

## Epidemic outbreak of acute haemorrhagic conjunctivitis caused by coxsackievirus A24 in Thailand, 2014

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Received 4 December 2014; Final revision 2 March 2015; Accepted 11 March 2015;  
first published online 31 March 2015

### SUMMARY

Acute haemorrhagic conjunctivitis outbreaks are often attributed to viral infection. In 2014, an unprecedented nationwide outbreak of infectious conjunctivitis occurred in Thailand, which affected >300 000 individuals over 3 months. To identify and characterize the virus responsible for the epidemic, eye swab specimens from 119 patients were randomly collected from five different provinces. Conserved regions in the enteroviral 5'-UTR and adenovirus hexon gene were analysed. Enterovirus was identified in 71·43% (85/119) of the samples, while no adenovirus was detected. From enterovirus-positive samples, the coxsackievirus A24 variant (70·59%, 84/119) and echovirus (0·84%, 1/119) were identified. Additional sequencing of full-length *VPI* and *3C* genes and subsequent phylogenetic analysis revealed that these clinical isolates form a new lineage cluster related to genotype IV-C5. In summary, the coxsackievirus A24 variant was identified as an aetiological agent for the recent acute haemorrhagic conjunctivitis outbreak in Thailand.

**Key words:** Conjunctivitis, coxsackievirus A24, enterovirus, epidemic, outbreak, Thailand.

### INTRODUCTION

Acute haemorrhagic conjunctivitis (AHC) is highly contagious and transmitted via direct or indirect contact with eye secretions [1]. Symptoms of conjunctivitis include ocular pain, swelling of the eyelids, irritation and eye discharge. Outbreaks are often associated with close contact in the community setting,

such as schools, prisons and swimming pools. Viral conjunctivitis generally persists for 3–7 days before resolving spontaneously. Major outbreaks of AHC are often attributed to adenoviruses, enterovirus 70 (EV70) and coxsackievirus A24 variant (CVA24v) [2, 3]. Many countries have reported extensive outbreaks of AHC due to CVA24v [4–8], a member of the enterovirus species C group initially isolated during an epidemic in Singapore [9, 10].

CVA24v is a non-enveloped plus-stranded RNA virus with a genome of ~7400 bp [11]. The virus belongs to the genus *Enterovirus* in the family Picornaviridae. The genomic RNA is translated into

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a single polyprotein, which is catalytically processed by the viral protease into four structural capsid proteins and seven non-structural proteins [12]. The capsid proteins (VP1–VP4) assemble to form an icosahedral virion. The external VP1 capsid protein is under constant evolutionary pressure to induce changes in the neutralization epitope for evasion of the host immune response.

Traditionally, antisera are used for viral neutralization detection of enterovirus serotypes, but this assay is time-consuming, costly, and requires large sample volumes. Moreover, new strains are often untypable due to accumulated changes on the capsid protein. Molecular methods, such as polymerase chain reaction (PCR) and reverse transcription (RT)–PCR, are feasible diagnostic tools that may replace conventional cell culture methods [6]. For molecular epidemiological analysis of enteroviruses, VP1 and 3C protease regions can be used to identify distinct genotypes, which would facilitate accurate and rapid determination of the virus species involved in outbreaks.

During the rainy season of 2014, an outbreak of AHC occurred throughout Thailand. The Ministry of Public Health documented a significantly greater than usual number of AHC cases, beginning in July. By August, >100 000 individuals had been affected. The number of affected patients (from 1 January to 31 December) with infectious conjunctivitis from all 77 provinces of Thailand reached 447 781 cases. Epidemiological data and molecular methods were used to determine viral aetiology in the current study.

## METHODS

### Epidemiological data

The number of conjunctivitis cases from different provinces of Thailand was compiled from infectious disease reports sent to the Ministry of Public Health from provincial hospitals and clinics in all 77 provinces. The data were retrieved from the Bureau of Epidemiology online database (<http://www.boe.moph.go.th/index.php?nphss=nphss>).

