

Short Report: Chikungunya Virus Isolated from a Returnee to Japan from Sri Lanka: Isolation of Two Sub-Strains with Different Characteristics

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Abstract. A large-scale epidemic of chikungunya (CHIK) fever occurred in several Indian Ocean islands in 2004 and spread to India and Sri Lanka. In December 2006, a returnee to Japan from Sri Lanka developed an acute febrile illness. The patient was confirmed to have CHIK fever after reverse transcription-polymerase chain reaction, and specific IgM and IgG detection. CHIK virus was isolated from the serum specimen collected at the acute stage. The isolated virus developed two different sizes of plaques. Two sub-strains with different genetic and biological characteristics were obtained by plaque purification from one isolate. The entire genome was sequenced and phylogenetic analysis of the E1 genome showed that the sub-strains were of the Central/East African genotype, and were closely related to recent isolates in India. This is the first report of CHIK virus genome sequences isolated from a patient infected in Sri Lanka.

Recent outbreaks of chikungunya (CHIK) fever occurred in coastal Kenya in 2004 and spread to several Indian Ocean islands including Reunion.¹ In Reunion Island, there were ~244,000 cases and at least 213 deaths in the elderly population between March 28, 2005 and April 16, 2006.^{2,3} In India, at least 1.4 million cases were suspected during 2005–2007, after a gap of 32 years.^{4,5} In Sri Lanka, there were > 40,000 suspected cases of CHIK fever between October 2006 and June 2008, after 40 years without any clinical or serologic reports.^{6–10} It has been reported that CHIK virus entered Sri Lanka from India, but there do not seem to be CHIK virus reference sequences from Sri Lanka in any accessible database.¹¹

CHIK fever patients have been reported in travelers returning to the United States, Europe, Australia, Hong Kong, Taiwan, and Japan.^{12–16} One of the main vectors in transmission between humans is *Aedes albopictus*, which is widely distributed in parts of Europe, the United States, and East Asia. This fact raises the concern that the virus could be introduced and become established in these areas.¹⁷ CHIK fever became epidemic for the first time in Italy, which has a temperate climate, with 205 cases occurring between July 4 and September 27, 2007, and *A. albopictus* was considered the vector.¹⁸ In this study, we confirmed CHIK virus infection in a woman returning to Japan from Sri Lanka.

A 59-year-old Japanese woman stayed in Colombo, Sri Lanka, from November 27 to December 3, 2006. She had lassitude on December 4, 2006 and was admitted with high fever (40°C), myalgias, and arthralgias to Nagaoka Red Cross Hospital in Japan on December 5, 2006. She showed generalized erythematous rash and epistaxis. She reported a history of mosquito bites during her 1-week stay in Sri Lanka. The patient's clinical condition gradually improved, and she was discharged on December 12, 2006, although slight lassitude still persisted.

A serum specimen was collected on December 6 (Day 2) and 12 (Day 8), 2006 and November 5, 2007 (Month 11). CHIK virus-specific IgM was negative for the Day 2 serum but positive for the Day 8 serum using IgM capture ELISA (Table 1).

The neutralizing antibody to CHIK virus strain S27 (GenBank accession AF369024) was undetectable in the Day 2 serum but was detected at the titer of 1:20 in the Day 8 serum (Table 2) by 50% plaque reduction neutralizing antibody assay.¹⁹ The Day 2 serum was positive for CHIK virus RNA according to RT-PCR targeted to the *E1* gene, using forward and reverse primers (10294f; 5'-ACGCAATTGAGCGAAGCACAT-3' and 10573r; 5'-AAATTGTCTCTGGTCTTCCTG-3') (Table 1). Direct sequence analysis of the reverse transcription-polymerase chain reaction (RT-PCR) product using an NCBI BLAST search showed that the genome sequence was 99.3% identical with CHIK virus strain IMT/6466 (DQ462746), which was isolated from cerebrospinal fluid collected from a patient with encephalitis in Reunion in 2006.²⁰ Real time RT-PCR analysis, using forward and reverse primers (5'-GCC RCCMTCTKTAACGGACAT-3' and 5'-GCCCCRAAGT CKGAGGAR-3') with a dual fluorophore-labeled probe (5'-FAM-TACCAGCCTGCACYC-MGB-3'), showed that the Day 2 serum contained 1.8×10^9 RNA copies/mL of CHIK virus RNA (Table 1). Dengue virus-specific IgG, IgM, and real-time RT-PCR were all negative (data not shown). Based on clinical symptoms and the laboratory results, the patient was diagnosed with CHIK fever.

