no RRV antibodies were found in sera collected in Vanuatu in 1963 and 1972, New Caledonia in 1963, Palau in 1961 and 1974, and American Samoa in 1962. In a study further conducted in American Samoa, 43.8% of the people sampled at the end of the 1979–1980 RRV outbreak had evidence of RRV infection, whereas those previously sampled in 1972 had no RRV antibodies.<sup>20</sup> In Fiji, seroprevalence was over 90% in some communities after the RRV outbreak in 1979–1980.<sup>14</sup> In the only study conducted among blood donors, seroprevalence for RRV antibodies was 8.4% from the 2,952 sera collected in South Australia in 1992.<sup>29</sup> To our knowledge, except for Australia where RRV is the most common and widespread arboviral disease with about 5,000 cases notified annually,<sup>11,30</sup> and to a lesser extent for Fiji,<sup>16,24</sup> there are no recent data about circulation of RRV in the Pacific area.

Due to the incidence of RRV infections in Australia and frequent population movements across the Pacific region, the risk of emergence of this virus in PICTs, as happened in the early 80s,<sup>14,19–23</sup> is high. More recently, the detection of sporadic cases of RRV infections from Fiji<sup>16,24</sup> shows that this pathogen remains a serious threat to the PICTs. In order to determine if RRV has already been

introduced and has circulated in FP, we conducted a serological survey on 593 blood donors sera collected from July 2011 to October 2013.

## 2. Materials and Methods

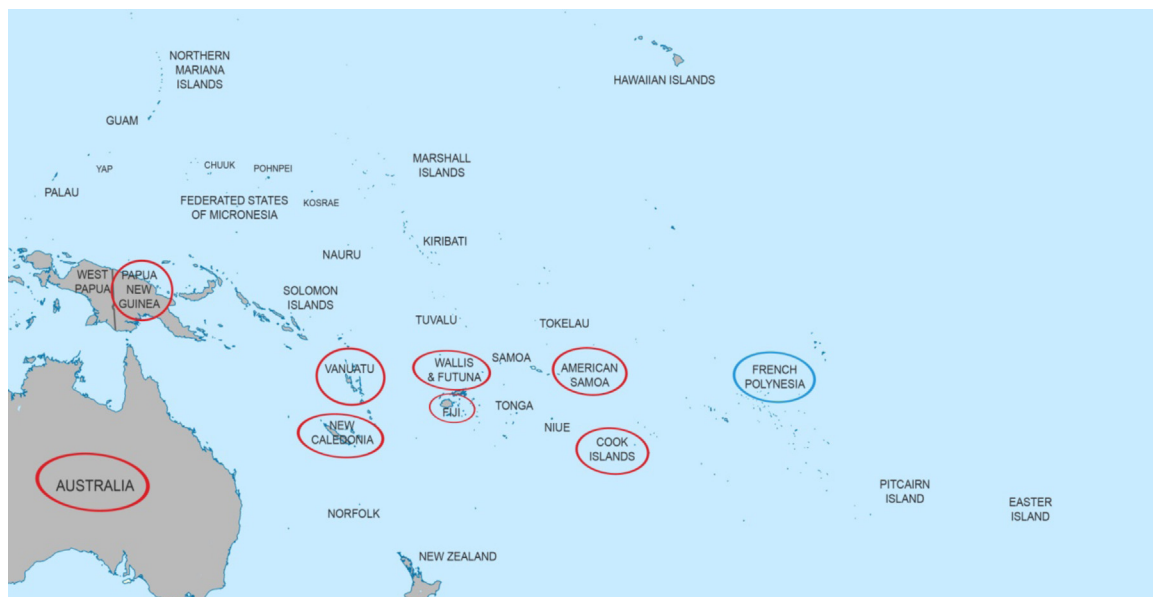
### 2.1. Participants

Blood donors were recruited by the French Polynesian blood bank centre (Tahiti, FP). All blood donors lived in Tahiti, the most inhabited island of FP, and were over 18 years old. Written consent for the use of a serum sample for serological testing was obtained before blood collection for all the blood donors included in this study. Volunteers to blood donation reporting a “dengue like syndrome” in the past 3 months, or any symptoms of acute or recent infection, were excluded from blood donation. None of the blood donors has been sampled specifically for the purpose of this study. A medical questionnaire was completed by the physician of the blood bank center before blood collection, in accordance with the recommendations for blood donation. The number of years of residence in FP and the past history of travel outside FP were recorded for all blood donors.

