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RESEARCH ARTICLE

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Molecular detection of Zika virus in blood and RNA load determination during the French Polynesian outbreak

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Zika virus (ZIKV) viremia is reported as low and transient; however, these estimates rely on limited data. We report RNA loads in sera collected from symptomatic patients during the 2013-2014 French Polynesian ZIKV outbreak. We performed molecular detection of ZIKV RNA in sera from 747 patients presenting with suspected acute phase ZIKV infection. Among patients with confirmed infection, we analyzed the duration of viremia, assessed viral RNA loads and recorded the main clinical symptoms. A total of 210/747 (28.1%) sera tested positive using a ZIKV-specific RT-PCR. Viral RNA loads in symptomatic patients that ranged from 5 to 3.7×10^6 copies/mL (mean 9.9×10^4 copies/mL) were not related to a particular clinical presentation, and were significantly lower than those previously obtained from asymptomatic ZIKV infected blood donors. The rate of detection of ZIKV RNA in sera from suspected cases of acute phase ZIKV infection was low. ZIKV RNA loads were lower in symptomatic patients compared to asymptomatic blood donors and were lower than RNA loads usually reported in dengue infections. As there is no abrupt onset of symptoms in ZIKV infections, we suggest that infected patients sought for medical attention when viremia was already decreasing or had resolved.

KEYWORDS
arbovirus, French Polynesia, RNA load, RT-PCR, Zika virus

1 | BACKGROUND

Zika virus (ZIKV), an arthropod-borne virus (arbovirus) of the *Flavivirus* genus in the *Flaviviridae* family[1] was discovered in Africa in 1947.[2] Only sporadic infections have been reported in Africa and Asia until the first ZIKV outbreak that occurred in the Pacific in 2007 in Yap (Federated States of Micronesia).[3,4] After, ZIKV caused a second large outbreak in French Polynesia in 2013-2014 and subsequently spread throughout the Pacific islands.[5–7] In 2015, ZIKV emerged in the Americas and rapidly spread throughout Latin America and Carribeans, and also caused an outbreak in Africa.[7] Between 2007 and September 2016, 72 countries reported evidence of active ZIKV transmission.[8] In August 2016, the first ZIKV autochthonous infections within the continental U.S. were reported in Florida (USA).[9] In September 2016, the virus was reported circulating in Asia with several autochthonous infections detected in Singapore.[10] ZIKV is principally transmitted by the bite of infected *Aedes* mosquitoes but non-vector-borne transmission is possible and includes sexual,[11–13] materno-fetal,[14,15] and blood transfusion transmissions.[16,17] As ZIKV has been detected in humans from urine,[18,19] saliva,[18] or breast milk,[20] other mode of transmission cannot be excluded. Like other arboviruses, most of the ZIKV infections are probably asymptomatic[7] but the ratio of asymptomatic/symptomatic infections is not yet established and may vary according to local condition and ZIKV strains. Symptomatic patients usually report a mild disease without acute onset of fever. Clinical manifestations of ZIKV infection mainly consist of maculopapular rash, arthralgia/myalgia, low fever, asthenia, headache, and conjunctivitis,[7] as reported in French Polynesia,[21] Yap,[22] and Brazil.[23]

At the time of the study, the recommendation for acute phase ZIKV infection was based on molecular detection of ZIKV RNA during the acute phase of infection using reverse transcription (RT)-PCR in blood samples collected within the first week post-symptom onset (PSO).[7,24] Since then, this recommendation has been enlarged to the second week PSO, and serodiagnosis is also performed if RT-PCR is negative.[25] Serology-based diagnosis is limited by the cross-reactivity of antibodies developed in response to infections with other flaviviruses, especially dengue virus (DENV)[7,25] and requires confirmation by sero-neutralization tests which can only be performed by highly trained and specialized laboratories.[4,7] However, cross-reactivity has been observed even using sero-neutralization assays, especially in secondary arbovirus infections.[24] In French Polynesia, as most of the patients were previously infected by DENV, ZIKV serodiagnosis was not implemented.[26] ZIKV viremia is usually reported as low and transient, making diagnosis by RT-PCR a challenge.[4,27] We report the data obtained using molecular based detection of ZIKV RNA from suspected cases of ZIKV infections and levels of ZIKV RNA loads observed for confirmed ZIKV infection cases. These viral loads were compared with those previously published for asymptomatic ZIKV RNA positive blood donors in French Polynesia.[28]