To isolate the virus for virus characterization, the serum specimens obtained on Day 2 and Day 8 were inoculated onto Vero cell cultures (American Type Culture Collection, Rockville, MD), and a cytopathic effect (CPE) was detected 4 days after inoculation with the Day 2 serum but not with the Day 8 serum (data not shown). The virus antigen was detected by indirect immunofluorescence only in cells inoculated with the culture supernatant fluid from Day 2 serum-inoculated Vero cells (Table 1). The culture supernatant fluid was passed on Vero cells one more time, and the collected culture supernatant fluid was designated as SL. The virus purified from the SL supernatant through the 30–60% (wt/vol) discontinuous sucrose gradient was analyzed by immuno-electron microscopy. Icosahedral-enveloped particles with a diameter of ~70 nm were labeled with anti-CHIK murine hyper-immune ascetic fluid. The morphology and immunology were compatible with that of CHIK virus, indicating that CHIK virus had been isolated (data not shown).

The isolated virus contained in the culture supernatant SL showed two types of plaques: large (5–6 mm in diameter) and

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TABLE 1

Results of laboratory diagnosis of the chikungunya fever patient serum

Serum	IgM	(P/N ratio)	IF	RT-PCR	Real-time RT-PCR (RNA copies/mL)
Day 2	—	(1.14)	+	+	1.8×10^9
Day 8	+	(3.44)	—	—	—
Month 11	—	(1.88)	ND	ND	ND

P/N ratio = positive to negative ratio (> 2.0 : positive); IF = indirect immunofluorescence staining; ND = not done; — = results are negative; + = results are positive.

small (1 mm) plaques (data not shown). These two variants were plaque-purified, cloned three times, and passaged once on a Vero cell monolayer to prepare a seed virus. The large plaque variant (SL10571, AB455494) and the small plaque variant (SL11131, AB455493) were established and used in the next experiments.

Two variants, SL10571 and SL11131, were inoculated at an m.o.i. of 0.001 PFU/cell onto Vero and C6/36 cells. The time of inoculation was defined as hour 0. Culture supernatant fluids were collected every 3 hours until 51 hours after inoculation. SL10571 and SL11131 were first detected in Vero cell cultures at 6 and 9 hours after inoculation, respectively (Figure 1A, B). The virus titers were consistently higher with SL10571 than with SL11131 during the first 24 hours (Figure 1A). In Vero cells at 51 hours after inoculation, the peak titers were 1.9×10^8 and 1.2×10^9 PFU/mL for SL10571 and SL11131, respectively. In contrast, in C6/36 cell cultures, CPE was not detected within either variant. The peak titers were 1.8×10^9 and 3.8×10^9 PFU/mL for the SL10571 and SL11131, respectively. These results suggest that SL10571 and SL11131 have different characteristics against Vero cells. Host humoral immunoresponse against SL11131 was four times lower compared with SL10571 at Day 8 (Table 2). The entire genes were sequenced, showing that the SL10571 exhibited four unique amino acid substitutions: nsP1-W456R, R501L, nsP2-M703L, and 6K-T58A; the SL11131 exhibited three unique substitutions, nsP1-R171Q, E3-A53V, and E2-G55R, compared with those of the other CHIK virus strains available in the GenBank database (Table 3). The

TABLE 2

Neutralizing antibody titer of patient serum against chikungunya virus strain S27, SL10571, and SL11131

Serum	Neutralizing antibody titer		
	S27	SL10571	SL11131
Day 2	< 10	ND	ND
Day 8	20	160	40
Month 11	5,120	10,240	10,240

S27 = S27 strain (African phenotype); ND = not done.

SL10571, SL11131, and other Indian-06, Italy-07, and Indian Ocean-05-06 isolates exhibited 99.7–99.9% and 99.7–99.9% for non-structural protein and 99.6–99.9% and 99.6–99.9% for structural protein at the nucleotide and amino acid levels, respectively.

A phylogenetic tree was constructed based on nucleotide sequences of the E1 region (Figure 2). Molecular virologic analysis showed that the sub-strains from Sri Lanka belong to the Central/East African cluster and that SL10571 and SL11131 formed a sub-cluster with those isolated in Seychelles 2005, Reunion 2005–2006, Mauritius 2006, India in 2006, Singapore 2006, and Italy in 2007. The two unique amino acids that were reported in all the India-06 and Italy-07 isolates responsible for genetic divergence of Indian viruses were also present in both SL10571, and SL11131: nsP1-M376, and C-S23 (Table 3).⁵ Taken together, the results of the phylogenetic analysis and the amino acid sequence analysis suggest that the CHIK sub-strains in this study are related to the recent epidemic strains.

