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Primary Isolation and Phylogenetic Studies of Chikungunya Virus from Surabaya, Indonesia

Kris C. Mulyatno[1], Helen Susilowati[1], Atsushi Yamanaka[1,2]*, Soegeng Soegijanto[1], and Eiji Konishi[2,3,4]

[1]*Indonesia-Japan Collaborative Research Center for Emerging and Reemerging Infectious Diseases, Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia;*
[2]*Center for Infectious Diseases, Kobe University Graduate School of Medicine and*
[3]*Department of International Health, Kobe University Graduate School of Health Sciences, Kobe, Japan; and*
[4]***BIKEN Endowed Department of Dengue Vaccine Development, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand*

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Chikungunya virus (CHIKV) is a mosquito-borne virus that causes Chikungunya fever (CHIKF) in humans. Although CHIKF was initially thought to be a mild disease, recent outbreaks in the Indian Ocean region showed that it can be lethal (1,2). The predominant mosquito vector for CHIKV is *Aedes aegypti*, but an amino acid substitution (from alanine to valine) at position 226 in the viral E1 envelope protein increases the susceptibility of Aedes *albopictus* to CHIKV, which may allow the virus to spread to temperate areas currently inhabited by this species (3). More recently, autochthonous transmission of CHIKV was reported in Italy (4) and France (5), raising concerns that CHIKF may no longer be restricted to cases imported from tropical countries, where CHIKF is endemic. In Japan, only imported CHIKF cases have been recorded, and more than a half of these were seen in patients returning from Indonesia.

CHIKV is indigenous in the tropical regions of Africa and Asia. However, reports of CHIKV from Indonesia are limited. Studies conducted in the 1990s in Yogyakarta (6) and Semarang (7) in Central Java showed the presence of CHIKV-specific antibodies in healthy inhabitants and/or viral RNA in patients. However, to our knowledge, only a single phylogenetic study has been conducted using CHIKV isolated from the Kalimantan and Anbian Islands of Northern Indonesia in the 1980s (8). There have been no reports on the isolation of CHIKV in Surabaya, the second-largest city in Indonesia, which is located in East Java. Therefore, we collected sera from recent dengue fever (DF)- or CHIKF-suspected patients and mosquito samples in Surabaya for CHIKV isolation and phylogenetic analysis.

A total of 773 serum samples were collected from patients diagnosed with DF or CHIKF at Dr. Soetomo Hospital in Surabaya from January to July in both 2010 and 2011. Samples were diluted 1:10 with Eagle's minimal essential medium (MEM) supplemented with 10% FBS, 1% L-glutamine, and 60 μg/ml kanamycin (Vero cell medium). For virus isolation, the Vero cell monolayers were inoculated with the serum samples and examined daily for cytopathic effects (CPE). The presence of the virus in CPE-positive culture fluids was confirmed using RT-PCR with CHIKV-specific primers (9), followed by determination of nucleotide sequences. Usage of human subjects in this study was approved by the Ethical Committees of Kobe University Graduate School of Medicine (Ethical Committee Approval Number 784) and Airlangga University (069/PANEC/LPPM/2009).

Of the 773 samples tested, 101 were CPE positive; 17 of these samples were also positive for CHIKV (Table 1). While the samples tested were collected from patients 3–7 days after the fever onset, CHIKV was isolated from patients only 3–5 days after the fever onset. These samples were sequenced to obtain the E1 gene sequences. The sequences of 12 isolates were identical to and representative of that of the CHIK/SBY8/10 isolate, while the sequences of 5 other isolates were identical to that of the CHIK/SBY59/10 isolate (boldface and underlined in Fig. 1). All the Surabaya CHIKV isolates were classified into the Asian group. The nearest phylogenetic neighbors of the Surabaya isolates were the Indonesian strains, which were previously reported as the causative agents for imported cases in Taiwan (10) and France (5). Our phylogenetic tree shows that the most recent common ancestor of the Indonesian strains might have existed approximately 46 years ago, and descendants of this strain are expected to have evolved in different geographical areas.

Isolation of CHIKV from the samples obtained in 2010–2011 prompted us to collect mosquitoes in May 2011. The collection was conducted in 2 houses containing patients with CHIKF-suspected patients and 10 adjacent houses in Surabaya. Adult mosquitoes were collected indoors, identified, and pooled. Pooled samples were homogenized, clarified, and filter-sterilized for virus isolation on C6/36 cells. CHIKV was isolated from

*Corresponding author: Present address: CRC-ERID, Institute of Tropical Disease, Airlangga University, Kampus C, UNAIR, Jl. Mulyorejo, Surabaya 60115, Indonesia. E-mail: paradios99@yahoo.co.jp

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Table 1. Number of CHIKV and DENV isolates in patient sera from Surabaya, 2010–2011

Year	Patients clinically diagnosed as:	No. patient serum samples	No. samples positive for CPE	No. isolates	
				CHIKV	DENV[1)]
2010	DF	596	54	10	44
	CHIKF	16	10	5	5
2011	DF	161	37	2	35
Total		773	101	17	84

1): DENV was exclusively dengue type 1 virus.