### Clinical samples

The study protocol was approved by the Ethics Committee of the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB 418/57). In total, 119 conjunctivitis swabs were collected from patients, who attended the outpatient

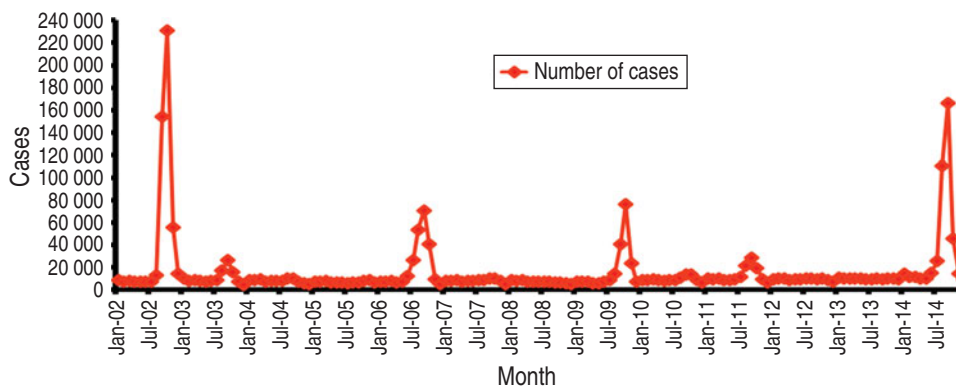
clinic between 8 and 19 September 2014, with a clinical diagnosis of AHC and sought medical care 2–5 days after the onset of symptoms. Specimens were collected from 50 males and 69 females at Bueng Kan Provincial Hospital (Bueng Kan), Chum Phae Hospital (Khon Khen), Thai Health Promotion Foundation of Roi Et (Roi Et), Thonburi 2 Hospital (Nakhon Pathom) and Bangpakok 9 International Hospital (Bangkok). Specimens from Nakhon Pathom and Bangkok were convenient samples sent to Chulalongkorn University for testing. Samples were obtained from individuals of all ages (infants to the elderly). The affected eyes were swiped with sterile cotton swabs that were subsequently placed in 1 ml viral transport medium containing antibiotics ( $2 \times 10^6$  U/l penicillin G and 200 mg/l streptomycin).

### Adenovirus detection

Viral genome extraction was performed using the Exgene Viral DNA/RNA kit (GeneAid, South Korea) according to the manufacturer's instructions. A 956 base-pair fragment of the human adenovirus (HAdV) hexon gene was identified using nested PCR. Primers for first-round PCR were ADV\_FO (5'-AYG CYA MCT TYT TYC CCA TGG C-3') and ADV\_R1 (5'-GTR GCG TTR CCG GCN GAG AA-3'). Primers for second-round PCR were ADV\_F2 (5'-TTY CCC ATG GCN CAC AAC AC-3') and ADV\_R2 (5'-GYT TCR ATG AYG CCG CGG TG-3'). PCR conditions were 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1.45 min, with final extension at 72 °C for 10 min. A stool sample containing adenovirus genotype 8 served as a positive control [13].

### Pan-enterovirus detection

Extracted RNA samples were subjected to cDNA synthesis using random hexameric primers and the ImProm-II Reverse Transcription System (Promega, USA). Pan-enterovirus real-time PCR was used for initial screening [14]. Additional pan-enterovirus semi-nested PCR was employed to amplify the highly conserved 5'-UTR using the first primer pair, CU-EVF2760 (5'-ATG GKT ATG YWA AYT GGG ACA T-3') and CU-EV3206 (5'-CCT GAC RTG YTT MAT CCT CAT-3'), and second primer pair, CU-EVF3029 (5'-TTC ATG TCR CCW GCS AGT GC-3') and CU-EV3206. Both amplification reactions were performed under the following



**Fig. 1.** Incidence of acute haemorrhagic conjunctivitis (AHC) in Thailand between January 2002 and December 2014. Data compiled by the Ministry of Health, Thailand were obtained from physicians and healthcare workers from all 77 provinces. The majority of AHC cases were reported between August and October. July–October coincides with the local rainy season.

conditions: 95 °C for 3 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

#### Additional characterization of CVA24

Specimens that tested positive for pan-enterovirus 5'-UTR were sequenced to identify the enterovirus genotype. CVA24-positive samples were further characterized by additional PCR and sequencing of full-length VP1 and 3C regions. The PCR primer sets used were CA24\_VP1\_F (5'-CACAGAGAACTTTGTTTGCG-3') and CA24\_VP1\_R3417 (5'-CCTC CAAAGTATTAATGTTTTC-3') for VP1 and CA24\_3C\_F (5'-ACCATTAGAACAGCAAAGG TG-3') and CA24\_3C\_R6047 (5'-CTTTTGATGG TCTCATCCATT-3') for 3C. Both amplification reactions were performed under the following conditions: 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1:30 min, with a final extension at 72 °C for 10 min.