In CHIK virus isolated in September 2005 after the Reunion outbreak, there was an amino acid change from alanine to valine at Position 226 in the E1 envelope glycoprotein. This may have led to an increased rate of CHIK transmission and an increase in severe non-classic symptoms.^{3,4} However, the codon specifying valine at Position 226 of E1 was absent in both CHIK SL11131 and SL10571 sub-strains (Table 3).

We isolated two CHIK virus sub-strains from a patient who returned to Japan from Sri Lanka. The patient had only visited

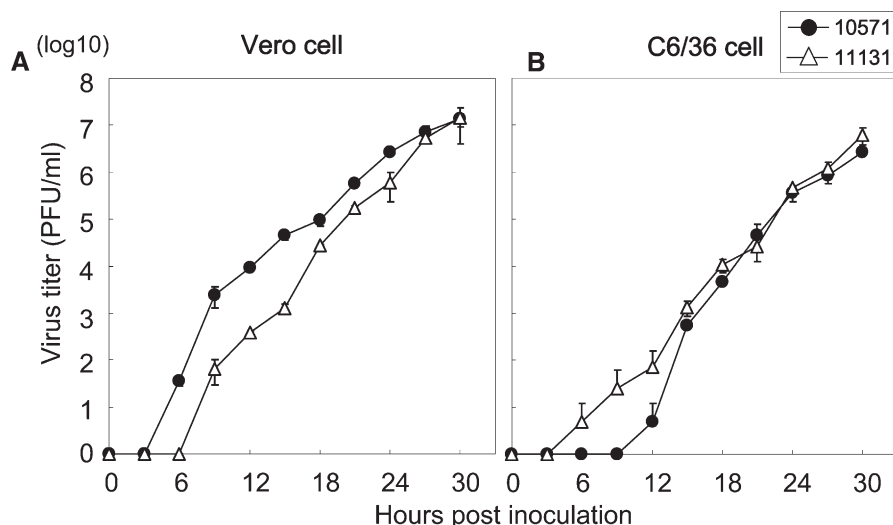


FIGURE 1. Comparison in growth kinetics between two CHIK variants: SL10571 and SL11131. Two variants, SL10571 and SL11131, were inoculated at m.o.i. of 0.001 pfu/cells on Vero (A) and C6/36 cells (B), respectively. Hour 0 was defined as the time of inoculation. Culture supernatant fluids were collected every 3 hours, and the virus titers of the samples were assessed by plaque assay on Vero cells. In Vero cell cultures, SL10571 (●) and SL11131 (Δ) were first detected at 6 and 9 hours after inoculation, respectively (A). The virus titers of SL10571 were consistently higher than those of SL11131 during the 24 hours after inoculation on Vero cells (A) compared with C6/36 cells (B).

TABLE 3
Amino acid substitutions identified in Sri Lanka isolates with respect to India 2006 (IND06AP3) strain

Protein	Non-structural proteins												Structural proteins				
	nsP1						nsP2			nsP3			C	E3	E2	6K	E1
Polypeptide position	128	171	230	314	376	456	488	501	1238	1664	1691	1709	23	27	314	380	471
Protein position	128	171	230	314	376	456	488	501	703	331	358	376	23	27	53	55	146
EF027134 India 2006	K	R	G	M	M	W	R	R	M	A	S	T	S	V	A	G	Q
AB455494 Sri Lanka																	
(Japan) 2006 'SL10571'*	R	Q	L	L	V	.	.	I	.	.	.	A
AB455493 Sri Lanka																	
(Japan) 2006 'SL11131'*	.	Q	Q	.	.	V	.	.	I	V	R	.	.
EF210157 India 2006	.	.	.	L	I
EU244823 Italy 2007	.	.	R	I	I	.	.	.	V
AM258990 Reunion 2005	T	.	.	.	T	P
AM258991 Seychelles 2005	T	.	.	.	T	P	.	P	.	.	R	.
AM258992 Reunion 2006	T	.	.	.	T	P	.	.	.	V
DQ443544 Reunion																	
(France) 2005	T	.	.	.	T	P	.	.	.	V
EF012359 Mauritius																	
(UK) 2006	T	.	.	.	T	P	.	.	.	V
AF369024 Tanzania																	
1953 'S27'	T	.	.	.	T	.	Q	.	.	V	.	I	P

* Isolates from a Japanese patient returning from Sri Lanka.

