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Isolation and complete genome sequencing of Zika virus imported from the Dominican Republic to Japan

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Summary

Zika virus (ZIKV) infection has been documented within Central and South America, Asia, and Africa. Here we report the isolation of virus from a patient infected with ZIKV returning to Japan from the Dominican Republic. The ZIKV strain was imaged by electron microscopy and its complete genome sequence was analyzed. Phylogenetic analysis and molecular characterization revealed that the strain was of the Asian lineage, and carried two unique mutations in its NS5 region. These mutations are characteristic of strains that originated in the Dominican Republic and the USA in 2016.

Text

Zika virus (ZIKV) belongs to the Flaviviridae family of viruses, and was first isolated in 1947 from a sentinel rhesus monkey in Uganda (1). In 2015, an outbreak of ZIKV was reported in Brazil (2), and in February 2016, the World Health Organization declared ZIKV infections a Public Health Emergency of International Concern (3). Many ZIKV infections are asymptomatic or cause low-grade fever, myalgia, conjunctivitis, ankle edema, and maculopapular rash; however, they can also be associated with Guillain-Barré syndrome (4-6). Additionally, ZIKV infection during pregnancy is linked to fetal death, central nervous system abnormalities, and
microcephaly (6, 7).

A previous study reported the isolation of ZIKV from urine and saliva using a Vero cell line (8). The first ZIKV strain identified in Japan was isolated from a patient returning from Fiji in 2016 (9). After February 2016, all ZIKV-infected patients in Japan were required to be notified by their clinician. All known ZIKV infections in Japan were derived from imported cases. In this study, we report the first ZIKV isolation from a patient returning to Japan from Central and South America, along with analysis of its complete genome.

The patient was a female in her twenties who returned from the Dominican Republic in May 2016. She presented with fever, pharyngitis, arthralgia, and myalgia, and was suspected to have ZIKV infection because of her travel history and obtaining a mosquito bite in the Dominican Republic. Samples of serum, plasma, and urine were collected 3 days after the onset of symptoms, and viral RNA was extracted from these samples using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Real-time reverse transcription PCR (RT-PCR) analysis of the RNA samples was performed using optimized ZIKV-specific primers ZIKV 1086 and ZIKV 1162c, along with probe 1107-FAM (10). Thermal cycler conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by 45 cycles of 94°C for 15 s and 57°C for 60 s. Real-time RT-PCR
analysis showed that all samples were positive for ZIKV, with Ct values of 29.7 (serum), 29.3 (plasma), and 36.1 (urine).

The serum sample was then used for Vero and C6/36 cell inoculations. The serum was first diluted 10-, 50-, 100-, and 500-fold in Eagle’s minimum essential medium containing 2% fetal bovine serum (2% FBS-MEM), and then used to inoculate confluent monolayers of each cell line. Following absorption of the diluted serum samples for 2 h at 34°C (Vero) or 28°C (C6/36), the sample medium was removed and replaced with fresh 2% FBS-MEM. Cells were checked daily under the microscope for the appearance of cytopathic effects (CPE). At 7 days post-inoculation, a blind passage of each infected monolayer was performed, and viral culture was continued for a further 7 days. CPE were observed in both cell lines at all serum dilutions. Viral RNA was extracted from cell culture supernatants for use in real-time RT-PCR assays and for next-generation sequencing (NGS). Two viral RNA samples isolated from the diluted 100-fold serum were positive for ZIKV, with Ct values of 19.0 (Vero) and 14.7 (C6/36). ZIKV sample isolated from the C6/36 cell line stored in phosphate-buffered saline supplemented with 30% sucrose were pelleted by ultracentrifugation at 40,000 rpm for 2 h at 4°C, and resuspended in sterile distilled water. The sample was then applied to collodion membrane grids, stained with 2% phosphotungstic acid (pH 7.0), and viewed
under an HT7700 electron microscope at 80 kV. The ZIKV virion size is ~50 nm (11), and virus particles observed by electron microscopy in the current study were ~50 nm in diameter, and had morphological features characteristic of flaviviruses (Fig. 1).