#### Sequence and phylogenetic analyses

Sequencing results were analysed with Chromas Lite v. 2.01 ([http://www.technelysium.com.au/chromas\\_lite.html](http://www.technelysium.com.au/chromas_lite.html)) and BioEdit v. 7.0.4.1 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and subjected to BLAST search (<http://blast.ncbi.nlm.nih.gov/>) to identify the viral sequence. Nucleotide sequences were submitted to the GenBank database under accession numbers KP 121936–KP122019 for 5'-UTR, KP122020–KP122090 and KP137044–KP137046 for

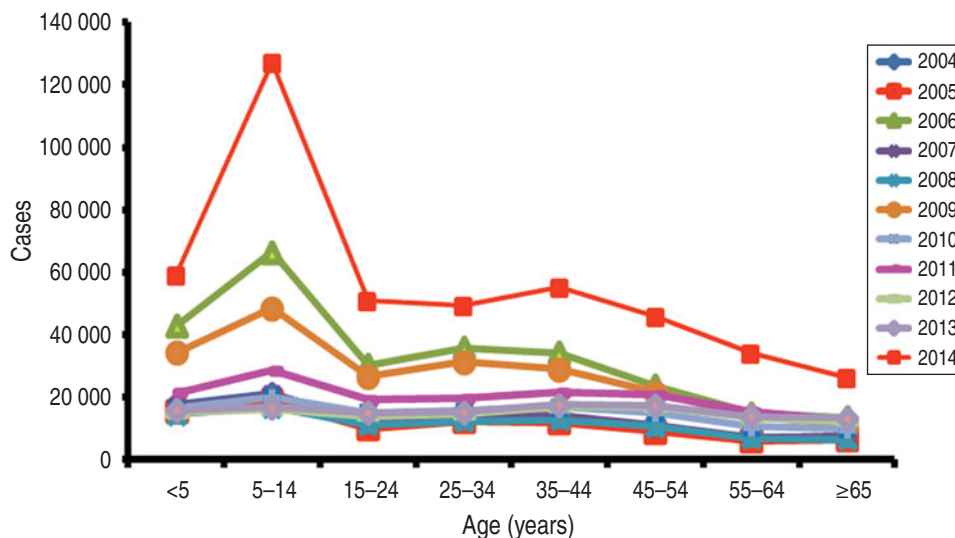
the VP1 gene, and KP122091–KP122162 and KP137042–KP137043 for the 3C gene.

Phylogenetic trees were generated using Clustal W alignments of nucleotide sequences. The neighbour-joining method was implemented in MEGA v. 5 (<http://www.megasoftware.net/>) with bootstrap resampling values of 1000 replicates.

## RESULTS

Outbreak reports of AHC in Thailand compiled by the Ministry of Public Health from 2002 to 2012 demonstrate a cyclical and seasonal pattern of 'pink eye', especially during the rainy season between July and October every 2–3 years (Fig. 1). In July 2014, however, the Ministry of Health in Thailand reported an unusually higher than expected monthly incidence of AHC compared to the last 10 years. In these years, monthly incidences were below 100 000 affected individuals and peaked between July and October. However, the number of AHC cases in August 2014 increased markedly, exceeding 160 000 in September. Although individuals of all ages were susceptible, the highest incidence was found in the 5–14 years age group (28.39%), followed by the <5 years (13.20%) and 35–44 years (12.30%) age groups (Fig. 2).

