Outbreak of Chikungunya Due to Virus of Central/East African Genotype in Malaysia

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SUMMARY

Chikungunya is an acute febrile illness caused by an alphavirus which is transmitted by infective *Aedes* mosquitoes. Two previous outbreaks of chikungunya in Malaysia were due to chikungunya virus of Asian genotype. The present outbreak involved two adjoining areas in the suburb of Ipoh city within the Kinta district of Perak, a state in the northern part of Peninsular Malaysia. Thirty seven residents in the main outbreak area and two patients in the secondary area were laboratory confirmed to be infected with the virus. The index case was a 44-year Indian man who visited Paramakudi, Tamil Naidu, India on 21st November 2006 and returned home on 30th of November 2006, and subsequently developed high fever and joint pain on the 3rd of December 2006. A number of chikungunya virus isolates were isolated from both patients and *Aedes albopictus* mosquitoes in the affected areas. Molecular study showed that the chikungunya virus causing the Kinta outbreak was of the Central/East African genotype which occurred for the first time in Malaysia.

KEY WORDS:
Outbreak, chikungunya virus, Central/East African genotype

INTRODUCTION

Chikungunya (CHIK) is an acute febrile illness caused by an arbovirus which is transmitted to human by the *Aedes mosquitoes*. It resembles dengue and is reported mainly in tropical and subtropical Africa and Asia, including India, Sri Lanka, Myanmar, Thailand, Indonesia, the Philippines and Malaysia. It occurs principally during the rainy season with high vector density. Chikungunya outbreaks typically result in large number of cases but deaths are rarely encountered[1].

Human infection is acquired by the bite of infected Aedes mosquitoes, which are day biters and epidemics are sustained by human-mosquito-human transmission[2-4]. These mosquitoes usually breed in collections of clean water in containers, tanks, disposables, junk materials present in domestic and peri-domestic environment. After an incubation period of 4 to 12 days, there is a sudden onset of flu-like symptoms including fever, chills, headache, nausea, vomiting, severe joint pain (arthralgia) and rash. The rash may appear at the onset or a few days into the illness; its development often coincides with defervescence, which takes place around the second or third day of the disease. The rash is most intense on the trunk and proximal part of limbs and may desquamate. Arthralgia which may proceed to frank arthritis usually affect joints of extremities and lower-back. The small joints of the extremities in particular become swollen and tender. Hemorrhage is rare and all but a few patients recover within 3 to 5 days. A full blown disease is most common among adults, in which the clinical picture may be dramatic. Chikungunya infection may be more severe in the extreme of age (elderly and young children especially newborns) and in those who are immunocompromised[1].

So far, Malaysia had experienced two CHIK outbreaks; one was in 1998/1999 (Klang) and the other was in early 2006 (Bagan Panchor, Taiping). In both of these outbreaks, CHIK viruses of Asian genotype were isolated. We report here an outbreak of similar illness due to CHIK virus of Central/East African genotype occurring for the first time in Malaysia.

MATERIALS AND METHODS

Epidemiological Investigation:

On 12th December 2006, a health officer of Kinta district received an unofficial information regarding the occurrence of fever and joint pains affecting some residents staying along Jalan Bendahara in the suburb of Ipoh city. Field investigation was carried out the following day and blood samples were collected from symptomatic residents. As soon as the CHIK virus infection was laboratory-confirmed, a house to house investigation was carried out in the outbreak areas in search of all suspected cases of CHIK virus infection. A suspected case was based on the working case definition, that is, a history of fever with and without any of the following symptoms: rash, joint pain or swelling in the last one month prior to the occurrence of the first confirmed case. Venous blood samples (5ml per case) were taken from all suspected cases and sent to the National Public Health Laboratory (NPHL) for laboratory confirmation.

A "search and destroy" surveillance for *Aedes* mosquito breeding sites in the affected areas was simultaneously carried out. Prior to the institution of chemical thermal fogging, adult mosquitoes were also collected by net catching and trapping for entomological identification and subsequently sent to the NPHL for virus study.

