Isolation of infective Zika virus from urine and saliva of patients in Brazil

Myrna C. Bonaldo[1*], Ieda P. Ribeiro[1¶], Noemia S. Lima[1¶], Alexandre A. C. dos Santos[1¶], Lidiane S. R. Menezes[1¶], Stephanie O. D. da Cruz[1¶], Iasmim S. de Mello[1¶], Nathália D. Furtado[1¶], Elaine E. de Moura[1¶], Luana Damasceno[4¶], Kely A.B. da Silva[2&], Marcia G. de Castro[2&], Alexandra L. Gerber[3&], Luiz G. P. de Almeida[3&], Ricardo Lourenço-de-Oliveira[2&], Ana Tereza R. Vasconcelos[3&], Patrícia Brasil[4¶].

[1]Laboratório de Biologia Molecular de Flavivírus, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil

[2]Laboratório de Mosquitos Transmissores de Hematozoários, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil

[3]Laboratório Nacional de Computação Científica, Petrópolis, Rio de Janeiro, Brazil

[4] Laboratório de Doenças Febris Agudas, Instituto Nacional de Infectologia Evandro Chagas, Fiocruz, Rio de Janeiro, Brazil

* Corresponding author

E-mail: mbonaldo@ioc.fiocruz.br (MCB)

[¶]These authors contributed equally to this work.

[&]These authors also contributed equally to this work.

ABSTRACT

BACKGROUND

Zika virus (ZIKV) is an emergent threat provoking a worldwide explosive outbreak. Since January 2015, 41 countries reported autochthonous cases. In Brazil, an increase in Guillain-Barré syndrome and microcephaly cases was linked to ZIKV infections. A recent report describing low experimental transmission efficiency of its main putative vector, *Ae. aegypti*, in conjunction with apparent sexual transmission notifications prompted the investigation of other potential sources of viral dissemination. Urine and saliva have been previously established as useful tools in ZIKV diagnosis. However, no evidence regarding the infectivity of ZIKV particles present in saliva and urine has been obtained yet.

METHODOLOGY/PRINCIPAL FINDINGS

Nine urine and five saliva samples from nine patients from Rio de Janeiro presenting rash and other typical Zika acute phase symptoms were inoculated in Vero cell culture and submitted to specific ZIKV RNA detection and quantification through, respectively, NAT-Zika, RT-PCR and RT-qPCR. Two ZIKV isolates were achieved, one from urine and one from saliva specimens. ZIKV nucleic acid was identified by all methods in four patients. Whenever both urine and saliva samples were available from the same patient, urine viral loads were higher, corroborating the general sense that it is a better source for ZIKV molecular diagnostic. In spite of this, from the two isolated strains, each from one patient, only one derived from urine,

42 suggesting that other factors, like the acidic nature of this fluid, might interfere with virion
43 infectivity. The complete genome of both ZIKV isolates was obtained. Phylogenetic analysis
44 revealed similarity with strains previously isolated during the South America outbreak.
45
46 CONCLUSIONS/SIGNIFICANCE
47
48 The detection of infectious ZIKV particles in urine and saliva of patients during the acute
49 phase may represent a critical factor in the spread of virus. The epidemiological relevance of
50 this finding, regarding the contribution of alternative non vectorial ZIKV transmission routes,
51 needs further investigation.
52
53 AUTHOR SUMMARY
54
55 The American continent has recently been the scene of a devastating epidemic of Zika
56 virus and its severe manifestations, such as microcephaly in newborns and Guillain-Barré
57 Syndrome. Zika virus, first detected in 1947 in Africa, only from 2007 started provoking
58 outbreaks. Zika, dengue and chikungunya viruses are primarily transmitted by *Aedes*
59 mosquitoes. Dengue is endemic in Brazil for almost 30 years, and the country is largely infested
60 by its main vector, *Aedes aegypti*. Chikungunya virus entered the country in late 2014 and Zika
61 presence was confirmed eight months later. Nevertheless, Zika notifications multiplied and
62 spread across the country with unprecedented speed, raising the possibility of other
63 transmission routes. This hypothesis was strengthened by some recent reports of Zika sexual

transmission in *Ae. aegypti*-free areas and by the description of a low transmission efficiency to Zika virus in local *Ae. aegypti*. We found Zika active particles in both urine and saliva of acute phase patients, and a finding that was promptly announced by Fiocruz via Press Conference on February 5, 2016. In this work, we bring up the potential alternative person-to-person infection routes beyond the vectorial transmission, that might have epidemiological relevance.

INTRODUCTION

Zika virus (ZIKV) is an emerging mosquito-borne virus of the family *Flaviviridae* and genus *Flavivirus* [1]. ZIKV was first reported in 1947 after isolation from a febrile sentinel rhesus monkey [2]. Since then, serologic evidence of human ZIKV infection in Africa and Asia was detected, but until 2005 only few human cases were reported [3]. The first well-described outbreak outside these geographic regions happened in 2007 in Micronesia, more specifically in Yap State, when the majority of the population was affected with Zika fever [4]. Intriguingly, the local mosquito vector was not confirmed by neither viral isolation nor molecular methods [4].

On October 2013, a second intense outbreak in Oceania occurred in French Polynesia (2013/2014), and soon after spread over to New Caledonia (2014), Cook Islands, (2014) and Ester Island, 2014 [5, 6]. In these outbreaks, approximately 80 % of ZIKV infections was asymptomatic [4, 7]. Commonly, Zika is considered to be a mild disease lasting one week with symptoms including fever, rash, conjunctivitis, arthralgia, myalgia, headache and malaise. However, during the French Polynesian epidemic, its association with severe neurological complications, the Guillain-Barré syndrome (GBS) was reported for the first time [8].

In April 2015, the first autochthone cases in the Americas was identified in Brazil [9, 10]. At present, Brazil suffers an explosive outbreak of ZIKV. Hence, in February 2016, Brazilian Ministry of Health (MoH) appraised the incidence of further than one million of ZIKV disease cases [11]. Notably, in addition of an increase of GBS cases as occurred in French Polynesia outbreak, the MoH of Brazil described a rise of microcephaly occurrence. Between 22 October 2015 to 5 March 2016, 6158 cases of microcephaly and/or central nervous system malformation were noticed in contrast to the estimated average number of 163 annual cases [12]. So far, 745 suspected cases of microcephaly have been confirmed as ZIKV-associated microcephaly in a total of 1927 investigated cases [11-13]. More recently, a case of ZIKV infection with vertical transmission demonstrated the association of severe fetal brain injury with fetal infection with ZIKV [14]. Moreover, ZIKV nucleic acid was detected in amniotic fluid of two pregnant women, whose fetuses were diagnosed with microcephaly, corroborating vertical transmission possibility [15]. Other abnormalities such as placental insufficiency, fetal growth restriction, CNS injury, and fetal death have also been reported in association with ZIKV infection [16]. This scenario of ZIKV infection linked to severe neurological complications as well as the establishment of ongoing ZIKV outbreaks in several countries in Latin America led to the WHO to declare ZIKV an international public health emergency [11, 17, 18].