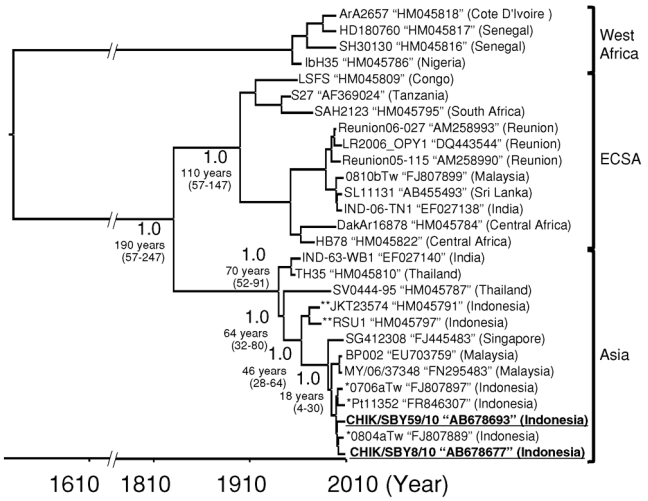

Fig. 1. Maximum clade credibility (MCC) tree of the E1 coding region of Chikungunya virus (CHIKV). The year of divergence for each clade was estimated using a relaxed molecular clock approach and the Bayesian Markov Chain Monte Carlo (MCMC) method available in the Bayesian Evolutionary Analysis by Sampling Trees (BEAST) software package v1.5.3. Horizontal branches are drawn to scale for the estimated year of divergence with tip times reflecting sampling date (year). The coalescent times of some key nodes, as well as their 95% Highest Posterior Density (HPD) values are shown. Posterior probability values of 1.0 are shown above nodes. The GenBank accession numbers are represented by quotation marks, followed by the country in which the strains were isolated (in parentheses). Single asterisks indicate Indonesian strains reported in the literature as cases imported from other countries. Double asterisks indicate an original isolate from Indonesia. Genotypes are shown in the right side: ECSA denotes the East, Central and South African group.

2 of the 5 pools of *Ae. aegypti* adult females with a minimum infection rate (MIR) of 38.5 (Table 2). Both the isolates had nucleotide sequences identical to those of CHIK/SBY8/10, which was isolated in 12 of the 17 patients, thus confirming CHIK/SBY8/10 circulation in Surabaya. In addition to adult mosquitoes, larvae were also collected to investigate the potential transovarial transmission of CHIKV in nature. However, although these larvae were reared to adulthood, the virus isolation was unsuccessful.

In this study, we also attempted to isolate dengue viruses (DENVs) from patient sera. DENVs belong to the genus *Flavivirus* and cause DF in humans, but are also transmitted by the *Aedes* mosquito species that can transmit CHIKV. Since CHIKF and DF show similar clinical symptoms, differential diagnosis between these diseases is usually problematic (11). Therefore, we determined the number of CHIKV infections in the patient population that was clinically diagnosed as having DF (Table 1). In the 757 samples, 12 and 79 of the isolates were determined to be CHIKV and DENV, respectively. This result suggests that DF-suspected patients include a relatively large number of patients with CHIKF. In contrast, in the 16 CHIKF-diagnosed samples, 5 isolates were CHIKV and 5 others were DENV. This also suggests that CHIKF-suspected cases include a relatively large number of DF cases. This is consistent with a previous study in Central Java, which showed that 118 cases of suspected DF included 2 cases of serologically confirmed CHIKF and 58 cases of serologically confirmed DF (7). According to statistics reported by the Surabaya Health Office, the annual number of CHIKF cases was less than 10 in the last 3 years (2008–2010), while the annual number of DF cases was more than 2,000, suggesting that the official number of CHIKF patients reported in Surabaya was underestimated.

Table 2. Number of CHIKV isolates obtained from mosquito samples from Surabaya, 2011

Aedes spp.	Mosquito stage collected	Sex	No. mosquitoes collected	No. pools[3)]	No. pools positive for CPE	No. CHIKV isolates	MIR[4)]
Ae. aegypti	Adult[1)]	Male	32	3	0	—	—
	Adult[1)]	Female	52	5	2	2	38.5
	Larvae[2)]	Male	210	11	0	—	—
	Larvae[2)]	Female	186	9	0	—	—
Ae. albopictus	Adult[1)]	Female	3	1	0	—	—

1): Collected by a sweep net.
2): Reared to adulthood for virus isolation.
3): 10 individuals/pool of adults collected and 20 individuals/pool of adults emerged from collected larvae.
4): Minimum infection rate calculated using: (number of positive pools for CHIKV/total number of mosquitoes tested) × 1,000.

In conclusion, the results of the present study show that CHIKV circulating in Surabaya belongs to the Asian group; it is distinct from viruses of the East, Central, and South African groups, which includes the pathogenic strain (amino acid substitution at position 226 in the E1 protein) (Fig. 1). Surveillance efforts are important to detect mutant strains of CHIKV. Our results also suggest that both CHIKF and DF are likely to be misdiagnosed at a relatively high frequency, confirming the difficulty that clinicians face while performing differential diagnosis on the basis of clinical symptoms alone. Use of viral RNA detection methods, such as RT-PCR, is considered to be critical for providing an accurate diagnosis of CHIKF in geographical areas where CHIKV and DENV coexist.

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Conflict of interest None to declare.

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