LOW-LEVEL DETECTION AND GENOME CHARACTERIZATION OF CHIKUNGUNYA VIRUS DURING COVID-19 MOBILITY RESTRICTION IN KLANG VALLEY, MALAYSIA

Shih Keng Loong[1], Mulya Mustika Sari Zulkifli[1], Fang Shiang Lim[2], Yik Zheng Lim[1], Pouya Hassandarvish[1], Kim-Kee Tan[1], Juraina Abd-Jamil[1], Sazaly AbuBakar[1] and Boon-Teong Teoh[1]

[1]Tropical Infectious Diseases Research and Education Centre, Higher Institution Centre of Excellence, Universiti Malaya, Kuala Lumpur, Malaysia; [2]Institute for Biosafety in Plant Biotechnology, Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Quedlinburg, Germany

Abstract. Chikungunya (CHIKV) and dengue (DENV) viruses co-circulate in Malaysia, where they share *Aedes* mosquito vectors and present overlapping early clinical features, making laboratory confirmation essential for accurate diagnosis. The study determined the presence of CHIKV infection among DENV-negative febrile patients at the Universiti Malaya Medical Centre, Kuala Lumpur, Malaysia, from May 2020 to May 2021, during the COVID-19 pandemic and Malaysia's Movement Control Order (MCO) period. Patients' serum specimens (n = 917) were screened for CHIKV using a validated in-house pan-*Alphavirus* RT-PCR to amplify the conserved *nsP4* gene, resulting in one positive specimen. Complete genome sequencing of the specimen revealed an 11.6 kb genome, the phylogenetic analysis of which placed the isolate within the East/Central/South African (ECSA) genotype, clustering closely with strains from Myanmar and Thailand. The isolate carried three amino acid substitutions (E1-E211, E1-A226 and E2-A264), characteristic of an ECSA lineage capable of efficient transmission by *Ae. aegypti* and indicative of a regional endemic lineage rather than an introduction from outside the country. The exceptionally low detection rate during the MCO period was consistent with the hypothesis that CHIKV transmission is influenced by human mobility and behavioral exposure patterns. Recovery of a complete genome during this period provided a valuable baseline for monitoring CHIKV evolution and/or re-emergence as human movements return to pre-COVID-19 pandemic levels. Integrating CHIKV testing into DENV-negative febrile illness clinical protocols and maintaining

genomic surveillance should contribute to a better understanding of the transmission and evolution of this mosquito-borne arbovirus in Malaysia.

Keywords: *Aedes* species, alphavirus, arbovirus, chikungunya virus, infection, tropical region

Correspondence: Boon-Teong Teoh, Tropical Infectious Diseases Research and Education Centre, Higher Institution Centre of Excellence, Universiti Malaya, Kuala Lumpur 50603, Malaysia
Tel: +60 3 7967 6670 E-mail: boonteong@um.edu.my

INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne alphavirus transmitted primarily by *Aedes aegypti* and *Ae. albopictus* (Sam *et al*, 2009). CHIKV infection typically presents as an acute febrile illness accompanied by rash and severe polyarthralgia (Sam *et al*, 2012; Loong *et al*, 2022). Owing to substantial overlap in early symptoms with dengue, chikungunya infections are commonly misclassified in the absence of laboratory diagnosis.

In Malaysia, multiple CHIKV outbreaks have occurred over the past two decades, with the East/Central/South African (ECSA) genotype consistently detected through genomic surveillance (Azman *et al*, 2024; Kalyanasundram *et al*, 2024). The CHIKV outbreak first occurred in 2008-2009 with the Indian Ocean lineage (IOL), a highly epidemic ECSA sublineage characterized by an E1-A226V mutation, which enhances transmission by *Ae. albopictus* circulating in the country (Sam *et al*, 2012; Kalyanasundram *et al*, 2024). Following this outbreak period, CHIKV infections in Malaysia occurred intermittently as reported through a national surveillance system, with sporadic cases and localized clusters, and occasional notifications in the central states, such as Selangor, located in the Klang Valley (Azman *et al*, 2024). Surveillance data from the Ministry of Health, Malaysia, indicate

