Isolation of infectious Zika virus from saliva and prolonged viral RNA shedding in a traveller returning from the Dominican Republic to Italy, January 2016

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We report the isolation of infectious Zika virus (ZIKV) in cell culture from the saliva of a patient who developed a febrile illness after returning from the Dominican Republic to Italy, in January 2016. The patient had prolonged shedding of viral RNA in saliva and urine, at higher load than in blood, for up to 29 days after symptom onset. Sequencing of ZIKV genome showed relatedness with strains from Latin America.

Case report

A young woman in her 20s was admitted to the Infectious Disease Unit of Venice City Hospital in Italy because of persisting fever (38 °C) associated with arthralgia, myalgia, and macular cutaneous rash, that had developed four days before, upon return from a two-week stay in the Dominican Republic, in January 2016. Clinical examination was remarkable for a mild macular erythematous skin eruption on the arms and the abdomen, and for conjunctival hyperaemia. There was no lymph node, liver or spleen enlargement. The abdominal ultrasound did not reveal pathological findings. Fever disappeared on the second day of hospital stay, and the skin eruption faded away completely after three days. The patient had no underlying diseases or important medical history and was not taking any medication.

None of the household contacts reported suspected symptoms similar to that of the patient.

Laboratory findings

Upon hospital admission, laboratory tests showed blood cell count, haemoglobin, liver and kidney function tests in the normal range. Real-time RT-PCR tests for dengue virus (DENV) [1] and chikungunya virus (CHIKV) [2] were negative, while real-time RT-PCR for Zika virus (ZIKV) [3] was positive in plasma, urine, and saliva, with estimated ZIKV RNA loads of 30 copies/mL; 0.5×10^6 copies/mL; and 3×10^6 copies/mL, respectively; IgM and IgG antibodies against DENV (ELISA, Focus Diagnostics Inc., Cypress, CA), CHIKV (immunofluorescence assay, IFA, IgM and IgG, Euroimmun AG, Luebeck, Germany), and ZIKV (IFA Mosaic Arbovirus 2 IgM and IgG and ELISA Zika virus IgM and IgG; Euroimmun AG) were negative.

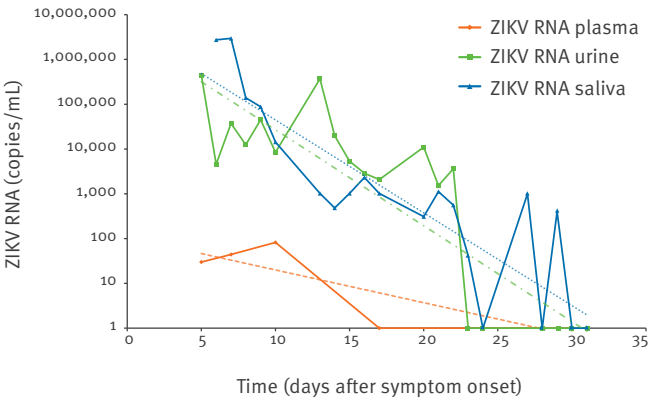
The patient was invited to collect saliva and urine samples daily and to return weekly for follow-up visits and blood sampling. Real-time RT-PCR testing of follow-up blood, urine, and saliva samples demonstrated persistent shedding of ZIKV RNA in saliva and urine for up to 29 days after symptom onset, while viral RNA was detectable in plasma up to day 10 after symptom onset. ZIKV RNA load in saliva and urine was higher than in blood also in follow-up samples (**Figure 1**). Anti-ZIKV IgM and IgG antibodies appeared on days 7 and 10, respectively, as demonstrated by IFA and ELISA.

Viral genome sequencing

Full ZIKV genome sequence was obtained with the Sanger method from nucleic acids purified from saliva and urine specimens collected on day 6 after symptom onset (GenBank KU853012). No nt sequence differences were observed between ZIKV in saliva and urine. Phylogenetic analysis demonstrated that the virus belonged to the Asian lineage and clustered with ZIKV strains from Latin America; it had›99.6% nt identity with ZIKV strains isolated in French Polynesia (2013) and Brazil (2015), 97.9% nt identity with a ZIKV strain isolated in Yap island in 2007, and 88.9% identity with the Uganda MR766 strain isolated in 1947 (**Figure 2**).

