



Short communication

Chikungunya virus of Asian and Central/East African genotypes in Malaysia

I-Ching Sam^{a,*}, Yoke Fun Chan^a, Shie Yien Chan^a, Shih Keng Loong^a, Hock Khim Chin^a,
Poh Sim Hooi^a, Rajasekaram Ganeswrie^b, Sazaly AbuBakar^a

^a Tropical Infectious Diseases Research and Education Centre, Department of Medical Microbiology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur, Malaysia

^b Department of Pathology, Hospital Sultan Aminah, 80100 Johor Bahru, Malaysia

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ABSTRACT

Background: Chikungunya virus (CHIKV) of the Central/East African genotype has caused large outbreaks worldwide in recent years. In Malaysia, limited CHIKV outbreaks of the endemic Asian and imported Central/East African genotypes were reported in 1998 and 2006. Since April 2008, an unprecedented nationwide outbreak has affected Malaysia.

Objective: To study the molecular epidemiology of the current Malaysian CHIKV outbreak, and to evaluate cross-neutralisation activity of serum from infected patients against isolates of Asian and Central/East African genotypes.

Study design: Serum samples were collected from 83 patients presenting in 2008, and tested with PCR for the E1 gene, virus isolation, and for IgM. Phylogenetic analysis was performed on partial E1 gene sequences of 837 bp length. Convalescent serum from the current outbreak and Bagan Panchor outbreak (Asian genotype, 2006) were tested for cross-neutralising activity against representative strains from each outbreak.

Results: CHIKV was confirmed in 34 patients (41.0%). The current outbreak strain has the A226V mutation in the E1 structural protein, and grouped with Central/East African isolates from recent global outbreaks. Serum cross-neutralisation activity against both Central/East African and Asian genotypes was observed at titres from 40 to 1280.

Conclusions: The CHIKV strain causing the largest Malaysian outbreak is of the Central/East African genotype. The presence of the A226V mutation, which enhances transmissibility of CHIKV by *Aedes albopictus*, may explain the extensive spread especially in rural areas. Serum cross-neutralisation of different genotypes may aid potential vaccines and limit the effect of future outbreaks.

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1. Background

Chikungunya virus (CHIKV) causes epidemic fever and arthralgia, and is transmitted by the mosquitoes *Aedes aegypti* and *Ae. albopictus*. There are 3 genotypes, West African, Central/East African, and Asian, initially named according to the regions where the strains were reported.¹ However, since 2005, CHIKV of the Central/East African genotype has caused large outbreaks in several countries worldwide and beyond the African continent, notably in the Indian Ocean and India.²

Malaysia, in Southeast Asia, has a population of approximately 27 million. Low-level CHIKV seroprevalence was noted in the 1960s,³ although the first 2 confirmed CHIKV outbreaks occurred only in the last decade, in Klang (Selangor state, 1998)⁴ and Bagan Panchor (Perak state, March 2006).⁵ Both were caused by the

endemic Asian genotype; of note, the second outbreak occurred during the global outbreaks of the Central/East African genotype. A third outbreak, in Ipoh (Perak, December 2006), was caused by a Central/East African strain imported from India.⁶ These 3 outbreaks were each restricted to a single area, affecting about 300 people in total. However, since April 2008, Malaysia has been experiencing a nationwide outbreak. Initial outbreaks in Johor state have spread to 14 out of 15 states and federal territories, involving at least 7100 people to date.⁷

2. Objectives

To study the molecular epidemiology and neutralising immune responses of this latest outbreak, the most extensive yet in Malaysia.

3. Study design

The study was carried out from April to December 2008 at the University Malaya Medical Centre in Kuala Lumpur, Malaysia.

Abbreviations: CHIKV, Chikungunya virus.

* Corresponding author. Tel.: +60 3 79492184; fax: +60 3 79675753.

E-mail address: jicsam@um.edu.my (I.-C. Sam).

Serum samples were collected from 83 inpatients and outpatients with symptoms of suspected CHIKV infection, such as fever, rash, and arthralgia. Clinical diagnoses and the decision to test for CHIKV were made at the discretion of the attending physicians. The choice of diagnostic tests was based on the timing of the sample with respect to the onset of disease.² Acute samples taken from day 3 onwards were tested by indirect immunofluorescence for IgM.⁴ If negative for CHIKV IgM, acute samples were cultured in Vero cells, and convalescent samples were requested for repeat IgM testing. Samples taken 6 days or less from onset of illness were tested by RT-PCR to detect a 257 bp fragment of the E1 gene,⁸ and virus isolation. Some PCR-positive samples and isolates were selected to span the 8-month period and different geographical sources. For these selected samples, published primers 10145F and 11158R were used to amplify and sequence a 1013 bp fragment of the E1 gene.⁹ Two representative CHIKV isolates of the Asian genotype from the Bagan Panchor outbreak, MY/06/37348 and MY/06/37350 were also sequenced.