fluctuating CHIKV infection in the Klang Valley before and during the COVID-19 pandemic, with reported cases decreasing from 185 in 2019 to 23 in 2020, followed by increases to 829 in 2021 and 501 in 2022 (Ministry of Health Malaysia, 2020a; Ministry of Health Malaysia, 2020b; Ministry of Health Malaysia, 2022; Ministry of Health Malaysia, 2023). Recent genomic investigations from Malaysia and across Southeast Asia showed that viruses carrying the ancestral E1-A226 have re-emerged as the predominant CHIKV clade (Chinnawirotpisan *et al*, 2020; Su *et al*, 2023; Azman *et al*, 2024; Kalyanasundram *et al*, 2024), reflecting a regional shift toward lineages more compatible with urban transmission by *Ae. aegypti*.

The emergence of the COVID-19 pandemic and the stringent public health measures implemented to mitigate SARS-CoV-2 transmission substantially altered human behavior and the dynamics of infectious disease transmission. Malaysia implemented a series of Movement Control Orders (MCOs) beginning in March 2020, imposing strict restrictions on travel and social interaction (Hashim *et al*, 2021). Reduction in population mobility results globally in an alteration in the prevalence of vector-borne diseases, particularly those that rely on human movement to sustain transmission (Liyanage *et al*, 2021). Although *Aedes* mosquitoes are most active during daylight hours, they can also bite indoors and under low-light conditions, making human behavior a key determinant of exposure risk (Mutebi *et al*, 2022).

In this context, dengue-negative febrile patients may serve as a useful sentinel population for detecting concurrent arboviral circulation, particularly infections such as CHIKV that might otherwise remain unrecognized. Thus, the present study aimed to determine whether CHIKV infection could be detected among dengue-negative febrile patients in the Klang Valley during the MCO period and to genetically characterize any identified viruses. By combining sentinel surveillance with genomic characterization, this

study provides valuable insight into CHIKV activity during a period of restricted human mobility and establishes a baseline for monitoring viral persistence and re-emergence in post-pandemic Malaysia.

MATERIALS AND METHODS

Location and specimens

A retrospective laboratory-based surveillance investigation was conducted on dengue-negative febrile cases presenting to the Universiti Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, during the COVID-19 MCO period from May 2020 and May 2021. Blood specimens (~5 ml) were collected from patients presenting with acute febrile illness clinically suspected to be dengue and stored at -80 ^{o}C until used.

Dengue virus (DENV) detection

Blood specimens were tested for dengue virus (DENV) RNA by qRT-PCR and for DENV NS1 antigen and anti- DENV IgM antibodies by ELISA as previously described (Teoh *et al*, 2016). All serum specimens that tested negative for these DENV biomarkers were used for CHIKV screening. Demographic and clinical information were not available because the specimens were anonymized before laboratory analysis.

RT-PCR CHIKV detection

Viral RNA was extracted from 140 µl aliquot of serum using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), and collected in 50 µl of RNase-free water. CHIKV screening was performed using an in-house pan-*Alphavirus* RT-PCR method targeting the conserved *nsP4* gene, yielding a 682-bp amplicon. The assay was validated for broad alphavirus detection and demonstrated adequate analytical sensitivity and specificity for the detection of medically important alphaviruses, including CHIKV (Sam *et al*, 2022). In brief, the assay used a forward (5′AAYTGCAAYGTIACICARATG3′) and reverse (5′RAACATICCIGAYTTCATCAT3′) primer pair, where I = Inosine,

R = G/A and Y = C/T, which was designed based on the genetic sequences of several alphaviruses, namely Barmah Forest virus, CHIKV, Eastern equine encephalitis virus, Getah virus, Mayaro virus, Middelburg virus, Ndumu virus, O'nyong-nyong virus, Ross River virus, Semliki Forest virus, Sindbis virus, Una virus, Venezuelan equine encephalitis virus, and Western equine encephalitis virus (Sam *et al*, 2022). RT-PCR was performed using the Access RT-PCR System (Promega, Madison, WI), with inclusion of positive (in-house CHIKV RNA) and negative (RNase-free water and in-house DENV RNA) controls. The identities of the *nsP4* RT-PCR amplicons were determined by Sanger sequencing (Thermo Fisher Scientific, Waltham, MA).