Figure 1

Kinetics of ZIKV RNA load measured by quantitative real-time RT-PCR in plasma, urine, and saliva samples of a patient with ZIKV infection, Italy, January 2016



ZIKV: Zika virus.

For real-time RT-PCR analysis, viral RNA was purified from 1 mL of plasma, saliva, or urine samples and eluted in a final volume of 50 µL by using a NucliSENS easyMag automated nucleic acid purification system (bioMérieux, Marcy-l'Étoile, France); 10 µL of purified nucleic acids were used for each real-time RT-PCR reaction, in a final volume of 30 µL. Real-time RT-PCR was performed using the primers and probe set 1086/1162c/1107-FAM developed by Lanciotti et al. [3] and AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher Scientific, Waltham, MA) on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific) for 45 cycles. ZIKV RNA load was estimated against a standard curve obtained by dilution of a plasmid in which the target sequence was cloned.

Viral isolation

Within the diagnostic workup for arboviral infections, viral isolation was attempted from serum, urine, and saliva specimens collected during the first week after symptom onset. In particular, ZIKV was isolated from a saliva sample collected on day 6 after symptom onset. For virus isolation, both Vero and Vero E6 cells were used, following the procedures described for WNV isolation, with slight modifications [4]. Briefly, saliva was diluted 1:3 in serum-free Dulbecco's modified Eagle's medium (DMEM), centrifuged at 1,200 x g for 10 minutes to separate cells from supernatant. Both saliva cells and supernatant were then inoculated into Vero and Vero E6 cells grown at 70% confluence in shell vials. After inoculation, shell vials were centrifuged at 290 x g for 30 minutes and incubated for 60 minutes at 37 °C in 5% CO_2; then, DMEM with 2% fetal bovine serum was added, followed by cell culture at 37 °C in 5% CO_2 for up to seven days. On day 4, a cytopathic effect appeared in all infection conditions, i.e. both Vero and Vero E6 cells infected with saliva cells or with saliva supernatant. Viral replication in cell culture was confirmed by increased ZIKV RNA load in cell supernatant (ca $330x10^6$ copies/mL). The ZIKV isolate was then propagated in Vero cells; a titre of $0.5x10^5$ TCID50 was obtained at the second passage in cell culture. Sequencing of the full ZIKV genome from the first passage of the viral cell culture (GenBank KU853013) identified only a G to A synonymous nt change in position 6971 in comparison with the ZIKV genome that was sequenced directly from urine and saliva specimens (**Figure 2**).

Background

ZIKV is a mosquito-borne flavivirus that generally causes asymptomatic infections in humans and, in an estimated 20% of cases, a mild and self-limited febrile illness associated with rash, arthralgia, and conjunctivitis. The virus, endemic in central and western Africa and in south and south-east Asia, was not considered a relevant human pathogen until outbreaks occurred in Yap, Federal States of Micronesia, in 2007 [5], in French Polynesia in 2013 [6], and in other countries in the Pacific Region in 2013–2014 [7]. In Brazil, the first cases of ZIKV infection were confirmed in March 2015 [8]; since then, the virus has spread exponentially also to other countries in South and Central America and has been estimated to have caused 0.5–1.5 million human infections [9].