Laboratory Investigation:

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For laboratory confirmation of CHIK infection, standard method of virus isolation in C6/36 cells (ATCC CRL-1660) and Vero cells (ATCC CCL-81), and molecular detection of CHIK virus genome by RT-PCR based on the published method by Hasebe *et al*.,[5] were carried on serum samples collected from patients and suspected cases with illness of four days or less at the time of blood collection. Only serological assay for anti-CHIK virus specific IgM (1:10 dilution) and IgG (1:20 dilution) was carried out on serum samples collected from patients and suspected cases with illness of more than seven days at the time of sample collection. All the above tests were performed on serum samples collected between 5 to 7 days.

All the mosquitoes in each container were pooled together and processed for virus isolation in C6/36 cells (ATCC CRL-1660) and Vero cells (ATCC CCL-81), and molecular detection of CHIK virus genome by RT-PCR.

Qualitative serological assay for anti-CHIK virus specific IgM and IgG was performed by indirect immunofluorescence test using in-house prepared Vero cells infected with known CHIK virus that were seeded on wells of Teflon coated slide. The procedure for carrying the tests was as described previously except in this assay, the CHIK virus infected cells was used as antigen[6].

For molecular detection and characterization of CHIK virus nucleic acids, CHIK virus RNA was extracted from 200μl of patient's serum sample or processed mosquito sample in viral transport medium using a viral RNA extraction kit (Roche Diagnostics, Germany). CHIK virus specific oligonucleotide primers was used for the amplification of CHIK virus genomic sequence fragments in accordance to Hasebe *et al*[5]. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in a single reaction tube using the Access RT-PCR Kit (Promega Corporation, USA). Each genomic fragment was amplified in a 50-μl reaction mix containing the respective CHIK virus forward and reverse primers of 20 pmol each and 2 μl of the extracted viral RNA as template. Each reaction mix was subjected to a 60-minute of reverse transcription at 42^{0}C, reverse transcriptase inactivation of 98^{0}C for 5 minutes, followed by 35 cycles of amplification at a denaturing temperature of 95^{0}C for 30 seconds, annealing temperature of 54^{0}C for 30 seconds and an extension temperature of 72^{0}C for 30 seconds per cycle. The amplified products were confirmed by electrophoresing 3 μl of each of the amplified products in a 1% agarose gel. The desired RT-PCR products were gel purified and extracted using QIAquick Gel Purification Kit (Qiagen, Germany), sequenced by ABI Prism Big-Dye (Pharmacia, USA) dideoxyl termination cycle sequencing using respective forward and reverse primers, and then analysed on an ABI 377 automatic sequencer (Applied Biosystems, USA). The sequence data derived from the PCR amplified fragments were routinely managed using the Clone Manager 5 and Align Plus 4 program package (S&E Software, USA). Multiple sequence alignments were done using standard linear scoring matrix with the following parameter settings: mismatch penalty of 1, open gap penalty of 15, extended gap penalty of 6 and similarity significance value cut-off of 60%. Multiple sequence alignments and neighbour-joining phylogram were generated using the Clustal X programme. Phylogenetic analysis was conducted using the MEGA version 3 programme[7].

RESULTS

Description of outbreak areas:

The main outbreak area was in the suburb of Ipoh city, along the Jalan Bendahara which is situated about 2km from the city centre. Along both sides of the road, there were more than 20 small companies dealing with the collection and trading of scrap metal. The scrap metal was stored temporarily at the site and intermittently sold to appropriate agencies. The scrap metal consisted of parts of cars and other vehicles which were kept in open spaces. Stagnant rain water could be easily seen; collected in parts of scrap metal. The residents and workers were mostly Indians who return to India fairly regularly to visit their families. They lived in small wooden dormitory-like houses within the scrap metal collection centre and each housed 5 to 15 people. An estimate of about 1423 people lived in this area. The solid waste disposal system at the site was poor and uncoordinated although the residents received tap-water supply from the Perak Water Board.

The smaller outbreak area was Sungai Tapah village within the district of Gugusan Manjoi, Ipoh city. The two outbreak areas were separated by a distance of 7km of good access road. There were about 1000 houses in Sungai Tapah village with a population of about 10,000 people. Residents staying in Sungai Tapah frequently visited the scrap metal collection centres in Jalan Bendahara to look for spare parts for their cars or vehicles.