Next-generation sequencing (NGS) and *de novo* genome assembly

RNA was extracted from CHIKV RT-PCR-positive specimens, followed by reverse transcription and amplification using random hexamers designed to achieve complete genome coverage. Library preparation, quality control and sequencing were performed on an Illumina NovaSeq 6000 platform (paired-end, 250 bp reads) by a commercial service provider (Apical Scientific, Selangor, Malaysia). One hundred and six raw reads were generated, filtered (to a Phred quality threshold score of 20) and adapters trimmed using Trim Galore! (v0.6.10) (Babraham Institute, Cambridge, UK) with default parameters and *de novo* assembled using Trinity (v2.15.2) (Grabherr *et al*, 2011). From the assembled transcripts, sequences longer than 10 kb were used to identify those representing near-complete CHIKV genomes. Subsequently, raw reads were aligned to the assembled genome to estimate mean coverage, confirming sufficient read depth across all genomic positions. Genome completeness was then evaluated using CheckV (version 1.0.3) and the CheckV database version 1.5 (Nayfach *et al*, 2021).

Phylogenetic analysis

The assembled genome was aligned with a representative dataset of CHIKV sequences from GenBank. A maximum-likelihood phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 12.1 to determine genotype and relatedness to other strains (King and Van Doorslaer, 2018). The best-fit nucleotide substitution model was the General Time Reversible with Gamma distribution and invariant sites (GTR+G5+I), as selected by model selection analysis available in MEGA. Statistical confidence of each tree node was determined using 1,000 bootstrap replicates.

Statistical analysis

A descriptive statistical analysis was used to estimate the detection proportion of CHIKV among dengue-negative specimens. The detection proportion was calculated as the number of CHIKV-positive specimens divided by the total number of dengue-negative specimens tested. The 95% confidence interval (CI) for the detection proportion was estimated using the Clopper-Pearson exact method.

Ethical considerations

The study protocol was approved by the Medical Research Ethics Committee, Universiti Malaya Medical Centre (MRECID no. 2021216-9834). No prior written consent was required as the study involved the collection of secondary patient blood specimens, which were anonymized before data acquisition.

RESULTS

Nine hundred and seventeen serum specimens from dengue-negative febrile patients were screened for CHIKV between May 2020 and May 2021. Specimen collection was performed throughout the majority of the months of the study period, with between 30 and 93 specimens tested per month, except in July 2020, when sampling was temporarily suspended due to

the increased COVID-19-related clinical workload (Table 1). Among the dengue-negative specimens screened, only one specimen (0.1%, CI: 0.003-0.61%), collected in May 2020, tested positive for CHIKV by the pan-*Alphavirus* RT-PCR assay. Demographic and clinical information for the positive case were not available because specimens were anonymized before laboratory analysis.

Sanger sequencing of the RT-PCR amplicon confirmed CHIKV infection, and the partial *nsP4* sequence was identified as belonging to the ECSA genotype (Genbank accession no. PX467505). Subsequent NGS generated ~10^6 paired-end reads, which, after quality filtering and assembly, resulted in ~11.6 kb consensus CHIKV genome. The average coverage was 67,457.8×, indicating high confidence in the assembly. CheckV analysis further confirmed the assembled CHIKV genome as being of high quality, with 100% completeness and no detectable contamination (not shown). Phylogenetic analysis indicated that the isolate indeed belonged to the ECSA genotype and clustered closely with strains previously reported from Myanmar and Thailand (Fig 1). This indicated that the CHIKV isolate most likely came from a preexisting regional lineage rather than from a recent introduction outside Southeast Asia. Analysis of the E1 and E2 coding regions revealed three amino acid substitutions of epidemiological relevance, namely E1-E211, E1-A226 and E2-A264. The genome sequence was deposited in the GISAID database (accession no. EPI_ISL_20199533).

