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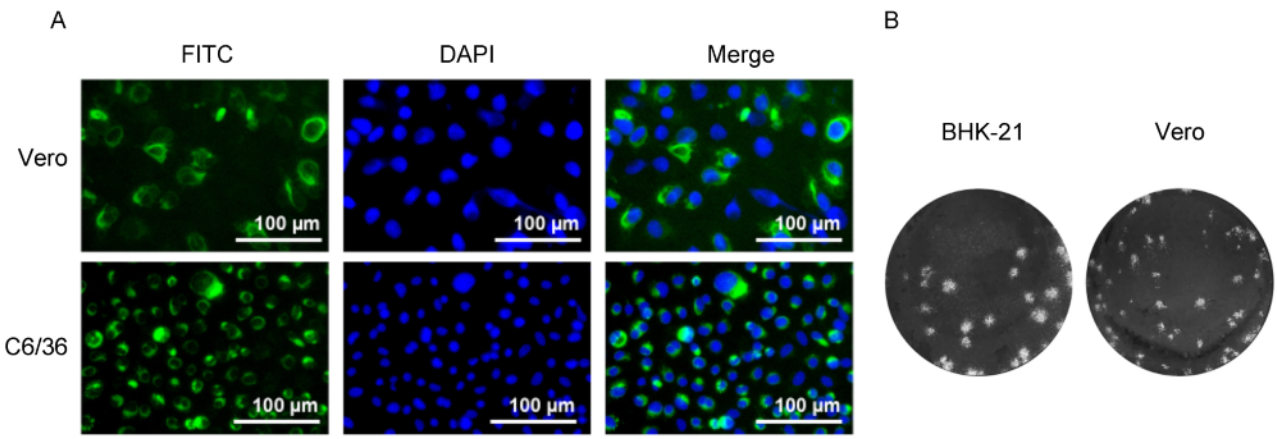
LETTER

Isolation and characterization of Zika virus imported to China using C6/36 mosquito cells

Dear Editor,

Zika virus (ZIKV) is a mosquito-borne flavivirus that usually causes asymptomatic infections or mild illness in humans. However, the unprecedented epidemics of ZIKV in Latin America since early 2015 have made this flavivirus an international health risk (Liu and Zhang, 2016). In particular, the potential association of ZIKV infection with the remarkable increase in the number of fetus microcephaly and Guillain-Barré syndrome cases has attracted worldwide concern (Triunfol, 2016; World Health Organization, 2016). In addition to the endemic and epidemic areas such as central and western Africa, south and southeastern Asia, and Latin America, imported cases of ZIKV infection are also frequently reported in many other countries (Barzon et al., 2016; Hills et al., 2016; Li et al., 2016; Shinohara et al., 2016). According to the Chinese health authorities, 10 imported cases of ZIKV infection in Zhejiang, Guangdong, and Jiangxi provinces were reported between February and March 2016. Because its vector, *Aedes spp*. mosquitoes (Gubler et al., 2007), is widely distributed in China (Kraemer et al., 2015), enhanced surveillance for imported cases of ZIKV is the first step in preventing a ZIKV epidemic in China.

Here, we describe a cell culture-based procedure for isolating the infectious ZIKV (GenBank KU963796) from a human serum sample (ca. 50 μL) with an extremely low viral load (*Ct* value = 32). This serum specimen was collected from a 38-year-old Chinese male patient who had traveled to Fiji and Samoa on 19 February 2016. In addition, the patient was clinically and experimentally confirmed as the third imported ZIKV case in mainland China. Recently, the virus was successfully isolated by intracranially inoculating 2-day-old BALB/c suckling mice using the same serum sample (Deng et al., 2016). In our study, C6/36 mosquito cells were used for virus isolation. The serum was inoculated directly into 35-mm culture dishes containing C6/36 cells at 80% confluence. Then, the infected cells were cultured at 28 °C in 5% CO_2 for up to 5 days. On day 5, the culture supernatant was collected and passaged on naïve C6/36 cells. Serial passages were conducted for three rounds. The viral RNA was extracted at each passage, and RT-PCR



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	Capsid	E	NS1	NS3	NS5
Nucleotide mutations	G433A	A2233G	G3460A	A9538G	A9676G
Specific non-synonymous substitutions	S109N	K419R	R324Q	N624S	K670R