A total of 593 blood donors recruited from July 2011 to October 2013 were included in this study. Based on their travel history, blood donors were divided into 3 groups: group 1 is composed of residents who were born in FP and have never travelled abroad (132 blood donors); group 2 of residents who were born in FP and have travelled at least once abroad (290 blood donors); and group 3 of immigrants (171 blood donors). Within groups 2 and 3, blood donors reporting past travel in countries with or without reported RRV autochthonous infections (Figure 1) were separated in 2 subgroups, a and b respectively (Table 1).

### 2.2. Sample Collection

Blood samples for serological testing were collected in BD Vacutainer® Blood Collection tubes and stored at + 4 °C for maximum 1 day at the blood bank centre before shipment to the “Institut Louis Malardé” (ILM), Tahiti, FP. At ILM, blood samples were centrifuged for 15 minutes at 1,300 g then sera were retrieved and frozen at – 20 °C until processing.



**Figure 1.** Circulation of Ross river virus in the Pacific. Countries with reported Ross River virus autochthonous infections are circled in red. French Polynesia is circled in blue.

**Table 1**  
Characteristics of the blood donors included in the study

Blood donors	Born in FP				Immigrants			Total
	Group 1	Group 2a	Group 2b	Total group 2	Group 3a	Group 3b	Total group 3	
Number	132	145	145	290	101	70	171	593
%	22.26	24.45	24.45	48.90	17.03	11.80	28.84	100
Age								
Range	18–59	18–69	18–63	18–69	19–75	19–72	19–75	18–75
Mean	32.80	38.13	31.93	35.03	44.78	41.11	43.28	36.91
Median	32.00	37.00	29.00	33.00	46.00	41.00	42.00	36.00
Years of residence in FP								
Range	18–59	12–69	15–63	12–69	0–49	0–50	0–50	0–69
Mean	32.80	37.47	31.65	34.56	17.16	11.41	14.81	28.47
Median	32.00	36.00	29.00	32.00	16.00	7.50	12.00	27.00

Group 1: residents who were born in FP and have never travelled abroad; group 2a: residents who were born in FP and have travelled at least once in a country with reported RRV infections; group 2b: residents who were born in FP and have travelled at least once but in countries without reported RRV infections; group 3a: immigrants who have travelled at least once in a country with reported RRV infections; group 3b: immigrants who have travelled at least once but in countries without reported RRV infections.

### 2.3. Production of recombinant antigens from RRV and CHIKV

Serum samples were tested for the presence of immunoglobulin G class antibodies (IgGs) against RRV by indirect ELISA using recombinant antigens from RRV. Given that CHIKV has circulated in the PICTs since 2011 and is antigenically related to RRV, recombinant antigens from CHIKV were also used to test serum samples.

Recombinant antigens were produced in *Drosophila* S2 cells using the *Drosophila* S2 expression system (Life Technologies, USA). The synthetic genes (GeneCust, Luxembourg) encoding the soluble ectodomain of the envelope E2 glycoprotein (sE2) from RRV strain QML 1 (GenBank accession no. GQ433354) or CHIKV La Reunion strain 06–49 (GenBank accession no. AM258994) were cloned into the shuttle vector pMT/BiP/His A in which the SNAP-tag sequence (Covalys Biosciences AG, Switzerland) had been initially inserted as a stabilizing protein. The resulting plasmids encoding either the chimeric protein RR.sE2-SNAP or CHIK.sE2-SNAP were transfected into S2 cells to establish stable cell lines S2/RR.sE2-SNAP, and S2/CHIK.sE2,<sup>31</sup> according to the manufacturer's recommendations (Life Technologies, USA). After a 10-days cadmium induction of the stable S2 cell lines grown in 1-Liter spinner, cell supernatants were recovered and secreted soluble His-tagged recombinant viral antigens were purified on chelating and size-exclusion chromatography columns. The protein amounts of highly purified RR.sE2-SNAP and CHIK.sE2-SNAP were determined using a BCA protein assay kit (Thermo Scientific, USA), according to manufacturer's instructions. At least 50 mg of each recombinant viral antigen (concentration: 0.5 to 1 mg/ml) were obtained from a single batch of 1-L of S2 cell culture. The samples of purified RR.sE2-SNAP and CHIK.sE2-SNAP proteins were kept at -80 °C.