The transmission of ZIKV has been associated with several *Aedes* mosquito species belonging to subgenus *Stegomyia*, notably *Ae. aegypti* [19, 20] and *Ae. albopictus* [21]. However, a recent study proposes that although susceptible to infection, *Ae. aegypti* and *Ae. albopictus* from the Americas display an unexpectedly low vector competence for ZIKV [22], suggesting other factors such as the large naïve population for ZIKV and the high densities of

human-biting mosquitoes contribute to the rapid spread of ZIKV during the current outbreak. Nonetheless, perinatal transmission [23] and potential risk for transfusion-transmitted ZIKV infections has also been demonstrated [24]. Most remarkably, ZIKV can be likely disseminated by sexual contact, due to its presence in semen [25, 26]. In addition, it was demonstrated the existence of ZIKV in urine [27, 28], breast milk [29] and saliva [30]. Indeed, ZIKV was more frequently detected in urine and saliva than in blood using ZIKV RT-PCR tests for diagnosis. It was considered that patients exhibit the highest concentrations of ZIKV in saliva at disease onset [30] while in urine, ZIKV possibly remains detectable for longer periods [27]. So far, no evidence has been obtained regarding the infectivity of ZIKV particles present in saliva and urine. In this study, we demonstrate that it is possible to recover infective ZIKV from both saliva and urine of acute phase patients by means of viral isolation in Vero cells. This achievement suggest that ZIKV may be transmitted between humans by infected saliva and urine.

METHODS

Study facilities and patients enrollment

The Acute Febrile Illnesses Laboratory and Molecular Biology of Flavivirus Laboratory conducted this study at Oswaldo Cruz Foundation, Rio de Janeiro. The institutional review boards at Fundação Oswaldo Cruz (Fiocruz) approved the study protocol. All subjects provided written, informed consent before participation, and a medical assistant filled a standardized medical questionnaire form, during an interview with the participants. Numbers of days from the first reported symptom (days after symptoms onset) and main signs and symptoms were

recorded. Urine and saliva samples investigated in this study were collected from January 14th to February 2nd, 2016. The samples were obtained only from patients clinically suspected with ZIKV infection, especially with pruritic maculopapular rash.

Clinical samples

Saliva and urine specimens were collected in 50 mL sterile certified, DNase-/RNase-free tubes, and after collection, in some cases, the pH was measured by a digital pH meter, in order to investigate the relevance of the pH for viral infection. Twenty five millimeter diameter sterile syringe filters with a 0.22 µm pore size were used to filter the specimens. The samples were aliquoted for subsequently analysis and assays, as infection in Vero cell culture and RNA isolation.

Primary viral isolation

The African green monkey kidney (Vero) cell line obtained from (ATCC) was grown in 37^0 C, under an atmosphere containing 5% CO_2, in Earle's 199 medium supplemented with 5% fetal bovine serum (FBS). The Vero cells were seeded at a density of 40,000 cells/cm^2 in 25 cm^2 culture flasks 24 hours before inoculation. The urine and saliva samples were diluted in Earle's 199 medium supplemented with 5% FBS (1:2 and 1:4), and 1 mL of each dilution was inoculated onto Vero cells monolayer. After 1 h incubation at 37°C, the inoculum was removed and replaced by 10 mL culture medium. As negative control for each experiment, Vero cells seeded

in one culture flask were mock inoculated with culture media. The presence of infectious viral particles was controlled by observation of cytopathic effects (CPE).

Plaque forming unit assay

Vero cells were seeded at a density of 40,000 cells/cm^2 in 6-well plates 24 h before inoculation. Dilutions of the biological specimens (1:2, 1:4 and 1:8) in culture media were used to infect monolayers (200 µL/well). After 1 h incubation at 37° C, the inoculum was removed and replaced by 3 mL of 2.4 % CMC (carboxymethyl cellulose) in Earle's 199 medium. After 7 days incubation at 37° C, cells were fixed with 10 % formaldehyde, washed, and stained with 0.4 % crystal violet for visualization of plaques.

RNA isolation

Viral RNA was isolated from 140 µL of each biological specimens and cell culture supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA was eluted in 60 µl of AVE buffer and stored at -80°C until use. The concentration and purity of each RNA sample were measured by Thermo Scientific NanoDrop 8000 Spectrophotometer and Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit according the manufacturer's instructions.

RT-PCR

The viral RNA was reverse transcribed applying the Superscript IV First-Strand Synthesis System (Invitrogen) using random hexamers according to the manufacturer's recommendations. The reverse transcription reaction was carried out at 23°C for 10 min, 55°C for 10 min and 80°C for 10 min. Further, the viral RNA was amplified by conventional PCR covering viral NS1 genomic region (genome position 3085-3385) using GoTaq Green Master Mix (Promega) according to the manufacturer's recommendations. The thermocycling program set up in a Veriti 96 Well thermocycler (Applied Biosystem) was 1 cycle of 95°C for 5min; 40 cycles of 95°C for 40 sec, 50°C for 40 sec, 72°C for 30 sec; 1 cycle of 72°C for 10min and hold of 4°C. 10 ml of Amplified products were detected by electrophoresis on a 2% agarose gel, visualized by ethidium bromide staining UV.

NAT and Quantitative RT-PCR

To discard co-infection of ZIKV with dengue and/or chikungunya viruses, we analyzed the urine, saliva samples and the viral strains isolated from Vero cell using he NAT- Dengue, Zika and Chikungunya discriminatory kit (Instituto de Biologia Molecular do Paraná and Fundação Oswaldo Cruz, Brazil). To measure genomic ZIKV load, viral RNA was reverse transcribed and amplified using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems) in an Applied Biosystems StepOnePlus Instrument. For each reaction we used 400 nM forward primer (5'-CTTGGAGTGCTTGTGATT-3', genome position 3451-3468), 600 nM reverse primer (5'-CTCCTCCAGTGTTCATTT-3', genome position 3637-3620) and 250 nM probe (5'FAM-AGAAGAGAATGACCACAAAGATCA-3'TAMRA, genome position 3494-3517). The sequences of this primer set were kindly provided by Isabelle Lepark-Goffart (French National Reference

Centre for Arboviruses, IRBA, Marseille, France). Samples were run in duplicate. The reverse transcription was performed at 50°C for 5 minutes. The qPCR conditions were 95°C for 20 seconds, followed by 40 amplification cycles of 95°C for 15 seconds and 60°C for 1 minute. Copy numbers of ZIKV genomic RNA were calculated by absolute quantitation using a standard curve for each run. To construct a standard curve, we cloned an amplicon comprising the genomic region 3085-4032 of the isolate Rio-U1 using pGEM-T Easy Vector (Promega) to serve as a template for in vitro transcription. The RNA transcript was made with mMessage mMachine High Yield Capped RNA Transcription Kit (Invitrogen) using T7 enzyme and purified using MEGAclear Kit (Ambion) according to manufacturer's instructions. The purity of the transcript was verified using NanoDrop 8000 Spectrophotometer (Thermo Scientific), the integrity was analyzed using 2100 Bioanalyzer (Agilent) using the RNA 6000 Nano Kit (Agilent), and the concentration of the RNA was accessed using Qubit 2.0 Fluorometer (Invitrogen). The standard curve was generated by a ten-fold dilution (ranging from 10 to 10^9 copies/reaction) of the transcript. The limit of detection under standard assay conditions was approximately 40 viral RNA copies/mL.

Nucleotide Sequence

Double-stranded cDNA libraries were prepared using the TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA). Briefly, the polyA containing mRNA purification step was not performed and the protocol was started with 25-35 ng of RNA in 5 ul of molecular biology grade water to which were added 13 ul of Fragment, Prime, Finish Mix. The remaining steps of the protocol were carried out without any modifications. Library quality

control was performed using the 2100 Bioanalyzer System with the Agilent DNA 1000 Kit (Agilent, Santa Clara, CA, USA). The libraries were individually quantified via qPCR using a KAPA Library Quantification Kits for Illumina platforms (KAPA Biosystems, Wilmington, MA, USA). The libraries were pooled together in equimolar quantities and sequenced. Paired-end reads (2 × 75 bp) were obtained using a MiSeq Reagent Kits v3 (150-cycles) in a MiSeq sequencing system (Illumina).