2 | METHODS

The study was conducted in French Polynesia from October 2013 to March 2014 and was approved by the Ethics Committee of French Polynesia (reference 66/CEPF). Serum samples from 747 patients presenting to health care facilities (hospitals, clinics, private physician, and dispensary) with suspected acute phase ZIKV infections were collected from venous blood puncture according to medical prescriptions. For each patient, a standardized symptom questionnaire inquiring about the number of days PSO and the main clinical symptoms was recorded. A suspected case was defined as a patient presenting with macula-papular rash and/or self reported or measured low grade fever (<38.5°C) and at least two of the following; conjunctivitis, arthralgia, myalgia, hand and/or feet oedema. A confirmed case was a suspected case with ZIKV RNA detected in serum and/or saliva samples.[21] Viral RNA was extracted from serum samples using the NucliSENS® easyMAG® System (BioMérieux) extractor. Detection of ZIKV RNA was performed by RT-PCR with the CFX96 Touch™ Real-Time PCR Detection System thermocycler using the iScript One-Step RT-PCR kit for Probes (Bio-Rad), and two primer and probe sets that specifically bind to partial M/E and E encoding genes, as previously reported.[4,5,26,29] ZIKV RT-PCR results were reported as positive when both genomic regions were amplified, equivocal if only one was amplified, and negative if no amplification occurred. Since DENV was co-circulating in French Polynesia during the ZIKV outbreak,[5] all sera were also tested using a DENV-specific RT-PCR, as previously reported.[30]

ZIKV RNA loads (number of RNA copies/mL) were determined from sera that tested positive by RT-PCR by comparison to a standard curve obtained from serial dilutions of a known concentration of ZIKV RNA synthetic transcript, as previously reported.[14,28,29] Viral loads assessed in sera from symptomatic patients were compared using the same quantification method to those previously obtained in sera from asymptomatic blood donors collected by the blood bank center in French Polynesia from November 2013 to February 2014 during the ZIKV outbreak.[29]

All statistical analyses were performed with the GraphPad Prism 6 software, using Mann-Whitney test.

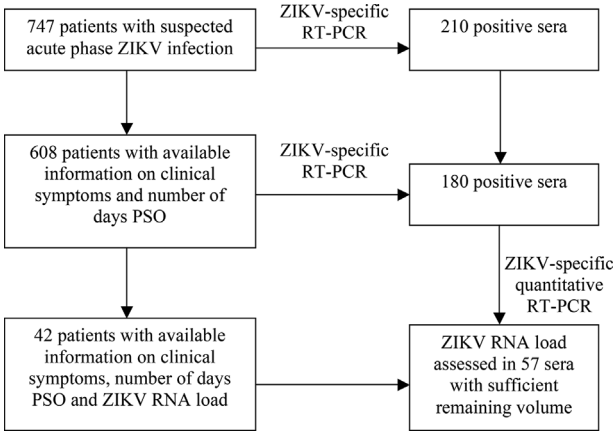

FIGURE 1 Characteristics of the studied population

3 | RESULTS

Characteristics of the studied population are reported in Fig. 1. Among the 747 serum samples tested by ZIKV-specific RT-PCR, 210 (28.1%) were reported positive, 68 (9.1%) equivocal, and 469 (62.8%) negative (Table 1). Equivocal results were reported for single detection of the partial M/E genes in 38 patients (5.1%) or partial E gene for 30 patients (4%). All sera tested negative for DENV RNA. For patients with suspected ZIKV infections, information on clinical symptoms and number of days PSO was available for 608 (81.4%), including 180 with confirmed ZIKV infections.

ZIKV RNA loads according to the number of days PSO are reported in Fig. 2. The percentage of positive samples was significantly higher for patients collected from day 1 to 7 PSO [30.8% (176/572)] compared to those collected later [11.1% (4/36)] ($P < 0.01$).

