THE ZIKA INFECTION, HISTORY AND EVOLUTIONARY STUDY IN MALAYSIA

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ABSTRACT

Zika virus (ZIKV) has been isolated in Malaysia from *Aedes aegypti* in 1966, however, not until recently, the infection has been reported in humans in a few states in Malaysia. This study aimed to determine the origin of the ZIKV detected throughout Malaysia by population-based Sanger sequencing. Sera and urine samples were received from eight positive patients for ZIKV from the Sungai Buloh Hospital, Queen Elizabeth Hospital, Sultanah Aminah Hospital and Miri Hospital, between 31st August 2016 to 13th December 2016. The samples were subjected to real-time Reverse Transcription PCR (rRT-PCR) and the partial E gene fragment was amplified and sequenced. Phylogenetic analysis of the ZIKV isolates was constructed. Of eight patients, sera and urine samples from seven of them were successfully amplified and sequenced. Phylogenetic analysis showed that all ZIKV strains from 2 patients were closely related to the Micronesia strain and the Malaysian strain that was isolated in the year 1966. Meanwhile, the ZIKV of other patients from various states within Malaysia were from Asian lineage. The findings have suggested a possible concurrent event between the recent introductions of the virus from the neighbouring country and the re-emergence of Zika in Malaysia. The need for continuous surveillance of ZIKV infection in Malaysia is crucial in understanding the epidemiology of ZIKV infection in Malaysia and comprehending the reasons behind the low ZIKV cases detection in Malaysia as compared to our neighbouring countries.

KEYWORDS: Zika, Malaysia, Partial E gene

INTRODUCTION

Zika virus (ZIKV), a member of the family Flaviviridae and the genus Flavivirus, is a mosquito-transmitted virus that was first described in 1947. The virus was isolated from a sentinel monkey in the Zika Forest of Uganda (1). From the 1950s to the 1980s, sporadic human infections were detected in several countries in Africa and Asia. Most of the infections are asymptomatic or difficult to be distinguished from dengue with symptoms such as fever, myalgia, maculopapular rash, arthralgia, headache and conjunctivitis (2,3). Since first described, ZIKV received far less attention in comparison to other mosquito-transmitted viruses such as dengue, West Nile and Japanese encephalitis viruses until ZIKV was introduced from south-east Asia across the Pacific causing large outbreaks in the Island of Yap, French Polynesia and Brazil (3). The ZIKV infection was given more attention when WHO declared microcephaly and other neurological disorders associated with ZIKV as a Public Health Emergency of International Concern (PHEIC) in February 2016.

ZIKV, dengue (DENV) and chikungunya (CHIKV) infections in humans pose similar clinical presentations and are very difficult to differentiate from one another especially in DENV and CHIKV endemic countries like Malaysia (4). Even though the diagnosis of ZIKV can be made by performing ZIKV serology test, cross-reaction with other flaviviruses has made the test limited only to non-endemic countries for dengue, chikungunya, Japanese encephalitis and West Nile infections (5). The results produced from each individual ZIKV serology test have to be interpreted with caution as many ZIKV serology test kits have been made available in the market. Therefore, nucleic acid detection of ZIKV is the most reliable method to detect the presence of ZIKV in clinical specimens. In Malaysia, since the first isolation of ZIKV in 1966 (4), no infection was reported until a German tourist was described to have ZIKV infection after returning from Malaysia in 2014.

As ZIKV infection has posed a significant public health threat, the need for the laboratory to perform tests to detect the presence of the virus was crucial. In April 2016, up to 14 hospital and public health laboratories in Malaysia were trained and currently have the capacity to perform the molecular test for detecting ZIKV in human samples. Meanwhile, genome sequencing of ZIKV identified from the outbreak was carried out only by the Virology Unit, Institute for Medical Research. Since 31st August 2016, there have been eight positive cases of ZIKV detected in a few states in Malaysia. The objective of this study was to identify the viral sequences of the ZIKV from all eight cases of ZIKV infection in Malaysia that were detected between August 31st to December 13th, 2016 in order to determine the origin of the detected ZIKV throughout the states in Malaysia, and phylogenetic analysis was reported.

MATERIALS AND METHODS

The first confirmed case of ZIKV infection in Malaysia (MyH318) was reported in Klang, a town 32 km to the west of Kuala Lumpur on 31st August 2016. The patient was referred to Sungai Buloh Hospital by a private clinic as suspected for ZIKV infection. The patient described having visited her daughter, who was hospitalised in Singapore due to a fever. Two days after the first identified case, a case (MyH319) was reported in Kota Kinabalu (East Malaysia), situated on the island of Borneo involving a 61 year-old patient. Initially, he was treated in Luyang Health Clinic and referred to Queen Elizabeth Hospital due to severe symptoms of fever, generalised body ache and diarrhoea. The patient reported having other co-morbid conditions including hypertension, chronic kidney disease, kidney stone and gout with no history of recent travel.