Assembly and annotation

A total of 17,413,830 reads was generated for Rio-U1 sample and 21,734,486 for Rio-S1 sample. Related reads to *Chlorocebus sabaeus* have been filtered using Bowtie2 and Samtools, remaining 12,614,062 reads of Rio-U1 and 12,943,134 of Rio-S1. Both genomes were assembled using Ray 2.20 (k = 31). The completed genome of Rio-U1 has 10,795bp (Acession number KU926309) and Rio-S1 has 10,805bp (Acession number KU926310). Gene prediction was performed by GenemarkS 4.17. Mature peptides were identified by blastp against the protein annotated in reference sequence NC_012532.

Phylogenetic analysis

Phylogenetic reconstruction was inferred by means Maximum Likelihood method based on the General Time Reversible model with a discrete Gamma distribution and invariable sites (GTR+G+I) using alignments of poliprotein genes from 39 Zika virus strains and 1 dengue virus serotype 4.

236 RESULTS

237

238 Patients, clinical and social demographic characteristics

239 We examined nine enrolled patients suspected of ZIKV infection. The initial medical
240 support and collection of urine and saliva samples were performed from January 14th to
241 February 2nd 2016. Out of seven women, six were pregnant with gestational ages varying from
242 18 to 33 weeks, median value of 20. 5 ± 5.8 weeks (Supplementary Table 1 and 2). The female
243 patient ages ranged from 20 to 42 years old (median value of 28.5 ±7.4 years) and the male
244 patient ages were 24 and 68 years old. All the patients live in the metropolitan area of Rio de
245 Janeiro (Supplementary Tables 3 and 4).

246 The most frequent sign of ZIKV disease was pruritic maculo papular rash which lasted in
247 average 4 days (Supplementary Tables 1 and 2). However, other clinical symptoms were also
248 prevalent, such as low grade fever (< 38°C), headache, myalgia and arthralgia of large and small
249 joints, present in 5 out of 9 patients.

250 We collected and analyzed urine from patients 1 to 4 and both urine and saliva samples
251 from patients 5 to 9. Vero cells cultures were inoculated at the same date of sample collection
252 and then daily observed through inverted microscopic examination until the appearance of
253 cytopathic effect (CPE). Within one week of incubation, only two samples exhibited CPE (2 out
254 of 14), the urine sample of patient 4 with CPE detected at 4th day of post- inoculation (1 out of
255 9) and the saliva sample of patient 6 at 5th day post-inoculation (1 out of 5). In this last
256 infection, we recognized small foci of rounded and refractive cells detaching from the
257 monolayer (Fig 1 A and B). After one-week incubation, we proceeded to split cells from

negative cultures by means of trypsinization when monolayer was confluent. This procedure was repeated for three consecutive times. Nevertheless, it was not possible to isolate ZIKV in these samples, neither by detecting CPE in Vero cell monolayers or ZIKV genome by RT-PCR (results not shown). We also analyzed these samples by plaque forming assay as a way to detect infectious virus particles. Unfortunately, we did not perform this analysis with urine of patient 1, because we received a small aliquot of this specimen. Nevertheless, we detected viral plaques from samples of patient 6 (Fig 1 C and D), in which the dilution 1:2 of saliva originated in 8 PFU resulting in an original viral concentration of 80 PFU/ml in saliva of patient 6. Interestingly, only one viral plaque was visualized by means of this methodology in urine sample of this patient 6, resulting in a titer of 10 PFU/ml.

Fig 1. Isolation of Zika virus in Vero cell from the saliva of patient 6.

Phase contrast optical microscopy of culture flasks containing (A) Mock-infected Vero cells and (B) saliva-infected Vero cells presenting a clear visible cytopathic effect. Viral plaque detection in saliva (C) and urine (D). The white arrow shows the unique viral plaque detected in the urine sample.

ZIKV diagnosis and genome detection

Furthermore, we analyzed all urine and saliva specimens by RT-PCR to confirm the detection of ZIKV (Fig 2A and B). In addition, we included RNA samples of ZIKV isolated from patient 1 and 6 in Vero cells. The set of samples of patient 1 and 6 were all positive and an expected-amplicon band of around 300 bp was seen in electrophoretic analyses, demonstrating

the presence of ZIKV genome in these samples (Fig 1 A and B). We also observed a fade band from urine and saliva of patient 9 (Fig 2 B). The ZIKV specificity of this approach was confirmed when we tested this protocol in RNA samples of Chikungunya (CHIKV), dengue (DENV) and yellow fever (YFV) viruses (Fig 2 C).

Fig 2. Detection of genomic RNA of Zika virus in urine and saliva samples by RT-PCR analysis. (A) Shows the profiles obtained from urine samples. The lane numbers indicate the patient code. The lane 1I is the amplicon obtained from the viral isolate from urine of patient 1 (isolate Rio-U1). (B) RT-PCR analysis from patients from 5 to 9 where S indicates saliva RNA samples and U, urine RNA samples. (C) Amplification of Zika virus genome of isolate Rio-U1 (1i) with ZK-specific primers that were too employed in the RT-PCR assay of Chikungunya virus RNA (CHIKV), dengue virus RNA (DENV) and Yellow Fever 17DD RNA (YFV). In all of these analyses, a negative control of amplification were included (C). The size marker migration is indicated on the left of the figures.

Notwithstanding, it was mandatory to confirm the result of Zika virus infections in patients and isolations in Vero cells, since ZIKV, DENV and CHIKV are co-circulating in Brazil and the diseases caused by them exhibit similar symptoms. So, each sample was tested for the presence of these three viruses by the ZIKV nucleic acid testing (NAT) of samples which was established to be routinely used in Brazil as diagnosis test since December 2015. (Table 1). All patients included in this study were negative for DENV and CHIKV (Ct > 40.0). Patient 1 was positive for ZIKV in urine (Ct of 30.02) and patient 6 in urine (Ct of 25.56) and saliva (Ct of 30.27) and the viral isolates derived obtained from specimens of these patients were also

302 positive and presented Ct of 12.62 and Ct of 20.88, respectively. Patient 9 was also positive for
303 ZIKV in urine specimen (Table 1), whereas urine from patient 7 presented amplification in a late
304 cycle and, therefore, this result was considered inconclusive. To validate negative results, the
305 ribosomal 18S RNA was detected in all samples showing that there was no inhibition of the RT-
306 PCR.

307 **Table 1. ZIKV RNA detection and quantitation**

Patient	Sample	Target	NAT Ct*	Result**	Viral load (vRNA copies/mL)***
1	urine	18 S ZIKV	24.98 30.02	Positive	2,677
	Vero cell	18 S ZIKV	11.98 12.62	Positive	1.24×10^{10}
2	urine	18 S ZIKV	19.63 Ud	Negative	< 40
3	urine	18 S ZIKV	21.49 Ud	Negative	< 40
4	urine	18 S ZIKV	24.57 Ud	Negative	< 40
5	urine	18 S ZIKV	24.38 Ud	Negative	< 40
	saliva	18 S ZIKV	22.04 Ud	Negative	< 40
6	urine	18 S ZIKV	21.54 25.56	Positive	252,836
	saliva	18 S ZIKV	17.40 30.27	Positive	74,449
	Vero cells	18 S ZIKV	11.53 20.88	Positive	2.88×10^{9}
7	urine	18 S ZIKV	24.57 40.96	Inconclusive	102
	saliva	18 S ZIKV	24.02 Ud	Negative	< 40
8	urine	18 S ZIKV	22.89 Ud	Negative	< 40
	saliva	18 S ZIKV	15.35 Ud	Negative	< 40
9	urine	18 S ZIKV	23.41 37.41	Positive	431
	saliva	18 S ZIKV	16.53 Ud	Negative	40

308 * Ud means undetermined (Ct> 40.00). **Diagnosis of the patients suspected to be infected with Zika
309 virus (NAT- Zika, dengue and chikungunya diagnosis test). *** Value determined by quantitative RT-PCR.