TABLE 1 Results of RT-PCR assays using two primers and probes sets for detection of partial M/E and E partial encoding genes of ZIKV in the 747 serum samples collected from French Polynesian suspected ZIKV infections cases

M/E partial gene	E partial gene	Total number (%)
Neg	Neg	469 (62.8)
Pos	Neg	38 (5.1)
Neg	Pos	30 (4)
Pos	Pos	210 (28.1)
Total		747 (100)

y/o, years old.

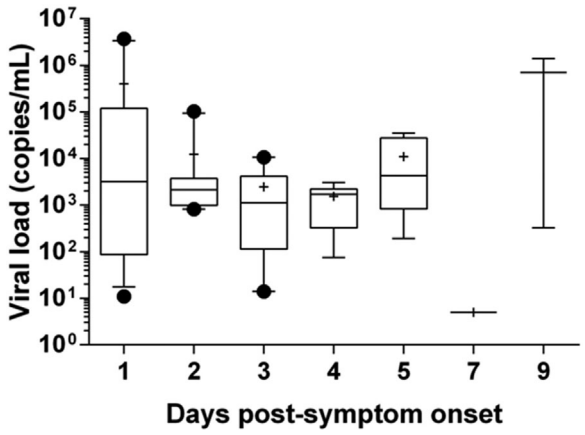

FIGURE 2 ZIKV RNA loads are showed by day post-symptom onset. Error bars are for 10-90 percentile interquartile ranges, middle bars are for median, and + are for means

ZIKV RNA loads were assessed in 57 sera found positive by RT-PCR for which remaining sera was available (Supplemental Table S1). Detailed results for number of days PSO, and clinical data (fever, rash, asthenia, conjunctivitis, arthralgia/myalgia) were available for 42 patients.

ZIKV RNA loads that ranged from 5 to 3.7×10^6 copies/mL (mean 9.9×10^4 copies/mL) were not significantly different between males and females ($P = 0.21$) and between patients aged under and over 18 years ($P = 0.12$) (Table 2) and were not significantly different from patients presenting with and without fever, rash, asthenia, conjunctivitis, or arthralgia/myalgia (Table 3). In contrast, ZIKV RNA loads were significantly lower compared to ZIKV RNA loads from 26 asymptomatic French Polynesian blood donors that were previously published (RNA loads ranging from 2.5×10^3 to 8.1×10^6 copies/mL, mean 7.3×10^5 copies/mL) ($P < 0.0001$) (Supplemental Table S2).[28]

The analysis of ZIKV RNA loads from five blood donors that became symptomatic post-donation (ranging from 3×10^3 to 5.2×10^6, mean 1.1×10^6 copies/mL) showed no significant difference with those obtained from the 21 blood donors who remained asymptomatic ($P = 0.95$), but they were significantly higher than those found in symptomatic patients ($P = 0.004$).

4 | DISCUSSION

Among the cases suspected to have ZIKV infection, only 28.1% tested positive for detection of both partial M/E and E ZIKV encoding genes. While patients presented with symptoms compatible with a ZIKV infection and tested negative for DENV, the high rate of equivocal and negative results may be explained by the low sensitivity of our method which may have been responsible for false negative results. Another explanation is the timing of symptom development relative to ZIKV viremia in serum with a potential delay between development of viremia and symptom onset. Indeed by the time patients developed symptoms and sought for medical attention, ZIKV viremia may have resolved in blood and RNA cleared in serum.

The sensitivity of our method was the same as previously reported on sera tested in Yap,[4] estimated at 25 and 100 copies per assay for partial M/E and E encoding genes, respectively.[29] As previously reported, using the same primers and probes, ZIKV RNA was more frequently detected in saliva than in blood collected at the same time during acute phase ZIKV infection, but the use of saliva did not increase the window of detection of ZIKV RNA.[31] A study conducted from persons with travel-associated ZIKV disease concluded that urine also increased the rate of ZIKV RNA detection during the acute phase of infection and extended the window of ZIKV RNA detection.[32] Our results and those previously published[32] suggest that, during the first days PSO, the higher detection rates by RT-PCR in urine and saliva compared to blood are probably related to higher ZIKV RNA loads in these fluids, suggesting that a higher sensitivity could be reached during the acute phase of ZIKV infection by testing urine or saliva instead of blood. Though the most important challenge to ZIKV diagnosis is the short window during which RNA can be detected in serum.