Epidemiological description of outbreak:

The first suspected case which was subsequently laboratory-confirmed was a 64-year old Indian man who visited his relatives in Paramakudi, Tamil Naidu, India on 28th June 2006. He returned to Malaysia on 9th November 2006 and came down with fever on 17th November 2006. The second such case was a 44-year Indian man who also visited Paramakudi, Tamil Naidu, India on 21st November 2006 and returned to Malaysia on 30th November 2006. On returning home, he developed high fever and joint pain on the 3rd of December 2006. The rest of the affected residents did not leave Kinta district for the last two month prior to the occurrence of outbreak.

Inclusive of the above two cases, of the 52 residents who were suspected of being infected with CHIK virus, 39 residents (37 from main outbreak area and two from Sungai Tapah village) were laboratory confirmed. The number of cases in each age-group, gender, racial, occupational category and types of presenting clinical features is as shown in Table I.

Laboratory results:

A total of 52 blood samples were received for laboratory confirmation of CHIK virus infection. The types of tests carried out for each of the patients' serum samples and their respective results are shown in Table II.

All the 80 adult mosquitoes in four separate containers were identified as *Aedes albopictus*. CHIK virus was isolated in Vero cells in two of the four mosquito pools and CHIK virus RNA was detected in all four Aedes albopictus mosquito pools. Alignment of nucleic acid sequences based on the 257-nts partial E1 gene of two representative CHIK virus isolates from patients' serum samples and a representative virus isolate

isolated from mosquito pool in this outbreak together with a similar CHIK virus genetic sequence detected earlier in the serum of a patient with CHIK in Batu Gajah, Perak in August 2006 is shown in Figure I[8]. Nucleic acid sequence information showed that CHIK nucleic acid sequence obtained from mosquitoes was identical to that of the nucleic acid sequence of CHIK virus isolated from both human cases in the same location but differed from the Batu Gajah CHIK virus nucleotide sequence by 2 nucleotides (thymidine → adenine; cytosine → thymidine). The phylogenetic relationship of the CHIK virus isolated in this outbreak with respect to other CHIK viruses is shown in Figure 2. Phylogenetic analysis of the 257-nts partial envelope (E1) gene shows that the CHIK virus isolated in this outbreak was closely related to the CHIK virus detected in the serum of a patient with CHIK in Batu Gajah in August 2006 and both are of Central/East African genotypes[8].

DISCUSSION

CHIK virus was first isolated from human serum and *Aedes aegypti* mosquitoes during an epidemic in Tanzania in 1953[1,2]. Hammon *et al* (1960) documented the first appearance of the virus in Southeast Asia by isolation during an intense epidemic of dengue fever in Bangkok, Thailand in 1958[9]. Since then, CHIK virus has caused occasional outbreaks and some larger epidemics throughout most of sub-Sahara Africa and tropical Asia[10-12]. In the last decade, the virus has shown not only increased activity but has expanded its geographical locations, thus classical delineation of various genotypes of chikungunya virus to specific geographic locales no longer holds true. Rapid mass movement of people and the constant presence of the right vectors in this region could have contributed to the change in virus ecology. Recently, the CHIK virus of Central/East African genotype has spread to India and caused extensive outbreak[13]. The CHIK virus of Central/East African genotype has not only spread to the Indian Ocean Islands, it has led to the emergence of new variant which may be of higher virulence[14].

In Peninsular Malaysia, a serological survey for alphaviruses conducted by Marchette *et al* (1978) showed that CHIK antibody was detected in persons older than 20 years with a proportionately larger number of seropositive individuals in the northern states bordering Thailand such as Perlis, Kedah and Kelantan. A follow up serological study by Marchette *et al* (1980) showed specific haemagglutination inhibition and neutralizing antibodies in a chicken in Kelantan and a pig in Kedah, further supporting CHIK activity occurred mainly along the Malaysia-Thailand border[15]. Despite the fairly high antibody prevalence in man in the surveyed areas, there was no report of clinical disease associated with the virus until 1998 where the first outbreak was reported involving residences staying in the suburb of Klang, a coastal city in the central western part of Peninsular Malaysia[16]. Following which, a second outbreak occurred in early 2006 involving residences staying in Bagan Panchor, a fishing village situated at approximately 15 kilometres from Taiping in the state of Perak, north western part of Peninsular Malaysia[17]. In both outbreaks, CHIK viruses of Asian genotype were isolated and nucleotide sequence analysis of the virus partial E1 gene strongly suggested that the 2006 virus strain most probably evolved from the 1998 outbreak strain which could have remained at low circulation between the outbreaks[17].