310

311 Viral loads of these samples were then measured by a RT-qPCR assay resulting in data
312 consistent with those obtained by the diagnosis assay kit (Table 1 and Figure 3). Accordingly,
313 the highest viral loads were obtained from those specimens that allowed us to isolate ZIKV by
314 Vero cell infections. The urine of patient 1 exhibited a ZIKV-genomic RNA copies of 2.68 x 10^3
315 per ml whereas the patient 6 displayed 2.53 x 10^5 ZIKV RNA copies per ml in urine and 7.44 x
316 10^4 ZIKV RNA copies per ml in saliva. As expected for isolated viral samples, we observed an
317 increase of genomic ZIKV RNA copies in Vero-cell- isolated samples, in which the isolated from
318 patient 1 presented 1.24 x 10^{10} copies/ml and patient 6, 2.88 x 10^9 copies/ml (Figure 3).
319 Furthermore, we confirmed positivity of the urine from patient 7 and the positive detection of
320 ZIKV RNA in saliva and urine of patient 9, although this established value is borderline localized
321 in the limit of detection.

322 **Fig 3. ZIKV Viral loads from urine and saliva specimens of infected patients measured by RT-**
323 **qPCR.**

324 Urine specimens are shown in black and saliva specimens are shown in grey. The limit of
325 detection is shown as a dotted line corresponding to 40 viral RNA copies/mL.

326

327 ZIKV genomic sequencing

328 The genomic sequences of Vero cell isolates ZIKV Rio-U1 strain (KU926309), isolated
329 from urine and Rio-S1 (KU926310) strain, isolated from saliva, were then determined. The
330 comparison between Rio-U1 and Rio-S1 yielded 99.61% identity, displaying six amino acid
331 variations in the viral proteins (Table 2). For phylogenetic analysis, we used nucleotide
332 sequences coding the complete ZIKV polyprotein. We observed that all sequences sampled in

the Americas form a robust monophyletic cluster (bootstrap score = 97%) within the Asian genotype and share a common ancestor with the ZIKV strain that circulated in French Polynesia in November 2013 and remained genetically isolated from African clusters (Fig 4). **Table 2. Differences in amino acid residues in ZIKV polyproteins of Rio-S1 and Rio-U1 isolates**

Polyprotein position	ZIKV protein (amino acid position)	Rio-S1	Rio-U1
625	E (335)	A	T
1143	NS1 (349)	V	M
1404	NS2B (32)	M	I
2039	NS3 (537)	K	R
2122	NS4A (3)	T	A
2688	NS5 (168)	A	V

Fig 4. Molecular Phylogenetic analysis by Maximum Likelihood method.

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.9645)). The rate

variation model allowed for some sites to be evolutionarily invariable ([+I], 37.8665% sites). The analysis involved 40 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 10247 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

Phylogenetic analysis of the isolated viruses exhibiting the highest identity of ZIKV strain Rio-U1 with KU501216.1 and KU501217.1 both from Guatemala (99.7 % identity), isolated also related with the first reported autochthonous transmission of ZIKV in Brazil [31]. Whereas Rio-S1 presented 99.7% of identity with KU527068.1, isolated in Brazil from a Zika-associated microcephaly case [14].

366 DISCUSSION

367 In this study, we demonstrate the occurrence of infectious Zika viral particles in urine
368 and saliva of patients. Besides, we also showed that the saliva of an acute phase patient may
369 have a viral concentration of 80 PFU/ml. The isolation of two ZIKV samples from urine and
370 saliva was associated with ZIKV load in infected patients during the acute phase. Actually, the
371 presence of ZIKV genome in urine is not a novelty. Hence, former studies preconized the use of
372 urine and saliva for ZIKV RNA detection and diagnosis [27, 30], since ZIKV genome was more
373 frequently identified in saliva and urine compared to blood. Furthermore, the finding of
374 flaviviral genome in urine was earlier described in Dengue [32], Yellow Fever [33], St. Louis
375 Encephalitis [34], Japanese Encephalitis [35], and West Nile viruses [36]. Dengue genome was
376 also detected in saliva of infected patients [32]. Interestingly, the existence of excreted-
377 infectious West Nile particles in the urine of acute phase patients was earlier described in
378 conjunction with their isolation in Vero E6 and in BHK21 cells [37]. Particularly, ZIKV isolation
379 was approached by many groups utilizing Vero cells (GeneBank: KJ776791; JN860885;
380 KU647676). Therefore, we adopted this cell model to detect, amplify and quantify viable ZIKV
381 straight from patient's samples of urine and saliva.

382 The recovery of ZIKV from these urine and saliva was effective in two of nine patients
383 whose viral load were clearly detectable. Interestingly, despite the fact that the viral load
384 found in the urine of patient 1 was considerably lower, around one hundred times, than the
385 equivalent sample in patient 6, we only recovered virus from urine of the former (Rio-U1
386 strain). On the other hand, recovering of infective ZIKV from patient 6, the Rio-S1 strain, was
387 successful using the saliva sample, but not with urine one, even though the highest number of

388 copies has been established in urine. Concordantly, we detected in this analysis a superior
389 number of plaques in plaque assay of saliva. Viral detection and recovery from urine and saliva
390 of ZIKV patients might be firstly related to the severity of infection as well as the period of
391 specimen collection after the onset of Zika symptoms. The detection of ZIKV RNA in saliva
392 improved the diagnosis in the first week from the disease onset [30]. But ZIKV viruria persists
393 for longer periods after disease beginning and, in some cases, for longer than two weeks from
394 Zika onset [27], as described in the two recently reported cases of Guillain–Barré syndrome
395 occurred in Martinica [38]. However, it is necessary to perform additional clinical studies
396 associating disease onset, severity of symptoms and viral persistence in urine and saliva to
397 better clarify this point.

398 Another aspect in viral recovering deals with the physiological pH found in saliva and
399 urine. Hence, pH in urine varies from 4.5 to 8.0 while saliva assumes values near neutral pH. It is
400 well known that the flavivirus envelope protein E undergoes irreversible conformational
401 changes at a mildly acidic pH (below 6.5), a process naturally occurring in the viral membrane
402 fusion in endosomes [39]. These structural changes are irreversible, and outside of cellular
403 environment, provoke loss of infectivity and hemagglutination activity as well as virus
404 aggregation due to increased hydrophobicity [40]. Thereby, we suggest that the failure of
405 recovering ZIKV strain in Vero cells propagation from the urine of patient 6 would be due to the
406 inactivation of most ZIKV due to exposition of the acidic pH value of 5.6 of this urine specimen.
407 The infectious virus number was lower, at least proportionally to the viral RNA copies
408 presented in this fluid, when compared to saliva of the same patient. We do not establish the
409 pH of patient´s 1 urine, due to volume sample limitations. The importance of ZIKV in urine for

410 human transmission is unexplored, but the effect of acidic pH on viral viability might represent
411 a serious restriction for viral spreading. In West Nile Virus when a similar urine excretion occurs,
412 it is considered that the presence of infectious particles would represent a real risk for inter
413 human transmission through kidney transplantation [37].