In contrast to other mosquito-borne diseases such as dengue, there is no abrupt onset of clinical symptoms in ZIKV infections.[7] In dengue, flushing is common on day 1 or 2 post-onset of fever and rash usually appears between day 2 and 6 after onset of fever.[33,34] DENV viremia peaks at day 2 before defervescence of fever, and only one third of infected patients have detectable RNA at the end of the febrile phase.[35] Fever is not a prominent feature of ZIKV infection, is low grade, and is not the main cause of consultation as reported in French Polynesia and in Brazil.[7,23] In French Polynesia, patients usually sought for medical attention during rash. Consequently, ZIKV infected patients might have sought for medical care later in the acute phase of infection and at that time, viremia was already decreasing or resolved. The finding that ZIKV RNA loads were significantly higher in pre-symptomatic blood donors compared to the 57 symptomatic patients confirms that blood samples from ZIKV infected symptomatic patients were probably collected during the resolving phase of viremia. Data about ZIKV RNA loads in symptomatic patients are scarce and rely on studies conducted in Yap[4] and from case reports,[14,20,36–39] with viral loads in blood up to 7.2×10^5 copies/mL. In Nicaraguan patients, the mean viral RNA load was 5.4×10^3 and 1.1×10^5 copies/mL in non pregnant patients and

TABLE 2 ZIKV RNA loads in serum samples from 57 symptomatic French Polynesian ZIKV infected patients according to gender and age

	≤18 y/o	>18 y/o	Total (range, mean ZIKV RNA load)
Female	13	23	36 ($5.0 - 1.4 \times 10^6$, 4.8×10^4)
Male	6	15	21 ($1.4 \times 10^1 - 3.7 \times 10^6$, 1.9×10^5)
Total (range, mean ZIKV RNA load)	19 ($5.0 - 3.7 \times 10^6$, 2.7×10^5)	38 ($5.0 - 1.2 \times 10^5$, 1.3×10^4)	57 ($5.0 - 3.7 \times 10^6$, 9.9×10^4)

y/o: years old.

TABLE 3 Comparison of ZIKV RNA loads according to the main symptoms of ZIKV

	With		Without		
Symptoms	Mean (SD)	Min-Max	Mean (SD)	Min-Max	*P*-value*
Rash	5.3×10^4 (2.4×10^5)	$5.0 - 1.4 \times 10^6$	4.7×10^5 (1.3×10^6)	$3.2 \times 10^2 - 3.7 \times 10^6$	0.132
Fever	1.8×10^5 (8.1×10^5)	$5.0 - 3.7 \times 10^6$	8.5×10^4 (3.1×10^5)	$1.4 \times 10^1 - 1.4 \times 10^6$	>0.99
Asthenia	1.8×10^5 (7.3×10^5)	$1.1 \times 10^1 - 3.7 \times 10^6$	2.2×10^4 (4.3×10^4)	$5.0 - 1.2 \times 10^5$	0.169
Arthralgia/myalgia	$1.5x10^5$ ($7.1x10^5$)	$5.0 - 3.7 \times 10^6$	1.0×10^5 (3.6×10^5)	$1.1 \times 10^1 - 1.4 \times 10^6$	0.546
Conjunctivitis	2.4×10^5 (9.3×10^5)	$1.1 \times 10^1 - 3.7 \times 10^6$	6.7×10^4 (2.7×10^5)	$5.0 - 1.4 \times 10^6$	0.658

*P values were calculated using the Mann-Whitney test.

pregnant women, respectively.[40] In French Polynesia, the ZIKV RNA loads detected in blood samples collected from symptomatic patients (ranging from 5 to 3.7×10^6 copies/mL) were lower than the viral loads usually reported during DENV infection (ranging from 10^5 to 10^9 copies/mL).[35]