### 2.4. Protocol of indirect ELISA

For the capture of anti-RRV and anti-CHIKV IgGs, RR.sE2-SNAP, CHIK.sE2-SNAP, and antigen control (SNAP) were diluted at the final concentration of 2 µg/ml in phosphate buffer saline (PBS, Biomérieux, France), and 100 µL of protein sample were coated in 96-well plates overnight at +4 °C. After incubation, antigen sample was removed and wells were blocked with PBS containing 3% of skimmed milk (Régilait, France) and 1% of sodium azide (Merck, Germany). Sera of participants, as well as negative and positive control sera, were diluted 1:400 in PBS with 3% skimmed milk, 0.05% Tween 20 and 1% sodium azide. Then, each diluted serum was added in 2 wells containing either RR.sE2 or CHIK.sE2 recombinant antigens, and in 2 wells containing only SNAP proteins. Plates were incubated for 1 hour at 41 °C and

subsequently washed using PBS with 0.05% Tween 20 (Merck, Germany). Peroxidase-conjugated goat anti-human IgG antibodies (Jackson ImmunoResearch, USA) were added in all wells at a dilution of 1:10 000 in PBS with 3% skimmed milk and 0.05% Tween 20. Plates were incubated for 1 hour at 41 °C and washed before addition of peroxidase substrate tetramethylbenzidine (TMB, KPL, USA). Plates were stored at room temperature in the dark for 5 minutes and the enzymatic reaction between the peroxidase and its substrate was stopped by the addition of TMB Stop Solution (KPL, USA). The absorbance was read at a wavelength of 450 nm (OD<sub>450</sub>) using a microplate photometer (Thermo Scientific, USA).

Sera found positive for RRV IgGs using recombinants antigens were also tested using the kit Panbio Ross River Virus IgG ELISA (Panbio diagnostics, USA), following manufacturer's recommendations.

### 2.5. Serological analysis

For each serum, average absorbance was calculated using the OD<sub>450</sub> values measured in the 2 wells containing the same recombinant antigen or SNAP proteins. Specific absorbance was then determined by deducting the mean absorbance value obtained with SNAP to the mean absorbance value found for each recombinant antigen. Sera with a specific absorbance value ≥ 0.2 were considered positive for the presence of IgGs against the virus tested.

### 2.6. Statistical analysis

To assess the impact of age and travel history on RRV seroprevalence among participants, Fisher's test was performed using the GraphPad Prism 6 version 6.03 software. P values less than 0.05 were considered as statistically significant.

### 2.7. Ethics Statement

The recruitment of participants and processing of blood samples were conducted in accordance with the Ethics Committee of French Polynesia under reference n°60/CEPF (06/27/2013). Blood sample and personal data of each participant were anonymized at the French Polynesian blood bank centre before sending to ILM.

## 3. Results

Among the 593 blood donors, 204 (34.40%), 18 (3.03%) and 10 (1.69%) were found seropositive for RRV, CHIKV or both viruses IgGs, respectively (Table 2). Seroprevalence in different groups and

**Table 2**  
Seropositivity for RRV and CHIKV in each group of blood donors

Blood donors	Born in FP				Immigrants			Total
	Group 1	Group 2a	Group 2b	Total group 2	Group 3a	Group 3b	Total group 3	
Seropositive for RRV								
Total	56 (2) <sup>a</sup>	49 (6)	52 (5)	101 (11)	32 (1)	15 (0)	47 (1)	204 (14)
%	42.42	33.79	35.86	34.83	31.68	21.43	27.48	34.40
Seropositive for CHIKV								
Total	2	6	4	10	6	0	6	18
%	1.51	4.14	2.76	3.45	5.94	0	3.51	3.03
Seropositive for both RRV and CHIKV								
Total	1	4	3	7	2	0	2	10
%	0.76	2.76	2.07	2.41	1.98	0.00	1.17	1.69

<sup>a</sup> Number of blood donors tested positive for RRV with the kit Panbio Ross River Virus IgG Elisa.