A combination of a constant presence of *Aedes* mosquitoes, the rapid and mass movement of people between India and Malaysia, and the recent extensive CHIK outbreak due to CHIK virus of Central/East African genotype in India, it is expected that CHIK virus of Central/East African genotype would gain its foothold in Malaysia in a matter of time. CHIK virus of Central/East African genotype was first detected in the serum collected on 27th August 2006 from a 49-year-old Indian housewife who visited her relatives in Chennai, India in mid-July 2006 and developed abrupt onset of high fever, a day prior to her return to Malaysia[8]. Epidemiological investigation carried out then did not show any cluster of similar illness in her immediate neighbourhood but it has been predicted that an outbreak due to this genotype may happen in a very near future.

This report documents the first outbreak of CHIK virus of Central/East African genotype in Malaysia. Epidemiological investigation in this outbreak showed that the virus was most probably introduced into Malaysia from India when relatives were visited in the outbreak areas in India. The index case for this outbreak was most likely the 44-year Indian man who visited Paramakudi, Tamil Naidu, India on 21st November 2006 and returned to Malaysia on 30th of November 2006. The first case the 64-year old Indian man probably acquired the infection and recovered from the illness during his long stay of four months in India. The outbreak most likely started in the scrap metal collection centre and subsequently spread to Sungai Tapah village. Once the aetiology of the outbreak was known, the outbreak was swiftly brought under control after instituting very intensive vector control measures.

Table I: Demographic data of residents infected with chikungunya virus types of clinical presentation

Category	Number of cases (Total = 39)
Age group (year)	
<10	1
11-20	2
21-30	8
31-40	9
41-50	6
51-60	13
Gender	
Male	34
Female	5
Race	
Indian	37
Chinese	-
Malay	2
Others	-
Occupation	
Labourer	18
Owner	16
Clerk	2
Housewife	3
Clinical presentation	
Fever	37
Arthralgia	11
Myalgia	8
Arthritis	2

Table II: The types of tests carried out for each of the patients' serum samples and their respective results

No	Lab ID	Sample age (day)	RT-PCR	Virus Isolation	CHIK IgM	CHIK IgG
1	CHIK/06/03	4	Detected	Positive	ND	ND
2	CHIK/06/08	>10	ND	Negative	Positive	Positive
3	CHIK/06/09	10	ND	Negative	Positive	Positive
4	CHIK/06/10	>10	ND	Negative	Positive	Positive
5	CHIK/06/12	3	Detected	Positive	ND	ND
6	CHIK/06/13	3	Detected	Positive	ND	ND
7	CHIK/06/14	1	Detected	Positive	ND	ND
8	CHIK/06/16	>10	ND	ND	Positive	Positive
9	CHIK/06/17	>10	ND	ND	Negative	Positive
10	CHIK/06/18	>10	ND	ND	Positive	Positive
11	CHIK/06/19	>10	ND	ND	Negative	Positive
12	CHIK/06/21	>10	ND	ND	Positive	Positive
13	CHIK/06/22	>10	ND	ND	Positive	Positive
14	CHIK/06/23	>10	ND	ND	Positive	Positive
15	CHIK/06/24	>10	ND	ND	Positive	Negative
16	CHIK/06/25	>10	ND	ND	Positive	Positive
17	CHIK/06/26	>10	ND	ND	Positive	Positive
18	CHIK/06/28	>10	ND	ND	Positive	Positive
19	CHIK/06/29	>10	ND	ND	Positive	Positive
20	CHIK/06/30	>10	ND	ND	Positive	Positive
21	CHIK/06/31	>10	ND	ND	Positive	Positive
22	CHIK/06/32	>10	ND	ND	Positive	Positive
23	CHIK/06/33	>10	ND	ND	Positive	Positive
24	CHIK/06/34	>10	ND	ND	Positive	Positive
25	CHIK/06/35	>10	ND	ND	Negative	Positive
26	CHIK/06/36	>10	ND	ND	Positive	Positive
27	CHIK/06/37	>10	ND	ND	Positive	Positive
28	CHIK/06/38	>10	ND	ND	Positive	Positive
29	CHIK/06/39	>10	ND	ND	Negative	Positive
30	CHIK/06/41	>10	ND	ND	Negative	Positive
31	CHIK/06/42	>10	ND	ND	Positive	Positive
32	CHIK/06/43	>10	ND	ND	Positive	Positive
33	CHIK/06/45	>10	ND	ND	Positive	Positive
34	CHIK/06/48	>10	ND	ND	Negative	Positive
35	CHIK/06/49	>10	ND	ND	Positive	Positive
36	CHIK/06/50	4	ND	Positive	ND	ND
37	CHIK/06/51	3	ND	Positive	ND	ND
38	CHIK/06/52	1	Detected	Positive	ND	ND
39	CHIK/06/54	3	Detected	Positive	ND	ND