414 In reference to the occurrence of viable ZIKV in saliva, a large range of viruses can be
415 identified in this specimen, such as Cytomegalovirus, Ebola virus, Enteroviruses, Hepatitis B
416 virus, Hepatitis C virus, Human herpesviruses, HIV, Human papillomavirus, Influenza virus,
417 Measles virus, Rhinoviruses and Rubella virus [41, 42]. As previously mentioned, Zika and
418 dengue virus were also discovered in saliva [30, 32]. Although, the presence of intact viral
419 particles in saliva do not distinguish viable virus from noninfectious virus. However, for the first
420 time, we could well identify ZIKV plaque forming units from saliva of an infected man in Vero
421 cell monolayers with a titer corresponding to 80 PFU per ml.

422 Essentially, another important subject is that the existence of viable virus in oral fluid
423 samples does not always indicate that the virus can be transmitted orally and become
424 epidemiologically relevant. Actually, viral infections of the oral cavity are relatively rare, since
425 saliva contains antiviral molecules and is relatively hypotonic being capable of lysing enveloped
426 viruses [43]. Perhaps, the established proportion of approximately 1 PFU to 1,000 ZIKV RNA
427 copies in saliva of one patient was modulated by these host factors.

428 Although saliva functions as a protective barrier for virus entry, some studies have
429 shown that a disruption in oral mucosa or periodontal disease can facilitate virus entry [44].
430 Since previous studies detected Flaviviruses as Dengue [45, 46] and Zika [30] virus in saliva, and
431 our study have demonstrated possible infectious ability of Zika viral particles in saliva, a

potential person-to-person Zika virus infection through this specimen, using a disrupted oral mucosa or periodontal pockets as virus entry, should be considered and investigated.

ZIKV is an emergent vector-borne disease, but fast growing evidence points to an increased relevance of its non-vector ways of transmission, as perinatal and transplacental transmission occurs from mother to child [14, 23]. Additionally, ZIKV genome was also detected in breast milk, followed by viral isolation of infective viral particles [29]. Moreover, cases of probable sexual transmission have been reported with association of ZIKV in semen [25, 26]. In addition, viral contamination linked to blood transfusion and organ transplantation have been previously discussed [47]. Furthermore, reports of laboratorial infection or bites of animals was associated to the transmission [48]. Finally, evidence of vertical and/or venereal transmission between mosquitoes was supported by the detection of ZIKV natural infection in males *Ae. furcifer* [19].

We compared the complete coding sequences obtained in this study with public sequence data from Zika virus representative of the isolates from three distinct genotypes in Asian, West African, and East African in addition to isolates from recent outbreak in Americans. Similarly to the sequences described in the recent widespread epidemic of ZIKV in the Americas, the sequences Rio-S1 and Rio-U1 from ZIKV isolated in this study clustered with the Asian clade, covering sequences from New World, Pacific, Micronesian and Malaysian strains.

Since surveillance programs have reported periodic circulation of the ZIKV virus since 1968, with high frequency activity varying an interval of 1 – 2 years added to fact that RNA virus evolve fast, their host and vector broad range, non-vector transmission, and particularly risk of

453 neurotropic and teratogenic outcomes, the molecular epidemiologic vigilance is crucial to solve
454 this questions.

455 In conclusion, the detection of infective ZIKV in saliva and urine of patients deserves a
456 more detailed study to establish whether or not these fluids contribute to viral transmission.
457 Surely, these findings will be extremely relevant to prevent and control ZIKV transmission.

458 Acknowledgments

459 The authors thank Denise Valle from Laboratório de Biologia Molecular de Flavivírus -
460 IOC/FIOCRUZ - RJ for for helpful discussion and comments about the manuscript and supporting
461 our studies on vector-borne-diseases. To Lais de Souza Soares from Laboratório de Biologia
462 Molecular de Flavivírus - IOC/FIOCRUZ - RJ, Anielly Alves Ferreira and Rafaela Miranda from
463 Laboratório de Mosquitos Transmissores de Hematozoários - IOC/FIOCRUZ – RJ, Aline dos
464 Santos Moreira, Renata Almeida de Sá and Beatriz de Lima Alessio Müller from Plataforma
465 Genômica–Sequenciamento de DNA/RPT01A/FIOCRUZ for technical support and Ricardo
466 Magrani Junqueira from Plataforma de Sequenciamento de Alto Desempenho/IOC-FIOCRUZ –
467 RJ, for sharing expertise and information on NGS. We are grateful to Miriã Alves Gonçalves
468 Trindade, Rosiléa de Souza Rosa dos Santos, Mirian da Conceição Sobrinho, Tulanne Yuriê da
469 Silva Santos from Instituto Nacional de Infectologia - Ambulatório de Doenças Febris Agudas -
470 INI/FIOCRUZ – RJ for helping with samples and data collection, Heloísa Diniz from Serviço de
471 Multimeios/CICT-FIOCRUZ and Gutemberg Brito from SEJOR/IOC-FIOCRUZ for the design of
472 photographs and figures. We also thank Ana Paula de Campos Guimarães from Unidade
473 Genômica Computacional Darcy Fontoura/ LNCC and Guilherme Loss and Rafael Guedes from
474 Laboratório Nacional de Bioinformática/ LNCC for *De novo* sequencing, assembly and
475 analysis of the genome of the Zika Virus.

