Journal of Infection and Public Health 16 (2023) 1435–1442

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: www.elsevier.com/locate/jiph

Original Article

# Epidemiological evidence of acute transmission of Zika virus infection in dengue suspected patients in Sri-Lanka

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ARTICLE INFO

*Article history:*
Received 5 June 2023
Received in revised form 11 July 2023
Accepted 18 July 2023

*Keywords:*
Acute Zika virus infection
Molecular and serological detection
Dengue suspected
Patients
Sri Lanka

ABSTRACT

*Background:* Zika Virus (ZIKV) is a re-emerging, arthropod-borne flavivirus transmitted by *Aedes mosquitoes (Ae. aegypti and Ae. albopictus)*. The coexistence of dengue virus (DENV) and ZIKV concurrently has been associated with a wide array of neurological complications, which may influence the clinical outcomes of infections. Sri Lanka witnessed a severe dengue epidemic in 2017, characterized by extraordinary and severe disease manifestations with considerable morbidity. Therefore, this study assessed the potential occurrence of ZIKV infection during DENV outbreak in Sri Lanka from 2017 to 2019, which could bear substantial implications for public health.

*Methods:* Five hundred ninety-five serum samples were procured from individuals suspected of dengue and admitted to Kandy National Hospital between 2017 and 2018 and the Negombo District General Hospital between 2018 and 2019. These samples underwent quantitative real-time RT-PCR (qRT-PCR) to identify the presence of the ZIKV gene, while enzyme-linked immunosorbent assay was employed to detect ZIKV-specific IgM and IgG antibodies. Focus reduction neutralization tests were subsequently conducted to confirm ZIKV infection.

*Results:* Among the 595 serum samples, 6 (1.0%) tested positive for ZIKV using qRT-PCR. Anti-ZIKV IgM and IgG were identified in 18.0% and 38.6% patients. Sixty-six (11.0%) samples demonstrated the presence of anti-ZIKV IgM and IgG. Within ZIKV IgM-positive samples, 2.2% exhibited neutralizing antibodies against ZIKV. Through the implementation of qRT-PCR, ZIKV IgM detection, and neutralization testing, 2% and 3.7% cases of ZIKV infections were confirmed in the Kandy and Negombo regions, respectively.

*Conclusion:* This study is the inaugural endeavor to substantiate the existence of ZIKV infection in Sri Lanka utilizing molecular and serological analysis. The findings of this investigation imply that ZIKV was circulating throughout the 2017–2019 DENV outbreak. These results underscore the necessity for improved preparedness for future outbreaks, fortifying governmental policies on public health, and establishing effective early warning systems regarding the emergence of these viruses.



## Introduction

Zika virus (ZIKV) is a re-emerging, mosquito-borne flavivirus of African origin that is transmitted by *Aedes* mosquitoes [1]. ZIKV was historically limited to Africa and Asia, however, major breakthroughs occurred in Latin America [2]. However, ZIKV has recently been

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https://doi.org/10.1016/j.jiph.2023.07.014

responsible for major global outbreaks associated with adverse neurological disorders [3], motor disability [4], and teratogenic events [5]. ZIKV remains a global threat, with an increasing risk of spreading to immunologically-naïve human populations [6], highlighting the importance of early detection of ZIKV infections [7]. In this regard, the continued surveillance will provide useful information of outbreak trends and contribute to establish predictive models for early outbreak detection [8].

The rise of ZIKV infection has been observed in areas endemic for dengue virus (DENV) infection, as these viruses are transmitted by a common mosquito *Aedes* species, *Ae. aegypti and Ae. albopictus* [9]. Simultaneous co-circulation of DENV and ZIKV is associated with a wide range of neurological complications that are postulated to affect clinical outcomes of infections [9]. Moreover, this co-circulation among flaviviruses has increased the risk of outbreaks and arboviral co-infections in the human population [10].

During the largest outbreak of dengue in Sri Lanka in 2017, several patients, both children and adults, presented with unusual neurological manifestations associated with DENV infection [11]. Despite mosquito-borne infections being abundant in Asia, only three confirmed cases of congenital ZIKV infection with microcephaly have been reported in this region: two in Thailand and one in Vietnam [12]. Due to the structural similarities of the envelope proteins of members of the *Flaviviridae* family, such as yellow fever, West Nile virus, Japanese encephalitis virus (JEV) and Hepatitis C virus [13], current available serological assays are affected by cross-reactivity of different flavivirus antibodies [14]. Although the differential diagnosis for flavivirus infection has been widely explored [15,16], the lack of diagnostic capacity for an emergence of new arboviruses has created a diagnostic gap, jeopardizing the patient's status. And this gap is exacerbated in regions with a wide distribution of arbovirus diseases, where often the clinical diagnosis used as a confirmation of arboviral infection rather than laboratory diagnosis [17].