**Table 3**  
Seropositivity for RRV according to the year of birth or the number of years of residence for blood donors that have never travelled in countries with reported RRV infections

Blood donors seropositive for RRV	Group 1	Group 2b	Group 3b
Born in FP			
Born before 1981	33/70 (47.14%)	28/68 (41.17%)	–
Born from 1982 to 1987	8/20 (40.00%)	12/37 (32.43%)	–
Born from 1988 to 1992	10/27 (37.03%)	10/31 (32.25%)	–
Born after 1993	5/15 (33.33%)	2/9 (22.22%)	–
Total born after 1981	23/62 (37.09%)	24/77 (31.16%)	–
Total born before and after 1981	56/132 (42.42%)	52/145 (35.86%)	–
Immigrants			
Arrived in FP before 1981	–	–	3/8 (37.5%)
Arrived in FP between 1982–2000	–	–	8/20 (40.00%)
Arrived in FP between 2001–2005	–	–	2/8 (25.00%)
Arrived in FP between 2006–2013	–	–	2/34 (5.88%)
Total arrived in FP after 1981	–	–	12/62 (19.35%)
Total arrived in FP before and after 1981	–	–	15/70 (21.43%)

subgroups ranged from 21.43% (group 3b) to 42.42% (group 1) for RRV and it ranged from 0% (group 3b) to 5.94% (group 3a) for CHIKV. Percentages of RRV seropositivity were not significantly different between groups except for group 3b against groups 1 and 2b (P values were 0.0032 and 0.0408, respectively). Among the 204 blood donors seropositive for RRV IgGs, 14 were also found positive using the kit Panbio Ross River Virus IgG ELISA.

Seroprevalence for RRV according to the year of birth or the length of stay in FP of blood donors that have never travelled in areas with known circulation of RRV is reported in Table 3. Among blood donors born after 1981 belonging to groups 1 and 2b, 37.09% and 31.16% were seropositive for RRV, respectively. In the two groups, the rate of positivity for RRV IgGs increased with age. Among blood donors of group 3b who arrived in FP during the years 1982–2000, 2001–2005 and 2006–2013, 40%, 25% and 5.88% were seropositive for RRV, respectively.

#### 4. Discussion

Arboviruses are emerging pathogens in Oceania<sup>8,32</sup> and, except for DENV, few data are available about their circulation, incidence and prevalence in the region. In particular, the spread of RRV in the Pacific region is unknown. In the present study, sample collection was conducted in FP from July 2011 to early October 2013. Even if the first CHIKV outbreak in FP was recorded in October 2014, CHIKV had begun circulating in other PICTs from 2011.<sup>8</sup> Consequently, a previous silent circulation of CHIKV in FP cannot be excluded. To insure that the detection of RRV IgGs was not due to cross-reaction of RRV recombinant antigens with CHIKV antibodies resulting from a non-detected previous infection, all sera were tested for the presence of CHIKV IgGs, in addition to RRV IgGs. The low rate of immunization against CHIKV measured among blood donors (3.03%) suggests that the virus did not

actively circulate in FP before 2014. This hypothesis is supported by the large magnitude of the CHIKV outbreak that started in October 2014, with at least 25% of the population estimated to have been infected.<sup>33</sup> Moreover, the small number of sera found positive for both RRV and CHIKV IgGs (10/593) suggests the absence or limited cross-reactions between the two *Alphaviruses* with our ELISA assay.

The 204 sera found positive for RRV IgGs using recombinant antigens were also tested with a commercial serological assay (kit Panbio Ross River Virus IgG ELISA), and 14 (6.86%) of them were found positive with both assays, confirming the circulation of RRV in FP. Discordant results between in-house and commercial serological tests have already been reported for other arboviruses. For instance, two commercial ELISA kits evaluated for the detection of CHIKV IgGs in serum samples previously tested positive with in-house ELISAs from 2 National Reference Centers for Arboviruses showed sensitivities of 52% and 88%.<sup>34</sup> Moreover, when comparing the performance of several serological tests for detecting JEV-specific IgGs in 29 vaccinated children, the in-house assay detected neutralizing antibodies in all children whereas the commercial ELISA assay showed no positive reaction.<sup>35</sup> In another study conducted on serum samples from 100 persons vaccinated against JEV, only 6.6% of the sera found positive with the in-house assay were also detected positive using a commercial IgG ELISA test.<sup>36</sup> The discrepancy between the results obtained with laboratory and commercial assays may be related to the cut-off used. For a diagnosis purpose (commercial kit), the cut-off is high because specificity is preferred to sensitivity. Moreover, diagnostic kits are dedicated to the detection of recent rather than past infections and should not be used to screen general population, which is the purpose of seroprevalence studies. According to the manufacturer's protocol, the cut-off of the commercial kit used in our study was determined on an Australian population and may require



adjustment depending on the population analyzed. Consequently, the cut-off of the commercial assay should be adapted for the analysis of the serological results obtained from the French Polynesian population.