The third positive case (MyH326PL) was reported on the 6th September 2016, from a pregnant lady residing in Johor Bahru, located approximately 25 km away from the centre of Singapore. She had the symptoms of infection on the 2nd September 2016 and sought medical treatment at a private general practitioner and was referred to Sultanah Aminah Hospital on the 5th September 2016. The lady travelled to Singapore 6 months earlier while her husband commutes daily to his workplace in Geylang, Singapore. The husband (MyH330) of MyH326PL also showed symptoms of ZIKV infection on the 2nd September 2016.

On the 9th September, we received a sample from a patient residing in Miri, located at the East Malaysian state of Sarawak, on the island of Borneo (MyH334PL) on 13th September 2016. The patient was reported to be 10 weeks pregnant, showing symptoms of generalised body rashes and red-eye. The next positive ZIKV case was a 39-year-old lady (MyH335PL), a sister to the previous case who lives in the Kuala Lumpur suburb of Setapak, who was hospitalised in Kuala Lumpur on 13th September 2016. She had shown symptoms of ZIKV infection and had a history of visiting her sister in Miri on August 26, before leaving for Kuala Lumpur on

September 6.

The seventh case of ZIKV positive case in Malaysia was confirmed on 1st October from a 60-year-old woman (MyH349PL) who resided in Kota Kinabalu, Sabah and attended a general practitioner after she showed signs and symptoms of ZIKV infection. She denied any history of travelling to any Zika-affected countries and had not contacted any patients infected with the virus. The last case of ZIKV infection in Malaysia was reported on 14th December 2016 by a 60-year-old man (MyH414PL) who was staying in Petaling Jaya, Selangor through a Zika virus surveillance system. The patient had undergone treatment at one of the local clinics on 30th November 2016 after showing symptoms of fever, nausea and diarrhoea for two days.

The Virology Unit, Institute for Medical Research Kuala Lumpur had received sera and urine samples from all the above-mentioned patients for further investigations and genome sequencing of the virus. A series of ante-mortem samples were also collected from the patients with no specific interval for the sample collection. A number of post-mortem samples were obtained from patient MyH319 and a vaginal swab was taken from MyH349.

RNA was extracted from the patients' sera and urine samples using QIAamp Viral RNA Kits (Qiagen, Germany) based on the manufacturer's instruction, and the extracted RNA was further subjected to real-time reverse transcription PCR (rRT-PCR). Two protocols were used; IMR-AFRIMS rRT-PCR targeting the prM-E region of ZIKV and CDC Trioplex rRT-PCR targeting 5'UTR region of DENV, nsP1 region of CHIKV and envelope region of ZIKV (18,19). All samples that had the ZIKV genome by both the rRT-PCR protocols were subjected to amplification of partial envelope (E) gene (577bp). The partial envelope (E) gene was amplified using primer ZV_E-F (5'- GTT YRC STG YWS YAR GAA GAT GAC-3') and ZV_E-R (5'- GCCAAGT GRT GGG TGA TYT CTT VTC SCC-3'). The partial E gene fragments were gel extracted and sequenced using Sanger Sequencing using the sequence primers as depicted in Table 1. The sequence analysis and phylogenetic relations of the sequences were examined using selected ZIKV strains obtained from GenBank. The sequences were aligned and analysed where a maximum likelihood (ML) phylogenetic tree was created in Molecular Evolutionary Genetics Analysis (MEGA) (12) using the Tamura–Nei model and 1,000 bootstrap step validation (6,7,13). TA number of samples were received from each individual patient with ZIKV infection and tested for the presence of ZIKV. The samples used in this study originated from our diagnostic services and all samples were anonymised and labelled with the specific laboratory numbers.

Table 1. Primers used in the sequencing of the partial E gene

Primer name	Sequence (5' to 3')
Primer used to amplify the E gene	
ZV_E-F	GTT YRC STG YWS YAR GAA GAT GAC
ZV_E-R	GCCAAGT GRT GGG TGA TYT CTT VTC SCC
Primer used for sequencing of the E gene	
ZV_E_SeqF	GCC CAC GCC AAG AGG CAR ACC G
ZV_E_SeqR	CAC GCC CTY CAR YCT MAG CTT GTCC

RESULTS AND DISCUSSION

Results of ZIKV PCR from serial sera and urine samples of each ZIKV positive patient are shown in Table 2. The pattern of ZIKV disappearance from the different samples collected was dissimilar for each individual patient. ZIKV infection persisted for a longer period in the serum of the pregnant patient, up to 15 days from the first day she was confirmed as having ZIKV infection compared to non-pregnant patients. Meanwhile, in post-mortem samples, ZIKV was detected in all samples sent to our laboratory, as depicted in Table 2. Results also showed that both serum and urine were suitable samples for ZIKV diagnosis.