The longer E1 sequences were used for phylogenetic analysis. Consensus sequences were generated using Geneious Pro version 4.0.4 (Biomatters Ltd., Auckland, New Zealand), and aligned against other CHIKV sequences retrieved from GenBank using ClustalX version 2.09.¹⁰ A phylogenetic tree was constructed using the neighbour-joining method and Kimura two-parameter distance model. The tree was rooted with O'nyong-nyong virus strain SG650 and displayed using MEGA version 4.1.¹¹ The strength of the phylogenetic tree was estimated by bootstrap analyses using 1000 random samplings.

Convalescent serum from 4 patients from each of the Bagan Panchor and current outbreaks were also assayed for cross-neutralisation activity against representative isolates from each outbreak. The Bagan Panchor serum samples were obtained from patients 1 year after the outbreak in 2006. For the current outbreak, convalescent serum was collected between 3 and 7 weeks after onset of symptoms. The isolates used were MY/06/37348 from Bagan Panchor, and MY/08/065 from Johor. A previously described neutralisation assay,¹² with minor modifications, was carried out in duplicate using an initial serum dilution of 1:10. The virus-serum mixtures were inoculated on 70%-confluent Vero cells and monitored for cytopathic effect for 7 days. The neutralizing titer was taken as the highest dilution of serum that inhibited virus growth.

4. Results

Of 83 patients tested, 34 (41.0%) had laboratory confirmation of CHIKV infection by detectable IgM (22 patients), PCR (18), virus isolation (16), or a combination of these. IgM was only detectable at least 5 days after symptom onset, while PCR and culture were positive at 5 days or less.

For phylogenetic analysis, longer E1 sequences were obtained for 11 patients infected at 8 different locations and time points throughout the outbreak, spanning 8 months in 2008. A phylogenetic tree was drawn using the partial E1 region of 837 bp, at nucleotide positions 10,264–11,100, numbered according to the CHIKV prototype S27 (Fig. 1). This confirmed that CHIKV isolates formed 3 distinct genotypes, West African, Central/East African, and Asian. The 11 isolates from the current Malaysian outbreak grouped together with recent Central/East African isolates from 2005 to 2008, sharing 98–99% nucleotide identity. All 11 Malaysian isolates had alanine replaced by valine at position 226 (A226V) of the E1 structural protein. There were also 2 nucleotide substitutions not seen in other Central/East African isolates, C300T and G939T, seen in 7/11 and 4/11 isolates, respectively (Table 1). Isolates MY/06/37348 and MY/06/37350 from the Bagan Panchor outbreak in 2006 were confirmed as the Asian genotype, and did

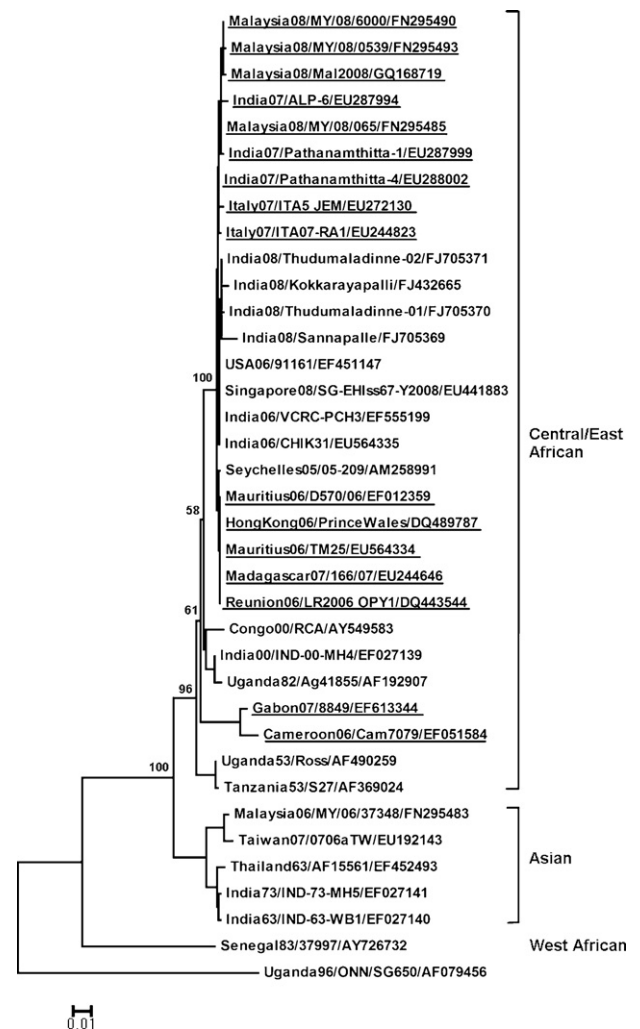


Fig. 1. Phylogenetic analysis of 837 bp partial E1 CHIKV sequences. Each CHIKV isolate is represented by the country of origin, year of isolation, isolate name, and accession number. Four representative isolates from this study (MY/06/37348, MY/08/6000, MY/08/0539, and MY/08/065) are included. The 13 CHIKV sequences from this study have been deposited in EMBL under accession numbers FN295483 to FN295495. Isolates with the A226V mutation are underlined. O'nyong-nyong virus strain SG650 was used as the outgroup.

Table 1

Nucleotide differences in E1 between CHIKV isolates of the current Malaysian outbreak compared to other CHIKV isolates.