476

477

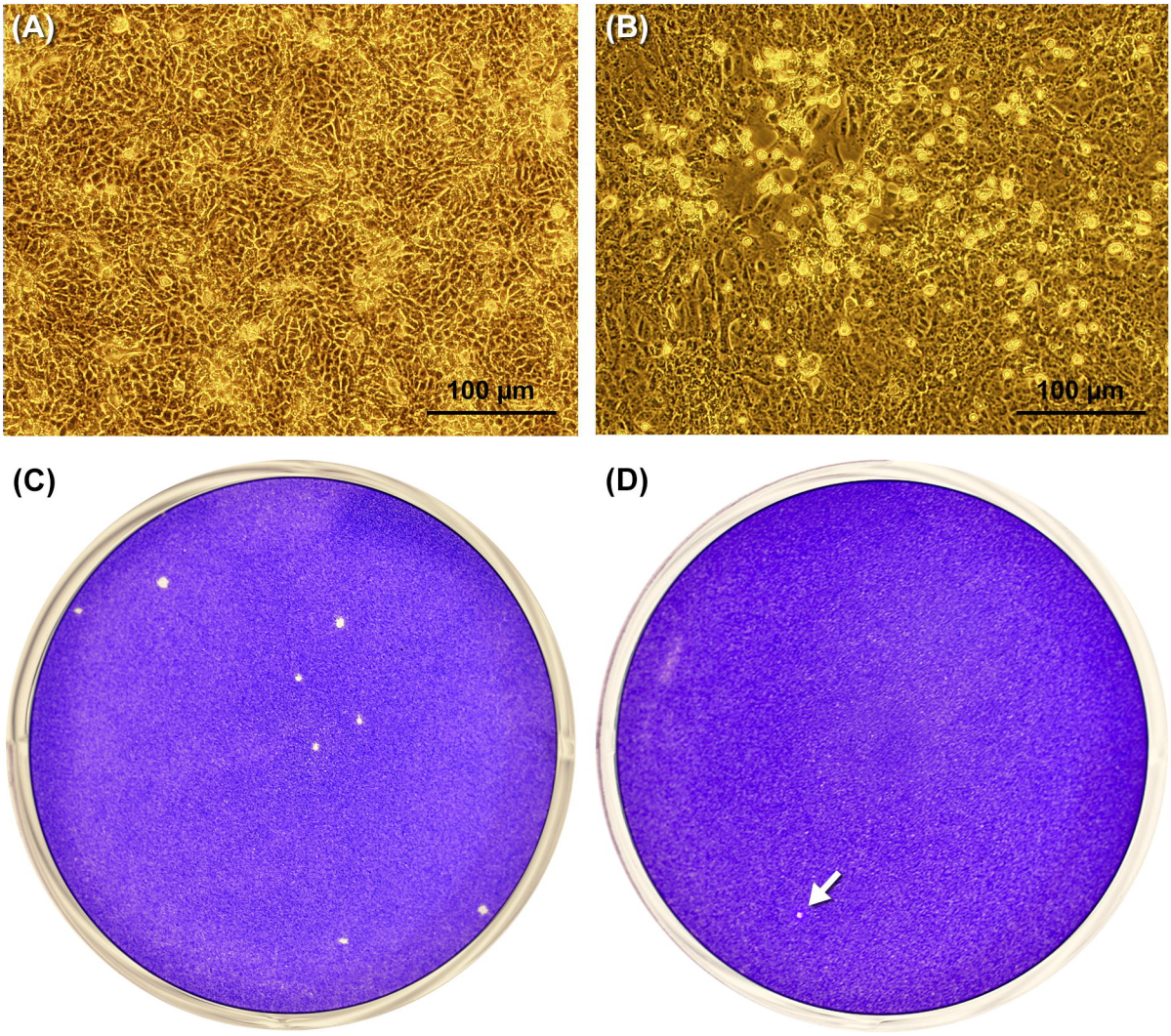
478 REFERENCES

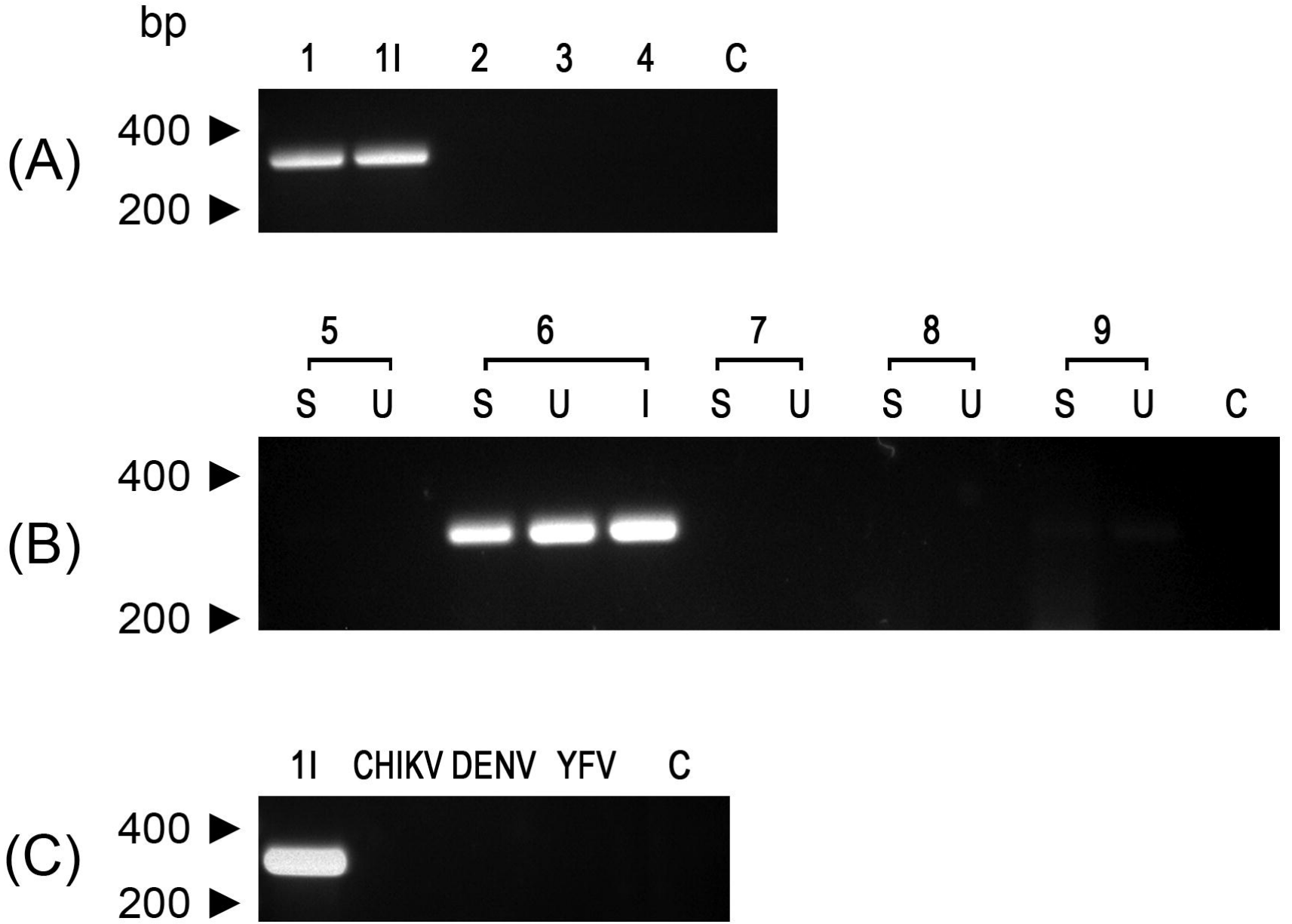
479

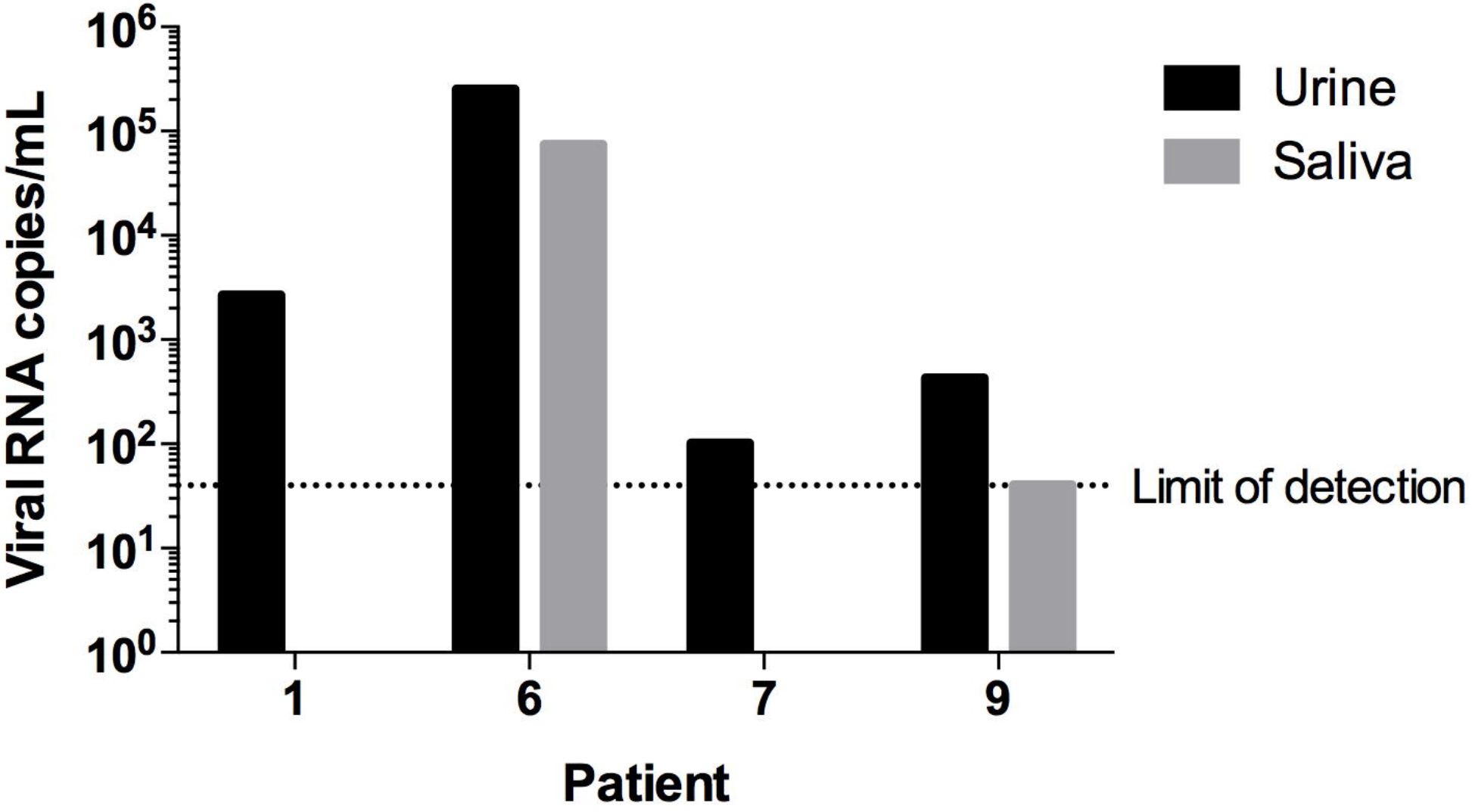
480

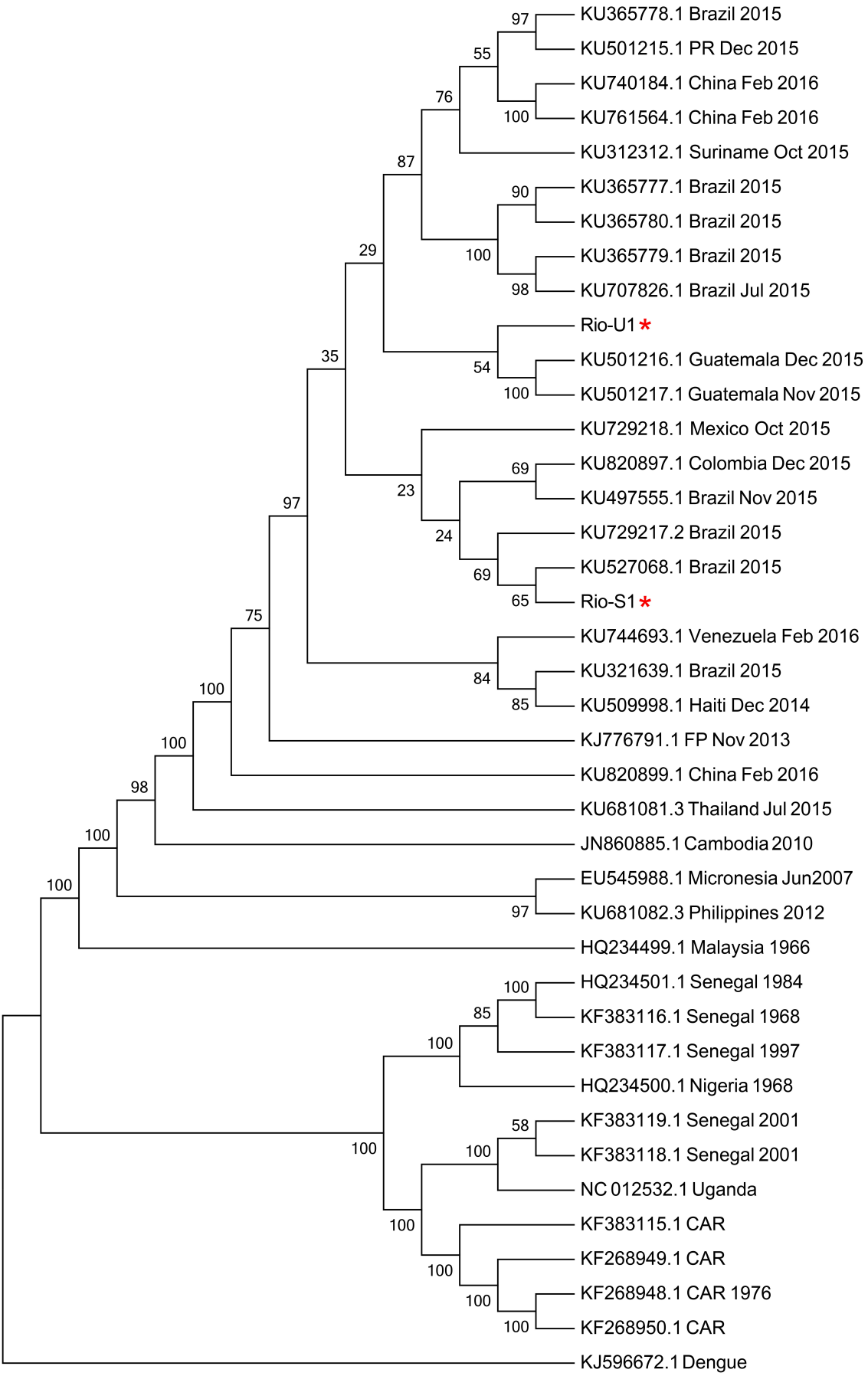
1. Kuno, G., et al., *Phylogeny of the genus Flavivirus.* J Virol, 1998. **72**(1): p. 73-83.
2. Dick, G.W., S.F. Kitchen, and A.J. Haddow, *Zika virus. I. Isolations and serological specificity.* Trans R Soc Trop Med Hyg, 1952. **46**(5): p. 509-20.
3. Hayes, E.B., *Zika virus outside Africa.* Emerg Infect Dis, 2009. **15**(9): p. 1347-50.
4. Duffy, M.R., et al., *Zika virus outbreak on Yap Island, Federated States of Micronesia.* N Engl J Med, 2009. **360**(24): p. 2536-43.
5. Musso, D., E.J. Nilles, and V.M. Cao-Lormeau, *Rapid spread of emerging Zika virus in the Pacific area.* Clin Microbiol Infect, 2014. **20**(10): p. O595-6.
6. Cao-Lormeau, V.M., et al., *Zika virus, French polynesia, South pacific, 2013.* Emerg Infect Dis, 2014. **20**(6): p. 1085-6.
7. CDC. *Zika virus*. 2016.
8. Oehler, E., et al., *Zika virus infection complicated by Guillain-Barre syndrome--case report, French Polynesia, December 2013.* Euro Surveill, 2014. **19**(9).
9. Zanluca, C., et al., *First report of autochthonous transmission of Zika virus in Brazil.* Mem Inst Oswaldo Cruz, 2015. **110**(4): p. 569-72.
10. Campos, G.S., A.C. Bandeira, and S.I. Sardi, *Zika Virus Outbreak, Bahia, Brazil.* Emerg Infect Dis, 2015. **21**(10): p. 1885-6.
11. WHO, *Zika virus infection: global update on epidemiology and potentially associated clinical manifestations.* Wkly Epidemiol Rec, 2016. **91**(7): p. 73-81.
12. Kleber de Oliveira, W., et al., *Increase in Reported Prevalence of Microcephaly in Infants Born to Women Living in Areas with Confirmed Zika Virus Transmission During the First Trimester of Pregnancy - Brazil, 2015.* MMWR Morb Mortal Wkly Rep, 2016. **65**(9): p. 242-7.