Different targets for rRT-PCR amplification were performed to ensure the integrity of the generated results. All samples reported in this study showed an amplification curve for ZIKV in the rRT-PCR (data not shown). The phylogenetic tree drawn based on the alignment of E gene sequences as illustrated in Figure 1

showed that the isolates from MyH318 and MyH326PL were clustered together, while MyH319 was grouped at a distance from the former cases. Other strains, MyH334 and MyH335 were grouped together and strains of MyH414 and MyH349 were classified in the other groups. The nucleotide sequences of the partial E gene of MyH318 and MyH326 were highly similar (99% - 100%) to the isolates from Singapore (GenBank accession number: KY241765 and KY921911). We were unable to amplify the partial E fragment directly from the sera of patient MyH330, the husband of MyH326PL, due to the very low virus titre obtained from the sample. In contrast to the former isolates, MyH334, MyH335, MyH349 and MyH414 strains were clustered more closely with the isolates from the Southeast Asia region. Meanwhile, the MyH319 strain was closely related to the Brazilian strain.

Table 2. Serial sera and plasma samples from each Zika positive patient

Case	Sample Types	Status of sample collection*	ZIKV PCR Results
ZK318/16	Urine	1st sample	+ve
	Plasma	1st sample	-ve
	Urine	D3	+ve
	Urine	D4	+ve
ZK319/16	Serum	1st sample	+ve
	Urine	1st sample	+ve
	2nd serum	D3	+ve
	Liver	D3	+ve
	Jejunum	D3	+ve
	Tracheal Swab	D3	+ve
	Nasopharyngeal swab	D3	+ve
	Tracheo-bronchial aspirate	D3	+ve
	Brain Stem	D3	+ve
	Frontal lobe of brain	D3	+ve
	Heart	D3	+ve
	Kidney	D3	+ve
	Stomach	D3	+ve
	Spleen	D3	+ve
	Colon	D3	+ve
	CSF	D3	+ve
	Lung	D3	+ve
ZK326/16	Serum	1st sample	+ve
	Urine	1st sample	+ve
	Serum	D3	+ve
	Urine	D3	+ve
	Serum	D4	+ve
	Urine	D4	+ve
	Serum	D10	+ve
	Urine	D10	-ve
	Serum	D11	+ve
	Serum	D12	+ve
	Serum	D13	+ve
	Serum	D14	+ve
	Serum	D15	+ve
	Serum	D17	+ve
	Serum	D41	-ve
ZK330/16	Urine	NA	+ve
	Urine	1st sample	+ve
	Serum	NA	-ve
	Serum	1st sample	-ve
	Serum	D55	-ve
	Urine	D55	-ve

ZK334/16	Serum	1st sample	+ve
	Serum	D3	+ve
	Urine	D3	-ve
	Serum	D13	+ve
	Urine	D13	-ve
ZK335/16	Serum	NA	+ve
	Urine	NA	+ve
	Serum	NA	+ve
ZK349/16	Serum	1st sample	+ve
	Urine	1st sample	+ve
	Serum	NA	-ve
	Vaginal Swab	NA	-ve
	Urine	NA	-ve
ZK414/16	Urine	NA	+ve
	Urine	NA	-ve
	Serum	NA	-ve
	Serum	NA	-ve

- NA – Not available, +ve – positive, -ve – negative; *Samples were labelled relative to first sample with confirmed positive ZIVK.