In view of its rapid spread, viral conjunctivitis was suspected. Sequence-specific PCR analysis of the 119 samples did not detect adenovirus nucleic acids. However, 71.43% (85/119) tested positive by pan-enterovirus PCR. Subsequent enterovirus species-specific PCR analyses led to the identification of CVA24 in 84 specimens and echovirus in one



**Fig. 2.** Reported cases of acute haemorrhagic conjunctivitis in Thailand between 2004 and 2014 classified by age group. The highest incidence was observed in patients aged between 5 and 14 years.

specimen. CVA24-positive samples were further confirmed by full-length amplification of *VP1* and *3C* genes. Phylogenetic analyses of the *VP1* and *3C* genomic sequences and comparison with other clinical isolates and reference strains for which sequences were available in the GenBank database were performed. In both *VP1* and *3C* phylogenetic trees, the Thai isolates grouped together with genotype IV (GIV) and shared highest sequence identities with GIV-C5A and GIV-C5B lineages (Figs 3 and 4).

## DISCUSSION

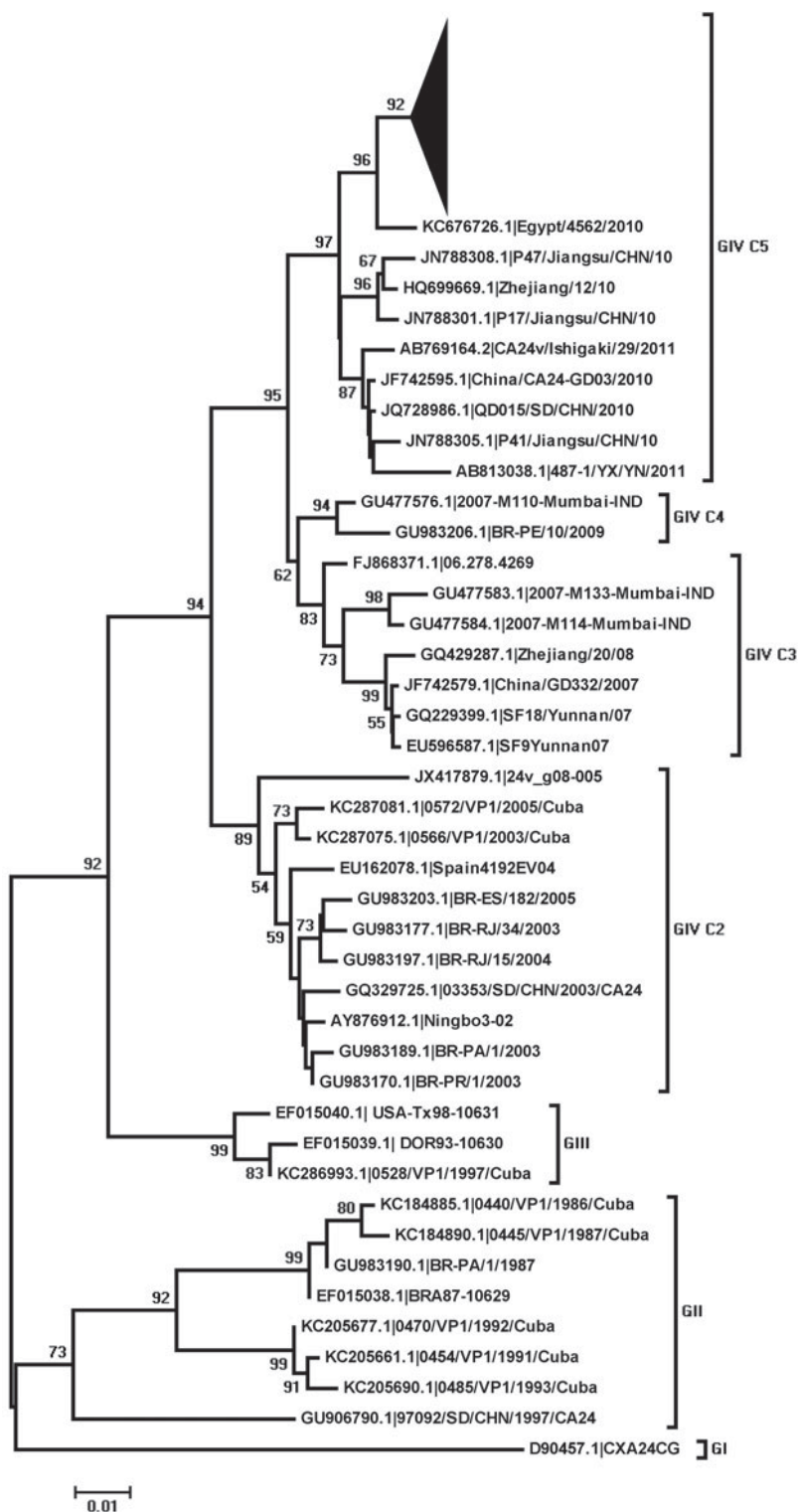
Viral conjunctivitis in Thailand occurs throughout the year, but increases during the rainy season. No vaccines or antivirals are available to prevent or treat conjunctivitis, but AHC generally self-resolves and requires no further treatment. The majority of patients from this study presented mild symptoms and were prescribed eye drops for redness relief. Overall, the calculated infection rate was 94.70/100 000 in the population and no associated mortality was reported. In decreasing order, the highest incidence rates of the 2014 conjunctivitis outbreak in Thailand occurred in the northeast, north, central, and south regions, respectively. The highest rates were in the provinces of Amnat Charoen (1900.16 cases/100 000), Prachin Buri (1600.35 cases/100 000), Buri Ram (1571.48 cases/100 000), Ubon Ratchathani (1348.39 cases/100 000) and Maha Sarakham (1321.27 cases/100 000) [15]. Four of these provinces are located in northeast