DISCUSSION

The detection of a single CHIKV-positive case among 917 dengue-negative febrile patients during Malaysia's MCO period was consistent with reports from other settings, which show reduced CHIKV prevalence during COVID-19 restriction periods (Mayilsamy *et al*, 2023). Although the present study was not designed to directly quantify

Table 1

Blood specimens from dengue-negative febrile patients, Universiti Malaya Medical Centre, Kuala Lumpur, Malaysia, May 2020 - May 2021

Month year	Number of collected blood specimen	Pan-*Alphavirus* RT-PCR result
May 2020	90	+ (1 specimen)
June 2020	80	-
July 2020	0*	-
August 2020	87	-
September 2020	93	-
October 2020	82	-
November 2020	83	-
December 2020	87	-
January 2021	87	-
February 2021	72	-
March 2021	83	-
April 2021	30	-
May 2021	43	-
Total	917	1 positive (1/917, 0.1%)

*Blood specimen collection was postponed in July 2020 due to a surge of COVID-19-related hospitalizations

+: positive; -: negative

COVID-19: coronavirus disease 2019; RT-PCR: reverse transcription-polymerase chain reaction

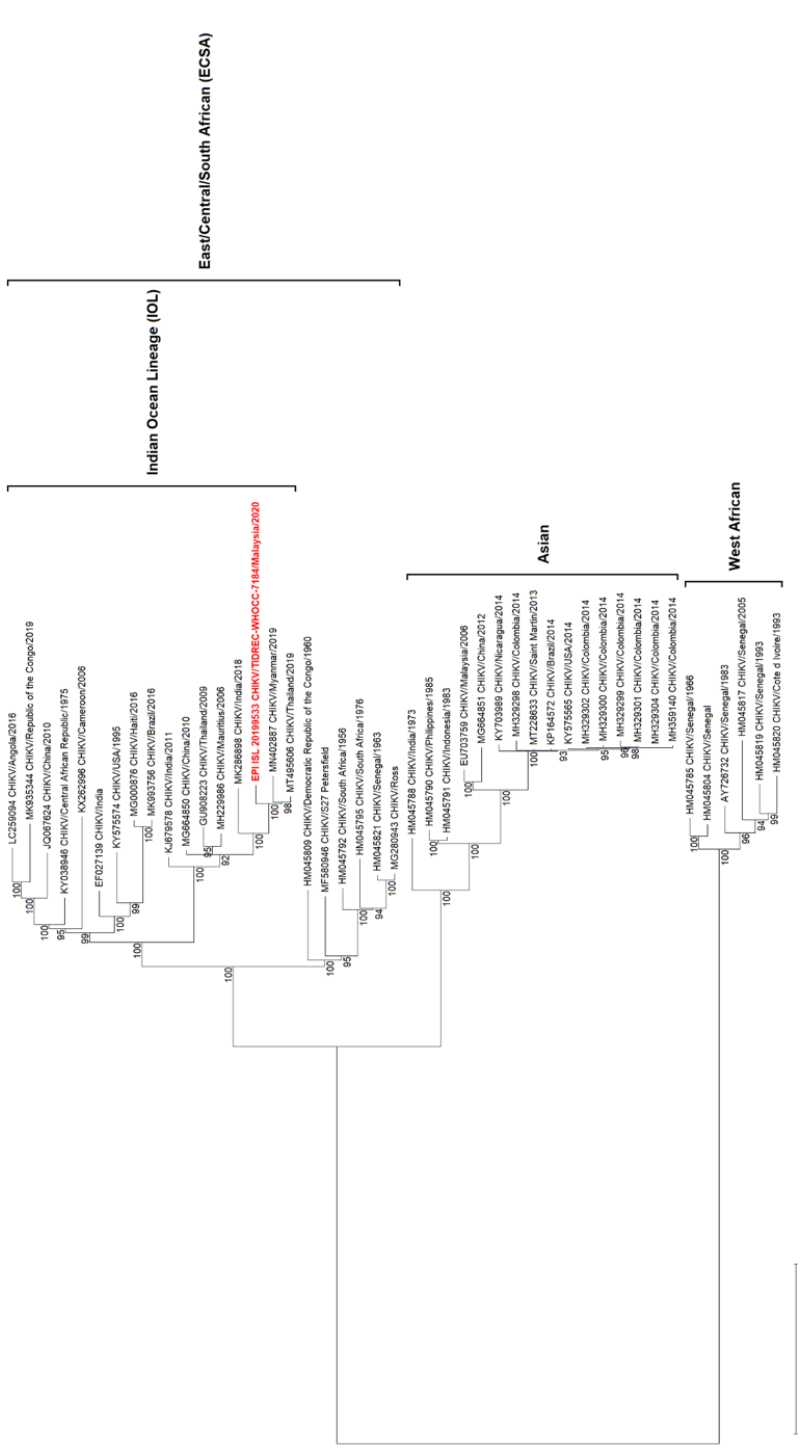

Fig 1 - Maximum-likelihood phylogenetic tree of chikungunya virus complete genome sequences, including reference sequences from GenBank

The tree was inferred under the General Time Reversible with gamma distribution and invariant sites (GTR+G5+I) model. Bootstrap values (1,000 replicates) are shown at the nodes. Scale bar indicates number of substitutions per site. The sequences are grouped into three distinct genotype clusters - East/Central/South African, Asian, and West African (indicated on the right). The isolate from the present study is shown in red.

the transmission dynamics, the detection of a single case suggested that CHIKV circulation, if present, occurred at very low levels within the study population during this MCO period. The very low CHIKV detection rate observed in the present study was consistent with the relatively low number of cases reported by the Ministry of Health, Malaysia, during the same period (n = 23 cases in 2020) (Ministry of Health Malaysia, 2020a).