13. Saúde, M.d., *INFORME EPIDEMIOLÓGICO Nº 15 – SEMANA EPIDEMIOLÓGICA (SE) 08/2016 (21 A 27/02/2016) MONITORAMENTO DOS CASOS DE MICROCEFALIA NO BRASIL*. 2016: Brasília, Brazil.
14. Mlakar, J., et al., *Zika Virus Associated with Microcephaly.* N Engl J Med, 2016.
15. Calvet, G., et al., *Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study.* Lancet Infect Dis, 2016.
16. Brasil, P., et al., *Zika Virus Infection in Pregnant Women in Rio de Janeiro - Preliminary Report.* N Engl J Med, 2016.
17. Gatherer, D. and A. Kohl, *Zika virus: a previously slow pandemic spreads rapidly through the Americas.* J Gen Virol, 2016. **97**(2): p. 269-73.
18. WHO. *WHO Director-General summarizes the outcome of the Emergency Committee regarding clusters of microcephaly and Guillain-Barré syndrome*. 2016; Available from: http://www.who.int/mediacentre/news/statements/2016/emergency-committee-zika-microcephaly/en/.
19. Diallo, D., et al., *Zika virus emergence in mosquitoes in southeastern Senegal, 2011.* PLoS One, 2014. **9**(10): p. e109442.
20. Faye, O., et al., *Quantitative real-time PCR detection of Zika virus and evaluation with field-caught mosquitoes*. Virol J, 2013. **10**: p. 311.
21. Grard, G., et al., *Zika virus in Gabon (Central Africa)--2007: a new threat from Aedes albopictus?* PLoS Negl Trop Dis, 2014. **8**(2): p. e2681.
22. Chouin-Carneiro, T., et al., *Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika Virus.* PLoS Negl Trop Dis, 2016. **10**(3): p. e0004543.
23. Besnard, M., et al., *Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014.* Euro Surveill, 2014. **19**(13).
24. Musso, D., et al., *Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014.* Euro Surveill, 2014. **19**(14).