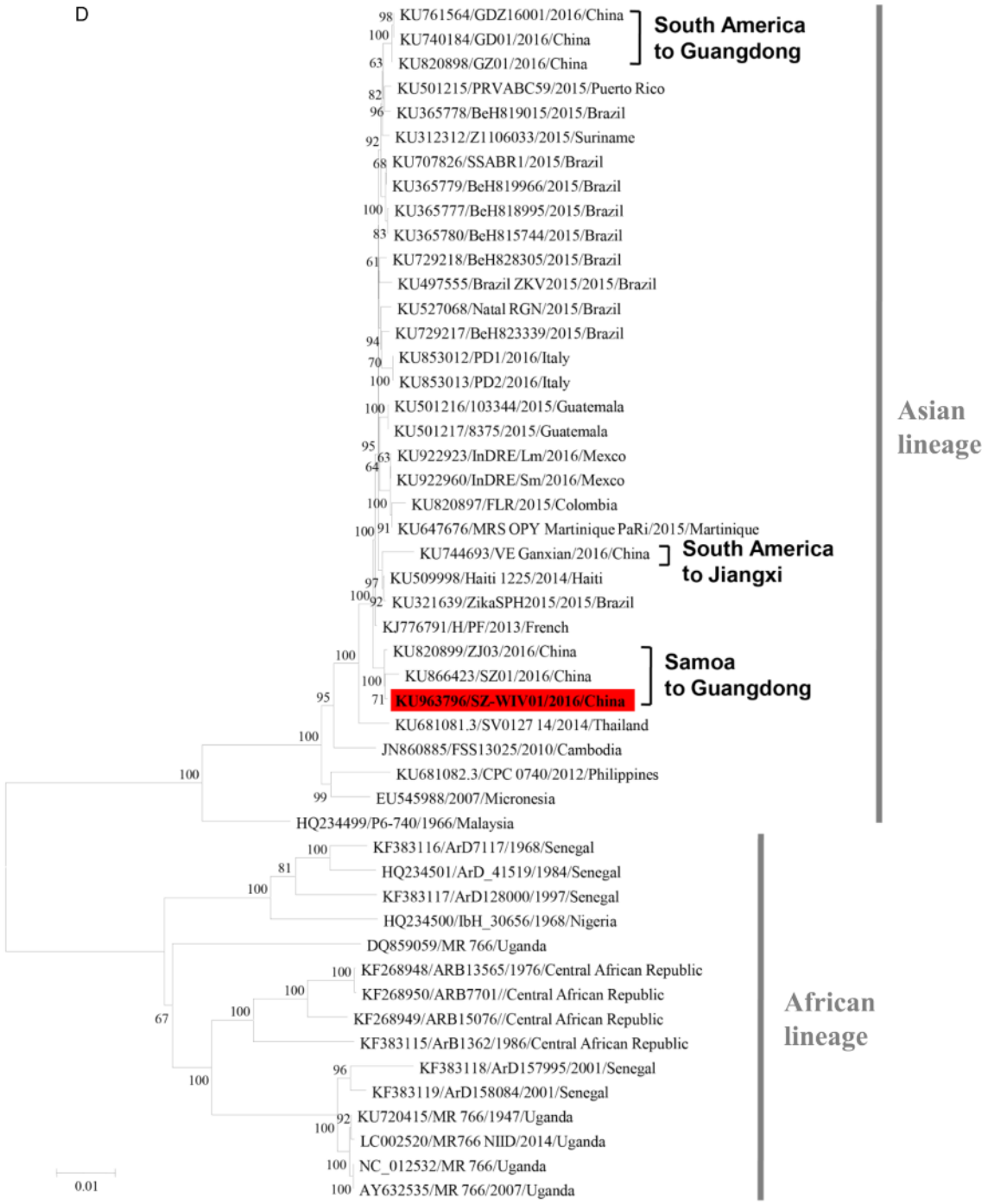

Figure 1. Phenotypic characterization of and specific substitutions in the ZIKV SZ-WIV01 strain. (A) Immuno fluorescence assay (IFA) of Vero and C6/36 cells infected with the third passage of SZ-WIV01 at 24 h post-infection. (B) Plaque morphology in BHK-21 and Vero cells infected with the third passage of SZ-WIV01 at 96 h post-infection. (C) Five specific non-synonymous substitutions in the Chinese imported strains (SZ-WIV01, SZ01, ZJ03) from Samoa. The residues 109N in the capsid, 419R in E, 324Q in NS1, and 624S and 670R in NS5 were different than in other ZIKV strains used in this report. (D) Phylogenetic analyses of ZIKV genome sequences using the neighbor-joining method. The newly isolated SZ-WIV01 strain is highlighted in red. The cluster consisted of the SZ-WIV01, SZ01, and ZJ03 strains named “Samoa to Guangdong”, and other Chinese imported ZIKV strains, including “South America to Guangdong” and “South America to Jiangxi”, are evolving along with different geographic branches within the Asian lineage.