Viral loads in asymptomatic and symptomatic patients infected with arboviruses are available for chikungunya (CHIKV) and West Nile (WNV) viruses. For CHIKV, viral loads (expressed in pfu/mL) were not significantly higher in symptomatic patients than in asymptomatic blood donors.[41] For WNV, a study conducted in 821 blood donors with confirmed WNV infection showed that 26% of WNV RNA positive patients developed symptoms and had higher RNA loads at index donation compared to those who remained asymptomatic.[42] It was previously reported that WNV RNA persists in blood compartments with detection of RNA in whole blood samples collected up to 3 months post-index donation in ~40% of blood donors who tested positive for WNV RNA in plasma at the time of blood donation.[43] Despite a trend for symptomatic WNV-positive donors to maintain higher levels of WNV RNA in whole blood compared to asymptomatic WNV-positive donors, the difference was not statistically significant.[43] For ZIKV, RNA loads were found higher in asymptomatic blood donors[28] than in symptomatic ZIKV-infected patients, but there was no difference in the levels of viral loads at the time of blood donation between blood donors who remained asymptomatic compared to those who developed symptoms.

In dengue, viral loads were found significantly higher in dengue hemorrhagic fever compared to dengue fever in some studies studies,[44–46] and not different in others.[47,48] However, high DENV viral loads appear to correlate with the severity of the disease.[49] For ZIKV-infected symptomatic patients, there was no correlation between viral loads and a particular clinical presentation.

Guidelines for laboratory diagnosis of ZIKV infection now recommend the use of RT-PCR assays to test blood samples collected the first 2 weeks PSO.[25] According to our results, blood samples collected during the first 7 days PSO gave the highest rate of positive reaction. However, we have detected ZIKV RNA up to 10 days PSO and in one case, with high ZIKV RNA load even at day 9 PSO (1.4×10^6 copies/mL). Viral loads were not significantly different according to sex and age. Prolonged viremia has been reported in infected pregnant woman[37] but information about pregnancy were not available for the women included in the study. Dating onset of symptoms is difficult in ZIKV infection as reported in French Polynesia[7] and New Caledonia.[50] Symptoms are sometimes only recorded after careful questioning of the patient and the number of days PSO is probably underestimated and subjective. Consequently, as long as there is no longitudinal studies that carefully characterize the timing of symptom development relative to the kinetics of viremia and antibody development, diagnosis of ZIKV infection by RT-PCR in blood should be attempted no matter the time PSO and should not only be restricted to the first days PSO.

According to the protocol implemented in Yap,[4] RT-PCR results were reported as positive if both M/E and E encoding genes were detected, and equivocal when only one gene was detected. Authors suggested that equivocal results could be false positive results or the consequence of low viral loads. In French Polynesia, similar proportions of samples tested positive for single detection of the partial M/E gene (5.1%) or partial E gene (4%) and detection of both encoding gene was equally sensitive. Equivocal results were interpreted as positive by practitioners since the patients presented clinical symptoms of ZIKV infection and tested negative for DENV. Equivocal results were probably related to low RNA loads at the time of blood collection rather than false positive results for the detection of partial M/E or E encoding genes.

5 | CONCLUSION

In symptomatic ZIKV-infected patients, the low rate of detection of ZIKV RNA in serum can be explained by low viral loads, delayed consultation due to mildness of symptoms, and delayed development of symptoms in patients that probably sough for health care provider attention while viremia was resolving in blood. At the acute phase of ZIKV infection, the detection rate of ZIKV RNA by RT-PCR in serum is low compared to urine and saliva. Consequently, ZIKV infection should never be excluded on the basis of a negative RT-PCR in serum, and saliva and urine samples should be also tested.

AUTHORS' CONTRIBUTIONS

D. Musso, M. Lanteri, J. Broult, E. Grange, T. Nhan, and M. Aubry participated to the design of the study and redaction of the manuscript. E. Rouault, A. Teissier, and K. Zisou performed analyses.

CONFLICTS OF INTEREST

Authors disclose any financial and other conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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