The overall rate of RRV seropositive blood donors found in our study was surprisingly high (34.40%) given that RRV infection cases had never been reported in FP. The absence of significant difference in prevalence between blood donors who were born in FP and have travelled or not in areas with or without RRV reported infections (groups 1 and 2), suggests that they have been infected in FP. The high rates of RRV seropositivity found among blood donors who were born in FP after 1981, whatever they had never travelled abroad (group 1, 37.09%) or had travelled only in areas without RRV reported cases (group 2b, 31.16%), support the idea that RRV has circulated in FP after the large outbreak that occurred in 1979–1980 in the PICTs. The lowest (21.43%) and highest (42.42%) RRV seroprevalence rates are found for the immigrant group that is supposed not to have been exposed elsewhere than in FP (group 3b) and for the group of residents who were born in FP and have never travelled abroad (group 1), respectively, and are significantly different between the two groups ( $P=0.0032$ ). Together with the median time of residence in FP for the immigrants (7.50 years) which is 4 times lower than the one found for the blood donors born in FP (32.00 years), these data suggest that the increase in RRV seroprevalence is linked to the time of residence in FP. The increase with age of the acquisition of RRV IgGs among the residents born in FP and that could have been infected only locally (groups 1 and 2b) is consistent with a silent continuous circulation of RRV during the last decades. This is confirmed by the detection of 12 RRV seropositive blood donors among immigrants who arrived in FP between 1982–2013 and who have never travelled in countries with reported RRV infections (group 3b). The finding that RRV has circulated in FP is concordant with the presence of several mosquito species that can potentially transmit the virus, including *Aedes aegypti*, *Aedes polynesiensis*, *Culex annulirostris*, *Culex quinquefasciatus* and *Culex sitiens*.<sup>12,19,37–41</sup>

RRV may have circulated in FP without being detected for several reasons. The main one is that 55% to 75% of RRV infections are asymptomatic,<sup>12,14–16</sup> and many symptomatic patients have mild symptoms that may not require to seek medical attention. In addition, RRV infections can have clinical presentations similar to DENV infections. However, with laboratory capacities for arboviruses detection mainly focused on DENV, patients presenting with a “dengue like syndrome” but tested negative for DENV are not systematically investigated for other arboviruses. At last, virus isolation is rarely achieved, probably because RRV does not persist beyond the early stages of disease,<sup>12</sup> which may explain why despite laboratory diagnosis, no RRV infectious cases were detected in FP between 1980–1984.

Only adults were tested in this study but since most infections occur between the ages of 20 and 60, peaking between 30 and 40,<sup>16,42</sup> it should be relevant. Thus, despite the exclusion of children and discrepant results in the percentage of RRV positive blood donors depending on serological methodology used, we can conclude to a silent circulation of RRV in FP. Irrefutable evidence of autochthonous RRV transmission in FP would require the isolation of the virus or of its RNA from a resident with no recent travel history. Attempts to isolate RRV will be performed in a second study on sera collected from patients presenting with symptoms suggestive of an arbovirus infection and tested negative for DENV, ZIKV and CHIKV.

Like RRV, it is possible that other arboviruses may also have circulated in FP and in other PICTs without being detected. Indeed, the occurrence of West Nile virus or JEV introductions respectively from the USA and Japan are possible since many PICTs have direct and frequent flights from these countries, and several mosquito

species established in the PICTs may transmit these viruses.<sup>38</sup> Due to the growing risk of emergence of new arboviruses in the Pacific, joint efforts should be done to improve local and regional surveillance,<sup>43</sup> as well as vector control.

## Acknowledgments

We are grateful to Jérôme Viallon from the Institut Louis Malardé, French Polynesia, for technical support, and to Philippe Buchy from the Institut Pasteur in Cambodia, for providing CHIKV positive control serum.

This study was supported by the “Contrat de projets 2012–2014” (Convention No. 7331/MSS/DSP, 08/31/2012).

**Conflict of interest statement:** None of the authors have any conflict of interest (financial or personal) in this study.

**Ethical approval statement:** This study was approved by the Ethics Committee of French Polynesia under reference n°60/CEPF (06/27/2013).

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