Thailand where the most severe outbreaks were reported.

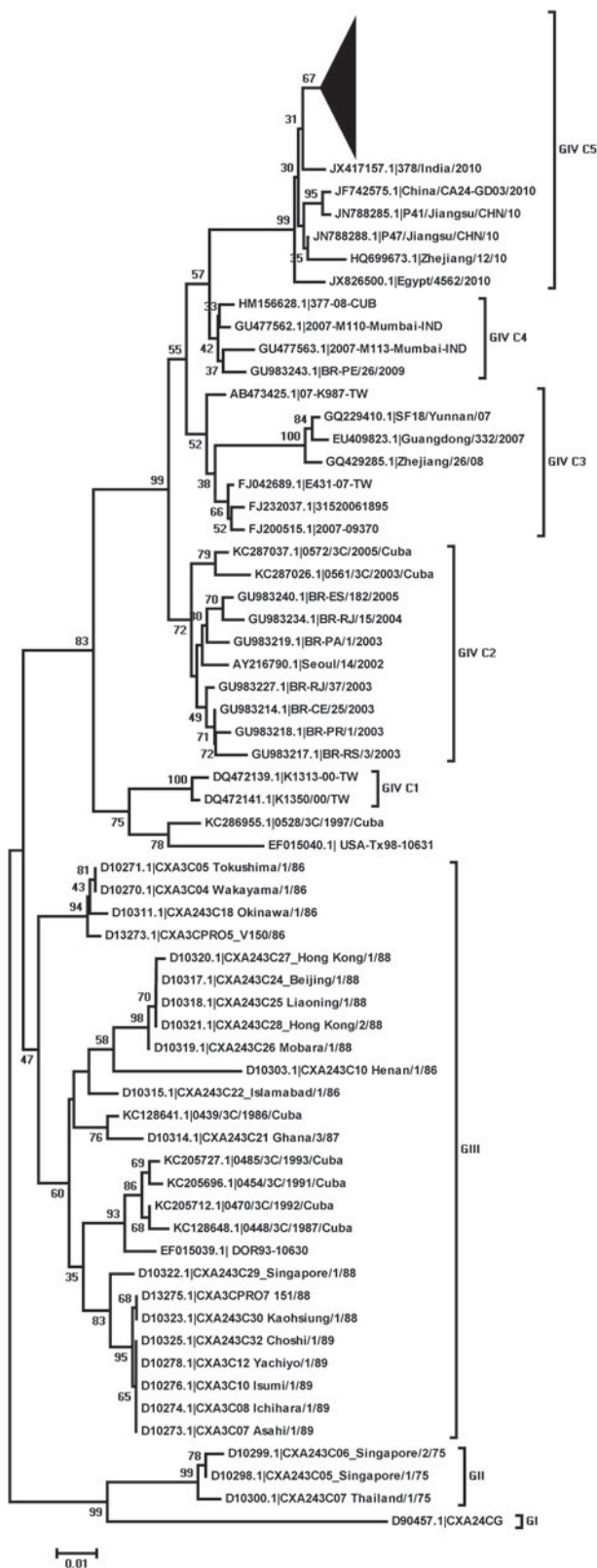
Previous AHC outbreaks appear to be cyclical. The morbidity rates of 842.58 (2002), 417.53 (2006) and 342.57/100 000 (2009) during the rainy months differed significantly to the mean morbidity rate of <200/100 000 during the rest of the year. Notably, the risk for conjunctivitis increased markedly for children. This observation coincides with the compulsory schooling of children beginning in the first grade, which places them in the community setting where the risk of disease exposure is high.