The transmission ecology of CHIKV provides a plausible explanation: both *Ae. aegypti* and *Ae. albopictus* have limited flight ranges (typically <200 m); therefore, CHIKV relies heavily on human movement to facilitate transmission (Mourad *et al*, 2022). Consequently, reductions in human mobility during the MCO period might have reduced opportunities for virus dissemination between transmission foci, although this inference remains indirect. A similar hypothesis was proposed in other settings where COVID-19 restrictions coincided with reduced arboviral transmission (Liyanage *et al*, 2021).

Although household transmission remained possible, the lack of repeated external introductions likely limited the outbreak during this period. In addition, CHIKV infection generally induces durable, long-lasting immunity, and reinfections are considered uncommon (Yoon *et al*, 2015). Pre-existing community-level seroprevalence from previous Malaysian outbreaks (Sam *et al*, 2009) might, therefore, have reduced the pool of susceptible individuals, contributing to the very low number of detected symptomatic cases during the MCO period.

The genome characterization of the detected ECSA lineage provided critical epidemiological information. The ECSA genotype has dominated Malaysian CHIKV outbreaks since its introduction in 2008 (Sam *et al*, 2012; Kalyanasundram *et al*, 2024). Its persistence at low levels during the COVID-19 pandemic suggested an ongoing low-intensity

transmission, likely sustained within urban or peri-urban settings. Importantly, the CHIKV isolate of the present study exhibited close genetic relatedness to ECSA-IOL strains previously circulating in Myanmar and Thailand, indicating that it likely descended from a preexisting regional lineage rather than a new introduction (Chinnawirotpisan *et al*, 2020; Su *et al*, 2023). However, given that only a single CHIKV case was detected, these conclusions regarding the extent of ongoing transmission within the Klang Valley should be interpreted with caution.