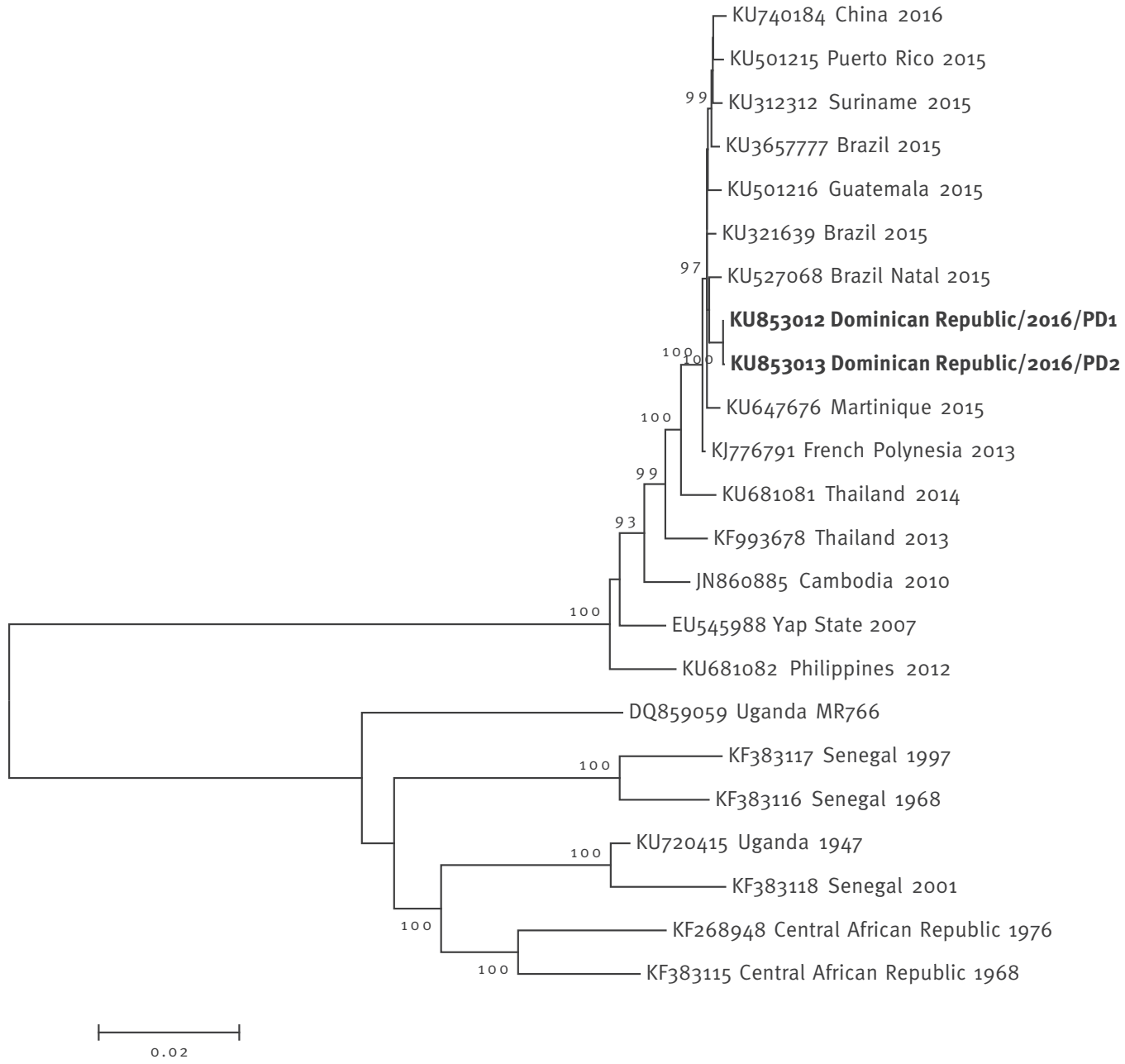
The association of the recent human epidemics of ZIKV infection in French Polynesia and Brazil with an increased incidence of Guillain–Barré syndrome and foetal microcephaly has led the World Health Organization (WHO) to declare a public health emergency of international concern on 1 February 2016 [9]. The aetiological link between foetal microcephaly and ZIKV infection has been recently supported by detection of the virus in the amniotic fluid [10] and in brain tissues of microcephalic foetuses [9,11,12], while the association with Guillain–Barré syndrome has been confirmed by a case–control study in French Polynesia [13].