Rapid dissemination of infectious conjunctivitis often implicates adenovirus or enterovirus in the outbreak [7, 16–17], but CVA24 was the only virus predominantly associated with conjunctivitis in the current study. Its initial isolation in 1970 and limited circulation in India and Southeast Asia prior to 1985 were followed by eventual worldwide spread [18]. In 1992, the variant was identified in Thailand, and shown to be the major cause (76.8%) of AHC via assessment of the neutralizing antibody [19]. Until now, no reports of CVA24 identification in Thailand using molecular methods have been documented.

Previous studies have identified CVA24 variants via phylogenetic analyses of the *VP1* capsid, *3C* protease, and RNA polymerase regions [20]. Both *VP1* and *3C* phylogenetic tree data showed that all clinical isolates from this study belonged to GIV, a recently diverged group separate from other previously characterized strains. CVA24 classified into four genotypes (I–IV)



**Fig. 3.** Phylogenetic analysis of full-length VP1 nucleotide sequences of coxsackievirus A24 (CVA24). Phylogenetic trees were produced using Clustal W alignments and the neighbour-joining method implemented in MEGA v. 5. Strains identified in this study are shown as one cluster located on the top of the tree (black arrowhead). Bootstrap resampling values are indicated at the nodes. The scale bar indicates the number of substitutions per site.



**Fig. 4.** Phylogenetic analysis of the full-length 3C nucleotide sequences of coxsackievirus A24 (CVA24). Strains identified in this study are shown as one cluster located on the top of the tree (black arrowhead).

and genotype clusters (C1–C5). The VP1 phylogenetic tree consisted of the prototype strain (GI, EH24/70), strains from 1987 to early 1990s (GII), late 1990s (GIII), and those isolated between 2003 and 2010 (GIV). Moreover, GIV was further subdivided into several clusters, depending on the isolation dates (GIV-C2 for 2003–2005 and GIV-C5 for 2006–2010). The phylogenetic tree of the 3C nucleotide sequences also showed four distinct genotypes. In addition to the GI reference strain, EH24/70 (accession no. D90457, [21]) and GII strains from Singapore and Thailand identified in 1975 [9], GIII included isolates from 1985 identified in Asia, Africa and France [22]. Strains from China (2007–2008) formed GIV-C3, while those from India (2007) and Brazil (2009) belonged to GIV-C4. The clinical isolates identified in this study clustered into GIV-C5 and subclusters A and B (97.4–98.3% identity), similar to strains involved in the outbreaks of AHC in Taiwan, China, India, and Egypt [4–5, 8, 16, 23].

The extensive outbreak of AHC in Thailand in 2014 may be attributed to the failure to recognize CVA24 as an aetiological factor or CVA24 variants may have evolved in virulence, which allowed rapid spread of the virus. Increased vigilance in control, response guidelines and prevention may help to reduce the incidence of AHC in the future.

**ACKNOWLEDGEMENTS**

The authors thank the National Research University Project, Office of Higher Education Commission (WCU-001, 007-HR-57), The Outstanding Professor of Thailand Research Fund (DPG5480002), Chulalongkorn University Centenary Academic Development Project (CU56-HR01), Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (PHD/0196/2556), Rachadapisek Sompote Fund for Postdoctoral Fellowship, Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University (RES560530093), Research Chair Grant, National Science and Technology Development Agency, The Center of Excellence in Clinical Virology, Chulalongkorn University, and King Chulalongkorn Memorial Hospital. We also thank The Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health Thailand for the epidemiological data and Ms. Kanchana Kongchak for collection of specimens. Finally, thanks are also due to Ms. Angkana Chirapanuruk and Siwanat Thongkomplew for their excellent technical assistance.

## DECLARATION OF INTEREST

None.

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