The single CHIKV isolate in the present study carried three amino acid substitutions, namely E1-E211, E1-A226 and E2-A264, that are of epidemiological significance. The E1-A 226 represents the ancestral clade. The paired mutations E1-K211E and E2-V264A were shown to increase CHIKV infectivity and dissemination in *Ae. aegypti* (Agarwal *et al*, 2016). This mutation profile was documented in ECSA strains circulating in India, Myanmar and Thailand over the past decade (Agarwal *et al*, 2016; Chinnawirotpisan *et al*, 2020; Su *et al*, 2023). The presence of these latter substitutions in the single CHIKV isolate was consistent with the previously reported regionally circulating ECSA lineages associated with urban transmission cycles in Southeast Asia.

Importantly, the detection of this lineage in May 2020 predated the genome sequences reported by Kalyanasundram *et al* (2024) from Malaysian cases in 2021, which also featured mutations characteristic of regionally circulating ECSA-IOL-associated strains. This suggested that the regionally adapted ECSA lineage was already present in the Klang Valley earlier than documented. Moreover, while Kalyanasundram *et al* (2024) reported substantial nationwide prevalence (5,024 cases) from 2019-2021, detection of CHIKV in dengue-negative febrile cohort in the present was extremely low (0.1%) during the strict MCO period. This discrepancy might

reflect spatial heterogeneity in transmission intensity, differences in surveillance design or a reduced healthcare-seeking behavior during the pandemic.

Complete genome sequencing enables differentiating between viral endemic persistence and viral reintroduction, identifying adaptive mutations that may influence transmissibility and establishing a molecular baseline for future outbreak investigations (Fischer *et al*, 2019). The CHIKV sequence detected in the present study contributed to Malaysia's virus genome reference dataset and provided a benchmark for tracking potential evolutionary changes in local CHIKV lineages in the post-COVID-19 pandemic period. As population movement normalizes, integrating molecular diagnostics, residential surveillance and genome sequencing into Malaysia's arbovirus monitoring framework will be essential for early detection and rapid response to future outbreaks.

The present study had several limitations that should be acknowledged. Firstly, the investigation was conducted at a single tertiary medical center, which may limit the usefulness of the finding to the overall Klang Valley or the nation. Secondly, the surveillance strategy focused exclusively on dengue-negative febrile patients, and, thus, might not represent asymptomatic or mildly symptomatic CHIKV infections occurring in the test community. Thirdly, the identification of only one positive case limits the ability to draw robust conclusions regarding transmission dynamics during the MCO period. And fourthly, demographic and epidemiological information for the detected case was not available because the specimens were anonymized prior to laboratory analysis, preventing further investigation of possible exposure or transmission sources.

Despite these limitations, the study highlighted the value of dengue-negative febrile illness surveillance as a practical approach

for detecting other circulating arboviruses with overlapping clinical presentations. Future surveillance strategies should benefit from expanding testing within dengue-negative febrile illness cohorts across multiple healthcare facilities, complemented by integrated vector surveillance and genome monitoring of circulating viruses. In addition, seroprevalence studies and longitudinal genome surveillance may help clarify the extent of silent CHIKV transmission and improve understanding of viral persistence and re-emergence following large-scale mobility disruptions.

In conclusion, CHIKV infection was extremely rare among non-dengue febrile patients at a hospital in Kuala Lumpur during Malaysia's Movement Control Order period, with only one confirmed case detected (0.1%) among the specimens tested. The identified ECSA strain indicated the low-level persistence of a regional endemic lineage rather than a new viral introduction. While definitive conclusions on transmission could not be drawn, the finding suggested that the reduced human mobility might have limited the opportunities for CHIKV spread, contributing to the observed low level of infection.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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