An RNA sequencing library was constructed from each of the ZIKV-positive RNA samples using a ScriptSeq v2 RNA-Seq Library Preparation Kit, and subjected to 150-mer single-end NGS using the Illumina MiSeq system with the MiSeq reagent kit v3. Complete genome sequences were obtained by de novo assembly using the VirusTAP analysis tool (12), and were subjected to phylogenetic analyses using Molecular Evolutionary Genetics Analysis (MEGA) 6 software, with 1,000 bootstrap replicates (13). The two ZIKV genomes were determined by NGS to have total lengths of 10,482 bp (Vero) and 10,786 bp (C6/36), although the nucleotide sequences of the two genomes were identical. Its complete genome sequence isolated from C6/36 cell line was deposited in GenBank under accession number LC190723 (ZIKV/Hu/Yokohama/1/2016).

A phylogenetic tree based on the entire genome revealed that the strain belonged to the Asian lineage, and clustered with several Central and South American strains (Fig. 2). The strain in our study also showed high similarity to ZIKV sequences derived from the Dominican Republic and the USA, with nucleotide sequence identities to PD2
(GenBank accession no. KU853013), FLUR007 (GenBank accession no. KY325468), and FL039U (GenBank accession no. KX922707) of 99.9% (10,605/10,618), 99.8% (10,721/10,738), and 99.8% (10,591/10,609), respectively. Two unique amino acid mutations in the NS5 region (NS5-322V and NS5-878E) were also detected in these four strains (Table 1), suggesting that the mutations were acquired by the strains in the Dominican Republic or the USA during 2016. The NS5 protein has RNA-dependent RNA polymerase activity in flaviviruses (14), and mutations in this region may be advantageous for ZIKV replication.

In conclusion, this study is the first to demonstrate isolation and complete genome sequencing of a ZIKV strain imported to Japan from Central and South America. Viral particles showing features characteristic of flaviviruses were observed by electron microscopy.

**Ethics Statement**

These analyses have been done as a part of the National Epidemiological Surveillance of Infectious Diseases, Japan (NESID) as stipulated under the Infectious Diseases Control Law. The ethics committee of Yokohama City Institute of Public Health approved this study.
Acknowledgements

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Conflicts of interest

None to declare.

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4.

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<td></td>
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<td>Rio-U1 Brazil 2016</td>
<td>M</td>
<td>H</td>
<td>I</td>
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*Protein position are relative to the MR766 (AY632535)
Figure legends

Fig. 1 Electron microscopy image of Zika virus particles (original magnification, 50,000×). Images are representative of multiple fields of view. Bar represents 50 nm.

Fig. 2 Phylogenetic analysis based on the complete Zika virus (ZIKV) genome. A phylogenetic tree was constructed based on the complete genome sequences of the ZIKV/Hu/Yokohama/1/2016 strain and reference strains using the maximum likelihood method. Only bootstrap values of >70% are shown. Strain labels show the GenBank accession number, the strain, country of origin, and year. The ZIKV strain isolated in this study is indicated by a filled circle.
Fig 2

- LC190723 ZIKV/Hu/Yokohama/1/2016
- KY325468 Zika virus/H. sapiens-wt/USA/2016/FLUR007 USA 2016
- KU853013 Dominican Republic/2016/PD2 Dominican Republic 2016
- KX922707 ZIKV/Homo sapiens/USA/2016/FL039U USA 2016
- KU527068 Natal RGN Brazil 2015
- KX879604 EcEs089 16 Ecuador 2016
- KX280026 Paraiba 01 Brazil 2015
- KU920309 Rio-U1 Brazil 2016
- KX856011 Aedes sp./MEX I-17 Mexico 2016
- KX377337 PRVABC-59 Puerto Rico 2015
- KX247646 Zika virus/Homo sapiens/COL/UF-1/2016 Colombia 2016
- KX702400 Zika virus/Homo sapiens/VEN/UF-1/2016 Venezuela 2016
- KU509998 Haiti/1225/2014 Haiti 2014
- KX447511 1 2015 PF French Polynesia 2014
- KJ776971 H/PF/2013French Polynesia 2013
- KX806557 TS17-2016 Australia 2016
- LC191864 ZIKV/Hu/Chiba/S392016
- KU681061 Zika virus/H. sapiens-t/THA/2014/SV0127-14 Thailand 2014
- KX94532 ZIKV/Homo sapiens/THA/PLCal ZV/2013 Thailand 2013
- KU681082 PHL/2012/CPC-0740 Philippines 2012
- KU955595 Zika virus/A.taylori-tc/SEN/1984/41671-DAK Senegal 1984
- KF383116 ArD7117 Senegal 1968
- KF268948 ARB13565 Central African Republic 1976
- KF383119 ArD158084 Senegal 2001
- AY632535 MR766 Uganda 1947
- NC 029055 Spondweni virus

Asian lineage

African lineage