ZIKV is transmitted between humans through *Aedes* spp. mosquito vectors, mainly the anthropophilic *Ae. aegypti* [14], which is widespread in tropical and subtropical regions in Africa, Asia, and Latin America, and is the main vector also for DENV and CHIKV. The virus has also been detected in *Ae. albopictus* [15], which has been shown to be a competent vector by experimental infection [16]. *Ae. albopictus* is established in Europe, especially in Mediterranean countries, including northern Italy [17], where the case reported in this study was imported. Due to the risk of emergence of outbreaks of vector-borne viruses following the introduction of a viraemic individual in areas where the vector is present [18], an integrated surveillance programme for imported dengue, chikungunya, and Zika virus infections has been implemented in Italy, along with veterinary and entomologic surveillance [17].

Although conceivably rare, non-vector-borne modes of ZIKV transmission may also occur, including trans-placental and perinatal transmission [11,19], blood-transfusion [20], and, potentially, organ donations. Unlike other arboviruses, sexual transmission of ZIKV is also possible and is of particular concern during pregnancy [21]. Actually, ZIKV has been detected and isolated in cell culture from semen samples of patients with infection and cases of probable sexual transmission of ZIKV

Figure 2

Phylogenetic tree of full genome sequences of Zika virus obtained directly from saliva and isolated in cell culture from saliva of a traveller returning from the Dominican Republic to Italy, January 2016



The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [36]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2745)). The analysis involved 23 nt sequences. All positions containing gaps and missing data were eliminated. There were a total of 10,092 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [37].

infection from males to their female partners have been documented [22-24].

Discussion and conclusions

In this report, we described the isolation of infectious ZIKV in cell culture from saliva collected from a patient during acute ZIKV infection. This finding poses questions on the potential risk of human-to-human transmission of the virus through saliva.

In particular, the virus was isolated from saliva collected on day 6 after symptom onset. It is conceivable that viral isolation is more successful from saliva samples characterised by high viral load and collected during the first week after symptom onset, before the appearance of antibodies. However, further analyses in other patients are required to assess the infectivity of ZIKV in saliva.

Shedding of ZIKV RNA in saliva has been reported in the literature. In particular, it has been observed in 48% of patients tested during the first week after symptom onset, i.e. more frequently, although not for a longer time, than in plasma [25]. For this reason, testing ZIKV in saliva by RT-PCR has been recommended as a non-invasive and sensitive method for the direct diagnosis of ZIKV infection during the first week after symptom onset [25]. In the case reported here, ZIKV RNA was present at high titre during the first week after symptom onset and remained detectable for a relatively long period, up to 29 days after onset of symptoms. Viral RNA was also excreted in urine for a long-time, in agreement with previous reports on ZIKV detection in urine for more than 10 days after onset of disease [26,27]. Shedding in saliva and urine has also been demonstrated for other vector-borne flaviviruses, i.e. DENV [28,29] and West Nile virus [30,31], and these samples are used for direct diagnosis based on viral nucleic acid or antigen detection. While isolation of ZIKV in cell culture from urine, semen, and breast milk has been described [22,32,33], to our knowledge, isolation of ZIKV from saliva has not been reported so far. Epidemiological data and experimental studies are needed to assess the potential risk of ZIKV spread and transmission through saliva. Interestingly, a human case of ZIKV infection following a monkey bite has been reported [34]. In addition, CHIKV, a mosquito-borne alphavirus, has been isolated in oral fluids of patients with severe infection and in the saliva of experimentally infected mice and monkeys, and mouse-to-mouse transmission of CHIKV without an arthropod vector was demonstrated [35].

Finally, from the laboratory perspective, the results of this study showed that saliva is a useful sample not only for ZIKV nucleic acids detection, but also for virus isolation.

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The study was approved by the local Ethics Committee and the patient provided written informed consent to participate in the study and for the publication of this case report.

Conflict of interest

None declared.

Authors' contributions

Coordinated the study: LB, MP, GP; managed the patient: PB; performed laboratory investigations: MP, EF, AS, AB, LB; performed bioinformatics analysis: EL; wrote the manuscript: LB, MP, PB, GP.

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