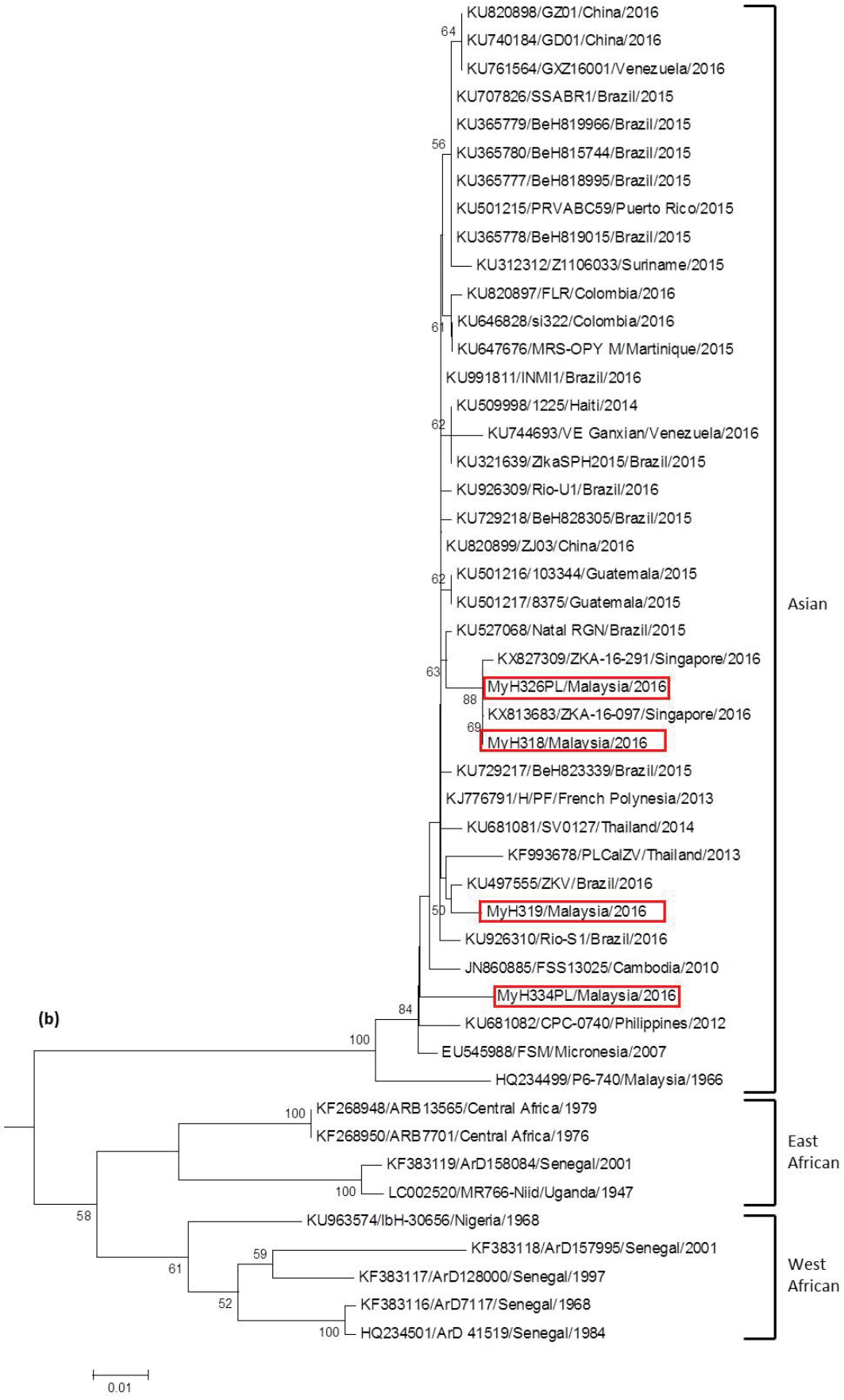

Figure 1. Evolutionary relationships of detected zika viruses in Malaysia

Limited phylogenetic analysis of Zika virus partial E gene sequences from 7 cases in Malaysia for 2016. The tree was rooted with Spondweni virus (not shown). The phylogenetic tree was constructed using the Neighbour-Joining method (6). The bootstrap values are shown as percentage derived from 1000 replicates and only value more than 50% was shown. The analysis of the isolates; MyH318 (GenBank accession number: KX906953), MyH319 (GenBank accession number: KX906954), MyH326PL (GenBank accession number: KX906955), MyH334PL (GenBank accession number: KX906956), MyH335 (GenBank accession number: MH130042), MyH349 (GenBank accession number: MH130043) and MyH414 (GenBank Accession number: MH130044) was performed against other nucleotide sequences available in the GenBank and conducted using MEGA6 (6).

The infection kinetic of ZIKV infection for each individual patient is shown in Table 1. Different pattern of ZIKV infection kinetics was seen between non-pregnant and pregnant patients with the presence of ZIKV lasting longer in the serum of the pregnant patient. Meanwhile, ZIKV was detected in all post-mortem samples collected from the patient MyH319.The duration of detectable ZIKV in the blood and urine varies, and many studies have been conducted to understand this newly re-emerging virus in a previously active region for ZIKV infection. The loss of ZIKV RNA detection was reported to be 8 and 39 days in serum and urine, respectively (8). The duration of detectable ZIKV in ante mortem specimens could be clearly elucidated if more samples were available for further analysis.