CHIKV isolate (accession number)	Nucleotide position in E1			
	300	363	677	939
MY/08/065 ^a (FN295485)	C	G	T	G
MY/08/066 ^a (FN295486)	C	G	T	G
MY/08/068 ^a (FN295487)	C	G	T	G
MY/08/5369 ^a (FN295491)	C	G	T	G
MY/08/6000 ^a (FN295490)	T	G	T	G
MY/08/7913 ^a (FN295492)	T	G	T	G
MY/08/6008 ^a (FN295495)	T	G	T	G
MY/08/2868 ^a (FN295488)	T	G	T	T
MY/08/2844 ^a (FN295489)	T	G	T	T
MY/08/2561 ^a (FN295494)	T	G	T	T
MY/08/0539 ^a (FN295493)	T	G	T	T
Other Central/East African genotype	C	G/A	T/C	G
Bagan Panchor, Malaysia (Asian genotype)	T	A	C	G

^a Isolates from the current Malaysian outbreak, all from the Central/East African genotype.

Table 2

Neutralisation antibody titres for patient sera against CHIKV from two Malaysian outbreaks, Bagan Panchor (Asian genotype, 2006) and current outbreak (Central/East African genotype, 2008).

Serum	CHIKV isolate	
	MY/06/37348, Bagan Panchor (Asian genotype)	MY/08/065, current outbreak (Central/East African genotype)
Anti-Asian genotype (Bagan Panchor)		
Patient 1	640	640
Patient 2	1280	1280
Patient 3	40	40
Patient 4	320	160
Anti-Central/East African genotype (current outbreak)		
Patient 1	1280	640
Patient 2	40	80
Patient 3	40	80
Patient 4	640	640
Non-CHIKV		
Patient 1	<10	<10
Patient 2	<10	<10

not possess the A226V mutation. Malaysian isolates of the Asian and Central/East African genotypes shared 94% and 96% nucleotide and amino acid identities, respectively.

The cross-neutralisation assays, performed to determine the role of genetic variation in antigenicity, showed that serum from CHIKV-infected patients from both outbreaks neutralised CHIKV from both Asian and Central/East African genotypes. The titres obtained ranged from 40 to 1280. Cross-neutralisation activity was observed, as heterologous neutralising antibody titres were within 1 dilution of homologous antibody titres (Table 2).

5. Discussion

Both Asian and Central/East African genotypes are now present in Malaysia, a situation similar to India.^{13,14} With increased international travel, genotypes are no longer restricted to the regions implied by their names. It remains to be seen if the Central/East African genotype replaces, or becomes established alongside the endemic Asian genotype in Malaysia.

The source of the current outbreak is unknown. The ongoing outbreak in India may be the source, as there is considerable traffic between the 2 countries, and previous infections of Central/East African genotype in Malaysia originated from India.^{6,15} Another possibility is spread from neighbouring Singapore, where an outbreak occurred 3 months earlier, but the Singaporean isolates did not possess the A226V substitution.¹⁶ Alternatively, the current outbreak may have arisen from the strain causing the 2006 Ipoh outbreak⁶ although this seems less likely as no CHIKV activity was reported in the intervening 16 months between outbreaks. The presence of 2 unique nucleotide differences in the current Malaysian strains compared to other Central/East African strains also suggests local microevolution.

It is unclear why the first 3 Malaysian outbreaks were limited, while the current outbreak has spread nationwide. The A226V mutation has not been described in the Asian genotype, which caused the first 2 Malaysian outbreaks. The third outbreak was caused by a Central/East African strain from India in 2006, for which E1 sequences are not available. However, the A226V mutation was not seen in Indian CHIKV isolates prior to May 2007.¹³ Thus, the A226V mutation probably occurred for the first time in Malaysia during the current outbreak.

The A226V mutation enhances infectivity and dissemination of CHIKV in *Ae. albopictus*.¹⁷ This selectively advantageous mutation has occurred independently on 3 occasions, in Reunion,⁹ India,¹⁴ and Cameroon/Gabon, an example of evolutionary convergence.¹⁸

The current Malaysian outbreak mainly involves rural areas, with numerous rubber and palm oil plantations, where *Ae. albopictus* predominates over *Ae. aegypti*.^{19,20} Therefore we suggest that in this setting, the current strain was able to cause an outbreak of unprecedented scale in Malaysia.

We also showed that serum from CHIKV-infected patients cross-neutralises CHIKV isolates from Central/East African and Asian genotypes. In an earlier study, sera from CHIKV-vaccinated mice and monkeys cross-neutralised different CHIKV strains from Africa, Asia, and India.²¹ Cross-neutralisation may limit the impact of a new CHIKV genotype being introduced into a population already immunised by natural infection or vaccination. Studies of potential vaccines^{22,23} should confirm this by testing efficacy against all CHIKV genotypes.

In conclusion, the largest ever reported outbreak of CHIKV in Malaysia is caused by an Central/East African strain with the A226V mutation in the E1 protein. Serum cross-neutralisation against Central/East African and Asian genotypes was shown, which has positive implications for vaccine development and future outbreaks.

Conflict of interest

None declared.

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