ND = Not Done

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Kinta14/06        1 ggcgcctactgcttctgcgacgctgaaaacacgcagttgagcgaagcacatgtggagaagtccgaatcat
Kinta13/06        1 ............................................................
Kinta/Mosq        1 ............................................................
BatuGajah2006     1 ............................................................a

Kinta14/06       71 gcaaaacagaatttgcatcagcatacagggctcataccgcatctgcatcagctaagctccgcgtccttta
Kinta13/06       71 ............................................................
Kinta/Mosq       71 ............................................................
BatuGajah2006    71 ..........................................t.................

Kinta14/06      141 ccaaggaaataacatcactgtaactgcctatgcaaacggcgaccatgccgtcacagttaaggacgccaaa
Kinta13/06      141 ............................................................
Kinta/Mosq      141 ............................................................
BatuGajah2006   141 ............................................................

Kinta14/06      211 ttcattgtggggccaatgtcttcagcctggacacctttcgacaacaa
Kinta13/06      211 ...........................................
Kinta/Mosq      211 ...........................................
BatuGajah2006   211 ...........................................
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Fig. 1: Nucleic acid sequence alignment of RT-PCR amplified products derived respectively from two representative chikungunya virus isolates from sera of patients (Kinta14/06, Kinta13/06) and an isolate from a mosquito pool (Kinta/Mosq) collected in this outbreak, together with chikungunya virus specific nucleotide sequence amplified directly from the serum of a patient in Batu Gajah who came down with the illness in August 2006 (BatuGajah2006). RT-PCR products were obtained using chikungunya virus specific primer-pair directed at the virus partial envelope (E1) gene of 257-nt as described by Hasebe *et al*. The nucleic acid sequences of both primers used in the amplification were not included in the alignment.