Analysis of the phylogenetic tree is crucial in determining the origin of circulating ZIKV in Malaysia as well as to determining the recent introduction of the virus to the region. Based on the limited phylogenetic analysis; the cases reported in Malaysia in the year 2016 were likely to have originated from two different sources. The cases reported in Peninsular Malaysia; MyH318 and MyH326PL are likely due to the introduction of the virus from the concurrent Singapore outbreak. This is further substantiated by the patients' history of travel to Singapore or having contact with a person who travelled to Singapore, that had ongoing epidemics of ZIKV infection since August 2016 (9). MyH319 and MyH349 were reported in East Malaysian states of Sabah and MyH334 case was detected in Sarawak, both of which are located on the Island of Borneo. The limited phylogenetic analysis indicates these isolates were closely related to the isolates reported in the Southeast Asia region, which further supports the hypothesis that the virus originated from a local strain. The geographic proximity of Borneo Island to Singapore further substantiated this hypothesis. Phylogenetic analysis of two ZIKV cases in Singapore also showed the virus was grouped under the Asian lineage (15).

The MyH319 strain appeared closely related to the strain from Brazil (KU497555), while the MyH334 and MyH335 isolates were clustered close to the Federated States of Micronesia (FSM) isolate (EU545988). These results indicate the recent introduction of ZIKV into Malaysia as well as the possibility of low-level endemic transmission that has gone undetected since 1966 (5). A similar incidence was reported on the re-emergence of CHIKV, which originated from a local strain (10).

To date, it is known that the virus can be passed from a pregnant mother to the foetus which can lead to certain defects (16). However, a lot more remains unknown, such as how ZIKV infection will affect pregnancy and the full range of disabilities, including the possible effect on neurodevelopment (11,17). Both My326 and My334 involved pregnant mothers in their early pregnancies. My334 delivered a healthy baby with no microcephaly. The baby is currently being monitored by the paediatrician until 2 years of age. The patient My326, however, decided to abort the pregnancy outside Malaysia, and we did not have access to the foetal tissue for further analysis. Currently, we are not sure how the differences in the ZIKV sequences of the partial E gene of these two pregnant cases have an impact on the occurrence of microcephaly or Guillian Barre Syndrome (GBS) and what other factors contribute to abating the ZIKV infection in Malaysia as we have not experienced a huge outbreak like in Singapore and Brazil. Factors that contributed to the low number of ZIKV infections remained unknown and a seroprevalence study of ZIKV infection in the community is warranted.

Due to the mild and self-limiting symptoms and the similarity between the symptoms caused by DENV and CHIKV, the infection might be misdiagnosed or not reported. There is also no active serological surveillance being conducted to monitor the exposure of the Malaysian population against ZIKV. Similar to Thailand, dengue and CHIKV are endemic in Malaysia. A team from Mahidol University reported the cross-reactive in their serologic surveillance to detect possible exposure to ZIKV in the Thai populations and the results needed to be translated with caution (13). Since ZIKV, DENV and CHIKV are transmitted by the same vectors, it is important to understand the factors that led to the re-

emergence of ZIKV within the Southeast Asia region. Due to the high potential for immunological cross-reactivity between flaviviruses (14), continued molecular surveillance of ZIKV within Malaysia and around the Southeast Asia region is crucial in understanding the molecular mechanisms that facilitate the spread of the virus and other factors that lead to microcephaly or GBS.

Further analysis of the ZIKV sequences was unavailable as our surveillance data showed that none of the ZIKV was detected in the samples sent to us for testing. For the year 2017, 101 samples were tested for the presence of ZIKV and none of the samples tested positive for ZIKV. In 2018, the ZIKV PCR test was performed on 66 samples, and all of them were negative. Furthermore, 72 and 26 samples were subjected to ZIKV PCR in the year 2019 and 2020, respectively and no positive case was reported. Similar findings were obtained from the state hospital laboratories, as any positive cases were required to be referred to our laboratory for further testing and sequencing. Thus, continued surveillance in detecting the presence of ZIKV infection is vital.

As to ensure continuous monitoring for ZIKV infection, ZIKV PCR is continuously available at the state hospital laboratories throughout Malaysia. Samples are only sent to Unit Virology, IMR for the detection of ZIKV for cases associated with GBS and microcephaly. The Virology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia is a National Reference Laboratory for outbreak investigation and is responsible for the further confirmation of ZIKV infection by performing Sanger sequencing.

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