S27 = strain (African phenotype); a dot indicates a match with the amino acid of the CHIK IND06AP3 strain.

Sri Lanka. A noteworthy characteristic of this CHIK case is a high level of viremia (Table 1). The vector mosquito of this virus, *Ae. albopictus*, infests most regions of Japan. Thus, the potential for the establishment of the imported CHIK virus in Japan is a cause for serious concern.²¹ The viremic patient returned to Japan during winter, when the activity of *Aedes* spp. was low. This probably reduced the risk of a CHIK outbreak in Japan.

In this study, the viremia did not last long, and CHIK virus-neutralizing antibody and IgM were detected 8 days after the onset of symptoms. Furthermore, the levels of CHIK virus-neutralizing antibody were as high as 1:10,240 at 11 months, but

IgM was negative (Tables 1 and 2). It is likely that a high level of viremia induced a rapid and strong host immunoresponse against the CHIK virus. These results are consistent with previous reports that CHIK IgM persisted for several weeks to 3 months and that CHIK virus infection elicited long-lasting protective immunity.²² Physicians should be aware of the presence of CHIK fever among travelers from disease-endemic areas such as Sri Lanka and India, especially during late summer. In addition to the usual tests for dengue virus, genetic and antibody detection tests for CHIK virus should be performed for patients with fever, particularly when they have joint pain.¹⁴ Furthermore, it should be stressed that imported cases

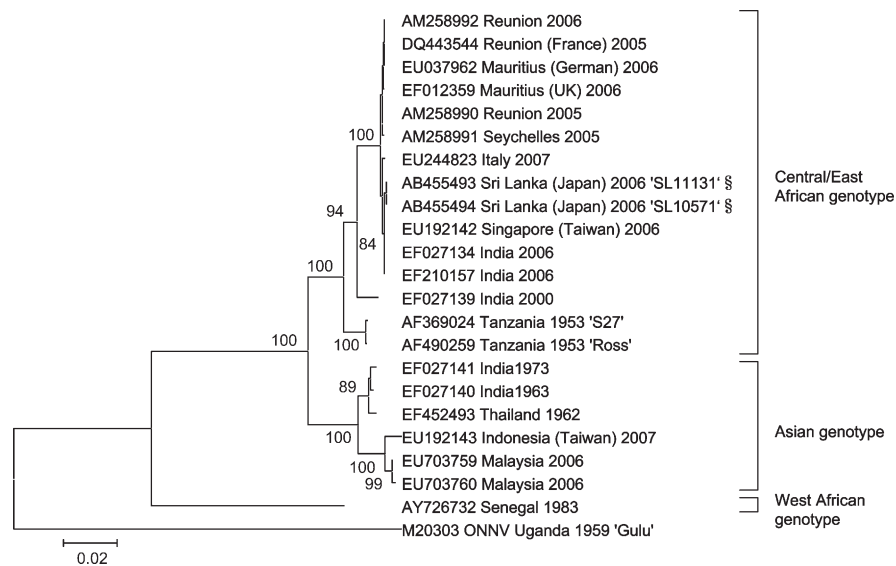


FIGURE 2. Phylogenetic analysis of the chikungunya virus isolates based on the E1 gene. The neighbor-joining tree was constructed using nucleic acid sequences of the envelope glycoprotein E1 gene with those of the other CHIK virus strains available in the GenBank database, including S27, Ross (GenBank accession AF490259), IND06AP3, DRDEHydISW06 (EF210157), ITA07-RA1 (EU244823), 0611aTw (EU19214), 05-115 (AM258990), 05-209 (AM258991), 06-021 (AM258992), Wuerzburg1 (EU037962), LR2006 OPY1 (DQ443544), D570-06 (EF012359), IND73MH5 (EF027141), IND63WB1 (EF027140), AF15561 (EF452493), 0706aTw (EU192143), MY002IMR06BP (EU703759), MY003IMR06BP (EU703760), and 37997 (AY726732) with o'nyong nyong virus (M20303) as the outgroup virus. Sub-strains from the Japanese patient returning from Sri Lanka are indicated with §. Bootstrap values, which are $\geq 75\%$, are indicated and derived from 1,000 samplings. The scale reflects the number of nucleotide substitutions per site along the branches.

may contribute to the occurrence of a CHIK fever epidemic wherever the competent mosquito vectors are distributed.

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