was performed with specific primers (primer-F: GCACCAATTTTAGTGTTGTCAG; primer-R: AGAAACCATGGATTTCCCCACAC) for ZIKV using the OneStep RT-PCR Kit (Takara). The specific DNA bands of the correct sizes were visualized using electrophoresis on 1% agarose gels, and the amount viral RNA increased from P1 to P3 passaged viruses. In order to confirm the isolation of ZIKV, an immunofluorescence assay (IFA) was performed using a home-made mouse monoclonal anti-flavivirus envelope antibody (kindly provided by Prof. Cheng-Feng Qin, Beijing Institute of Microbiology and Epidemiology). IFA-positive cells were observed in both C6/36 and Vero cells infected with P3 passaged virus at 24 h post-infection (Figure 1A). In order to quantify the isolated ZIKV, both real-time RT-PCR and plaque assays were performed for P3 passaged ZIKV. In-house-designed primers specific for ZIKV were used. The genomic RNA copy number in the third passage was significantly higher than that in the first passage (more than 6 *Ct* values decreased), which was consistent with the result of a high virus titer quantified by plaque assay (8×10^7 PFU/mL). However, the plaque morphology of this ZIKV isolate in BHK-21 cells was slightly smaller than that formed in Vero cells (Figure 1B). The successful virus isolation and subsequently efficient amplification indicated that this C6/36 cell culture-based method is appropriate for ZIKV isolation from very low viral load specimens.

To identify any genomic variations between the new ZIKV and circulating strains, genome sequencing of the new isolate from the third C6/36 cell culture passage was then conducted. Alignment of the full genome sequences of our ZIKV isolate (named SZ-WIV01), suckling-mice-isolated ZIKV (SZ01) (Deng et al., 2016), and other strains retrieved from the NCBI database (total of 49 strains, genome length > 10, 250 bp) revealed that at least two non-synonymous substitutions could have emerged during the viral passages in mammals. These two non-synonymous substitutions in the SZ01 strain occurred in the NS3 (R525K, 6, 186 nt) and NS5 (V124I, 8, 036 nt) proteins, while all other Asian strains, including SZ-WIV01, appeared to be unchanged and identical regarding these two residue positions. In addition, we found five specific non-synonymous substitutions in these Chinese strains imported from the South Pacific Samoan islands compared with the remaining ZIKV genomes (Figure 1C). This result indicated that the Asian lineage has genetically diverged in the different epidemic regions.

Based on analyses of the available genome sequences of ZIKV using the neighbor-joining method, the phylogenetic results showed that ZIKV strains can be separated into two distinct lineages: the African and Asian lineages (Figure 1D). The SZ-WIV01, SZ01, and ZJ03 strains, all isolated from individuals who traveled from Pacific Samoa back to China, formed a single cluster belonging to the Asian lineage (Figure 1D, Samoa to Guangdong). Interestingly, the ZIKV strains that were confirmed as imported ZIKV cases in individuals who traveled from South American countries (e.g., Venezuela) to Guangdong (strains GDZ1, GD01, GZ01) and Jiangxi (strain VE_Ganxian) provinces were incorporated into another cluster (Figure 1D, South America to Guangdong and South America to Jiangxi). This result demonstrated that the evolution of ZIKV within the Asian lineage is closely associated with the geographic distribution of the virus.

Here, we successfully isolated infectious ZIKV in a C6/36 cell culture system from the serum of an imported ZIKV case with a very low viral load. This ZIKV isolate has registered in China Centre for General Virus Culture Collection (CCGVCC) (Accession number: IVCAS 6.6110). Genome sequencing and alignment analysis indicated that cell culture with C6/36 cells could reduce the possibility of non-synonymous substitutions in the ZIKV genome during the process of virus isolation. In addition, the findings of this study shed light on the infectious features and genomic variability of ZIKV. The phylogenetic analyses demonstrated that the prevalent Asian lineage is probably undergoing lineage differentiation associated with its geographic distribution. Further studies are needed to investigate the relationship between evolutionary dynamics and epidemiology, and the results of these studies will facilitate the development of antiviral strategies for ZIKV.

FOOTNOTES

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