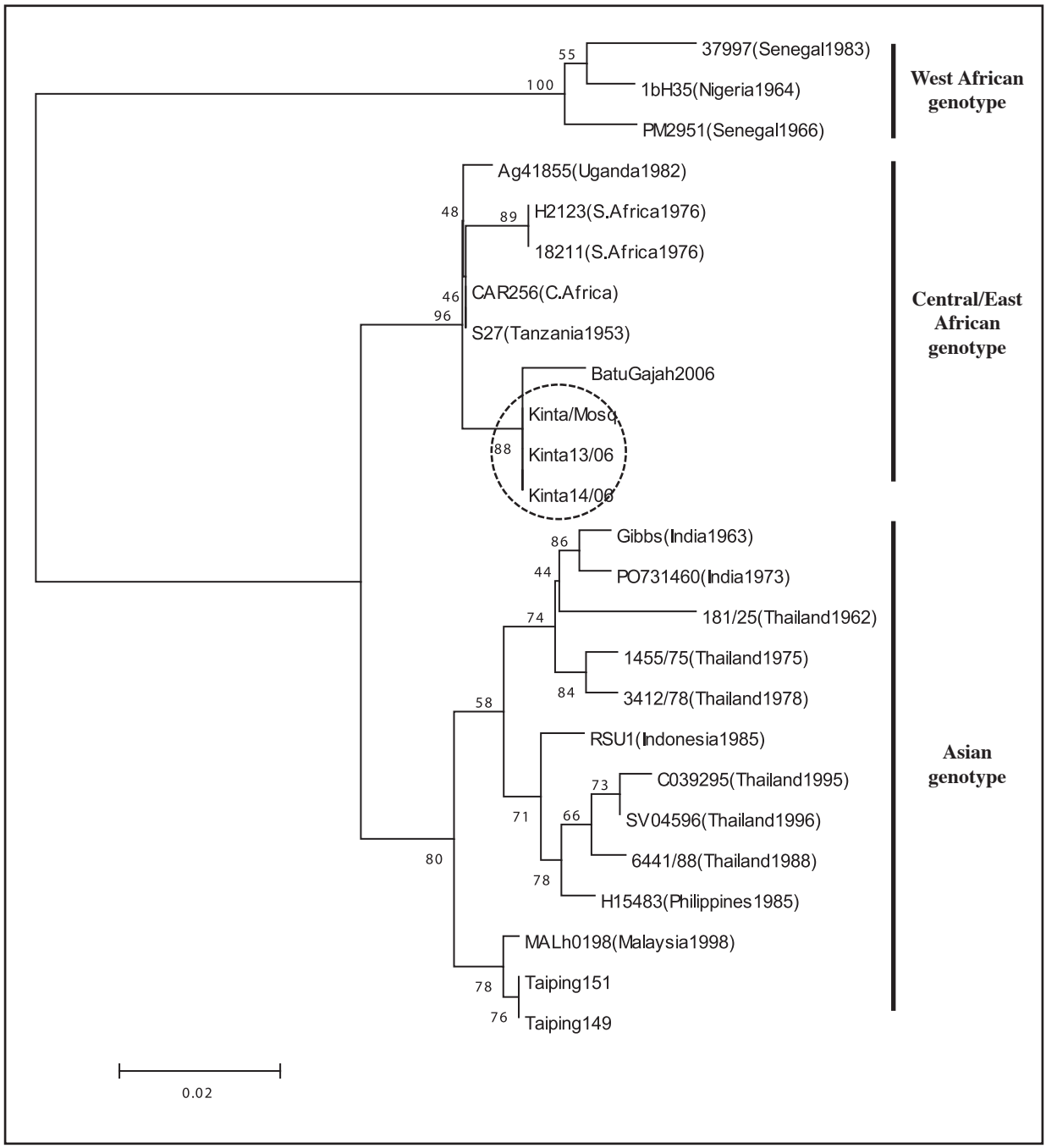

Fig. 2: Phylogenetic analysis of chikungunya (CHIK) virus based on 257-nt-long virus partial envelope (E1) gene sequence derived from three chikungunya virus isolates isolated in this outbreak [2 humans (Kinta14/06, Kinta13/06) and 1 mosquito pool (Kinta/Mosq)] with respect to similar gene segments detected in the acute serum sample of a patient in August 2006 (BatuGajah2006), 2 strains of CHIK virus isolated in the 2006 outbreak in northern part of Peninsular Malaysia (Taiping 149, Taiping 151) and sequences of 19 CHIK virus strains deposited in the GenBank. The bootstrapped consensus tree was constructed for a 257-nt-long E1 gene sequence using MEGA3 programme. Identity of each isolate, location and year of isolation was indicated in the figure. Their respective Genbank accession numbers are as followed: 37997 (AF192892), PM2951 (AF192891), IbH35 (AF192893), MALh0198 (AF394210), H15483 (AF192895), 6441/88 (AF192896), SV045196 (AF192900), C039295 (AF192897), RSU1 (AF192894), 3412/78 (AF192899), 1455/75 (AF192898), 181/25 (AF192908), PO731460 (AF192902), Gibbs (AF192901), Ag41855 (AF192907), S27 (L37661), CAR256 (AF192906), 18211 (AF192903), H2123 (AF192904).

Clinically, it is not easy to differentiate acute dengue from acute CHIK, especially if it is an isolated case and the patient's illness does not progress to dengue haemorrhagic fever or dengue shock syndrome. However, a high index of clinical suspicion of CHIK should be considered in situation where there is a cluster of patients with acute febrile rash illness and a proportion of the patients develop joint symptom. We recommend virus isolation and molecular detection of CHIK virus specific nucleic acid sequence for laboratory confirmation of CHIK for those patients with duration of illness of four days or less and serological assay for anti-CHIK specific IgM for those with illness of more than seven days. All the above three types of diagnostic tests are recommended for those patients with duration of illness between 5 to 7 days.

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