25. Musso, D., et al., *Potential sexual transmission of Zika virus.* Emerg Infect Dis, 2015. **21**(2): p. 359-61.
26. Foy, B.D., et al., *Probable non-vector-borne transmission of Zika virus, Colorado, USA.* Emerg Infect Dis, 2011. **17**(5): p. 880-2.
27. Gourinat, A.C., et al., *Detection of Zika virus in urine.* Emerg Infect Dis, 2015. **21**(1): p. 84-6.
28. Kutsuna, S., et al., *Two cases of Zika fever imported from French Polynesia to Japan, December 2013 to January 2014 [corrected].* Euro Surveill, 2014. **19**(4).
29. Dupont-Rouzeyrol, M., et al., *Infectious Zika viral particles in breastmilk.* Lancet, 2016.
30. Musso, D., et al., *Detection of Zika virus in saliva.* J Clin Virol, 2015. **68**: p. 53-5.
31. Lanciotti RS, L.A., Holodniy M, Saavedra S, del Carmen Castillo Signor L., *Phylogeny of Zika virus in Western Hemisphere, 2015 [letter].* Emerg Infect Dis., 2016.
32. Mizuno, Y., et al., *Confirmation of dengue virus infection by detection of dengue virus type 1 genome in urine and saliva but not in plasma.* Trans R Soc Trop Med Hyg, 2007. **101**(7): p. 738-9.
33. Domingo, C., et al., *Detection of yellow fever 17D genome in urine.* J Clin Microbiol, 2011. **49**(2): p. 760-2.
34. Luby, J.P., et al., *Antigenuria in St. Louis encephalitis.* Am J Trop Med Hyg, 1980. **29**(2): p. 265-8.
35. Mathur, A., et al., *Viruria during acute Japanese encephalitis virus infection.* Int J Exp Pathol, 1995. **76**(2): p. 103-9.
36. Tonry, J.H., et al., *West Nile virus detection in urine.* Emerg Infect Dis, 2005. **11**(8): p. 1294-6.
37. Barzon, L., et al., *Isolation of West Nile virus from urine samples of patients with acute infection.* J Clin Microbiol, 2014. **52**(9): p. 3411-3.
38. Roze, B., et al., *Zika virus detection in urine from patients with Guillain-Barre syndrome on Martinique, January 2016.* Euro Surveill, 2016. **21**(9).
39. Stiasny, K. and F.X. Heinz, *Flavivirus membrane fusion.* J Gen Virol, 2006. **87**(Pt 10): p. 2755-66.
40. Heinz, F.X., et al., *Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM.* Virology, 1994. **198**(1): p. 109-17.
41. Corstjens, P.L., W.R. Abrams, and D. Malamud, *Saliva and viral infections.* Periodontol 2000, 2016. **70**(1): p. 93-110.
42. Malamud, D., *Saliva as a diagnostic fluid.* Dent Clin North Am, 2011. **55**(1): p. 159-78.
43. Shugars, D.C., *Endogenous mucosal antiviral factors of the oral cavity.* J Infect Dis, 1999. **179 Suppl 3**: p. S431-5.
44. Sufiawati, I. and S.M. Tugizov, *HIV-associated disruption of tight and adherens junctions of oral epithelial cells facilitates HSV-1 infection and spread.* PLoS One, 2014. **9**(2): p. e88803.
45. Andries, A.C., et al., *Value of Routine Dengue Diagnostic Tests in Urine and Saliva Specimens.* PLoS Negl Trop Dis, 2015. **9**(9): p. e0004100.
46. Anders, K.L., et al., *An evaluation of dried blood spots and oral swabs as alternative specimens for the diagnosis of dengue and screening for past dengue virus exposure.* Am J Trop Med Hyg, 2012. **87**(1): p. 165-70.
47. Marano, G., et al., *Zika virus and the never-ending story of emerging pathogens and Transfusion Medicine.* Blood Transfus, 2016. **14**(2): p. 95-100.
48. Leung, G.H., et al., *Zika Virus Infection in Australia Following a Monkey Bite in Indonesia.* Southeast Asian J Trop Med Public Health, 2015. **46**(3): p. 460-4.


(A)
100 µm
(B)
100 µm
(C)
(D)


bp
1
1I
2
3
4
C
400
200
(A)
5
6
7
8
9
S
U
S
U
I
S
U
S
U
S
U
C
400
200
(B)
1I
CHIKV
DENV
YFV
C
400
200
(C)


Viral RNA copies/mL
10^6
10^5
10^4
10^3
10^2
10^1
10^0
1
6
7
9
Patient
Urine
Saliva
Limit of detection


KU365778.1 Brazil 2015
KU501215.1 PR Dec 2015
KU740184.1 China Feb 2016
KU761564.1 China Feb 2016
KU312312.1 Suriname Oct 2015
KU365777.1 Brazil 2015
KU365780.1 Brazil 2015
KU365779.1 Brazil 2015
KU707826.1 Brazil Jul 2015
Rio-U1*
KU501216.1 Guatemala Dec 2015
KU501217.1 Guatemala Nov 2015
KU729218.1 Mexico Oct 2015
KU820897.1 Colombia Dec 2015
KU497555.1 Brazil Nov 2015
KU729217.2 Brazil 2015
KU527068.1 Brazil 2015
Rio-S1*
KU744693.1 Venezuela Feb 2016
KU321639.1 Brazil 2015
KU509998.1 Haiti Dec 2014
KJ776791.1 FP Nov 2013
KU820899.1 China Feb 2016
KU681081.3 Thailand Jul 2015
JN860885.1 Cambodia 2010
EU545988.1 Micronesia Jun2007
KU681082.3 Philippines 2012
HQ234499.1 Malaysia 1966
HQ234501.1 Senegal 1984
KF383116.1 Senegal 1968
KF383117.1 Senegal 1997
HQ234500.1 Nigeria 1968
KF383119.1 Senegal 2001
KF383118.1 Senegal 2001
NC 012532.1 Uganda
KF383115.1 CAR
KF268949.1 CAR
KF268948.1 CAR 1976
KF268950.1 CAR
KJ596672.1 Dengue