Currently, there exists limited data on ZIKV infection in Sri Lanka. However, ZIKV cases have been reported in neighbouring countries, including India, Maldives [18], Thailand and Vietnam [19]. The first ZIKV case was reported in 2017 in India, with significant outbreaks in 2018 in Rajasthan, Madhya Pradesh and Gujarat [20]. Additionally, ZIKV cases were recently confirmed in 2021 in Kerala, Maharashtra and Uttar Pradesh [21]. Thus, this study aimed to understand the impact of recent and past ZIKV infections of dengue-suspected patients who were admitted to (1) the Kandy National Hospital during the unprecedented 2017 dengue outbreak and (2) the Negombo District General Hospital during the 2018 – 2019 dengue outbreak in Sri Lanka.

## Materials and Methods

### *Virus strains and cell lines*

The virus strains used for the serological tests, namely IgM capture enzyme-linked immunosorbent assay (ELISA), IgG ELISA and neutralization tests, were as follows: MR 766 (ZIKV), 99St12A (DENV-1), 00st22A (DENV-2), SLMC50 (DENV-3), SLMC318 (DENV-4) and JaOrS982 (JEV). These viruses were propagated in C6/36-E2 mosquito cells to generate working stocks and were used to inoculate in Vero (African green monkey kidney epithelial cell line ATCC, CCL81) cell line for virus titration and neutralization tests, as previously described [15].

### *Study participants and sample collection*

Patients with less than seven days of fever and at least one symptom listed following; headache, joint pain, myalgia, abdominal pain, vomiting, rash, nausea, fatigue or diarrhoea, were recruited in this study. Patients who presented connective tissue disorder, severe anaemia and bleeding disorders were excluded in this study. From March 2017 –January 2018, 295 serum samples were collected from dengue-suspected patients who were admitted to the Kandy National Hospital during the largest dengue outbreak in Sri Lanka (Fig. 1). During April 2018 –November 2019, 300 serum samples were collected from dengue-suspected patients who were admitted to the Negombo District General Hospital in Sri Lanka (Fig. 1). Kandy National Hospital is 2nd largest hospital in Sri Lanka and located in central part of country. Negombo District general Hospital is located in west coast of Sri Lanka. It covers part of Gampaha District, which is highly populated area. Both Hospitals accepted to attend children and adult patients and it has specialist unit for management of dengue suspected patients.

### *Real time and conventional RT-PCR*

For ZIKV RNA extraction, total RNA was purified from 70 µl of serum sample by using the QIAamp®viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer protocol. Quantitative real time PCR (qRT-PCR) was conducted using the TaqMan Fast Virus 1-step master mix kit. A total of 20 µl qRT-PCR mixture consisted of 5 µl of RNA, 5 µl of Taqman Master Mix, 8.75 µl of nuclease water, 0.5 µl of 10 µM forward and reverse primers, 0.25 µl of 10 µM probe (ZIKV 835 (forward): TTGGTCATGATACTGCT GATTGC, ZIKV 911(reverse): CCTTCCACAAAGTCCCTATTGC, ZIKV 860-FAM (probe): CGGCATACAGCATCAGGTGCATAGGAG) [22]. A cycle threshold value of < 40 was considered as ZIKV positive for qRT-PCR. Conventional RT-PCR was done by using Prime Script One step RT-PCR kit (Takara Bio, Shiga, Japan) following the manufacturer's instructions. A total of 25 µl of PCR reaction contained 5 µl of RNA, 1 µl of Prime Script enzyme mix, 13 µl of water and 1 µl of 10 µM forward and reverse primers (ZIK-X1 4939: AGAGTGATAGGACTCTATGG, ZIKV-X2 5778: GTTGGCRCCCATCTCTGARATGTCAGT) [23].

### *IgM capture ELISAs*

In-house IgM capture ELISAs were performed following the previously-described protocol [15]. Briefly, all wells of the 96-well microplates, excluding the blank (Nunc Maxisorp, Roskilde, Denmark) were coated with 5.5 µg/100 µl of anti-human IgM Goat IgG antibody (Cappel, Aurora, USA) in ELISA coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6, containing 0.02% sodium azide) at 4 °C overnight. Block ACE reagent was added to all wells, excluding blank wells, for 1 h at room temperature (RT). Wells were then washed three times with PBS-T (phosphate-buffered saline containing 0.05% Tween 20), and serum samples as well as negative and positive controls (NC and PC, respectively) prediluted 1:100 in PBS-T were added to the 96-well plate in duplicate and incubated at 37 °C for 1 h. After incubation, plates were washed as previously described. ZIKV or tetravalent DENV antigens were added, after which the plates were incubated at 37 °C for 1 h. After washing, 100 µl of horseradish peroxidase (HRP)-conjugated anti-flavivirus mouse monoclonal antibody (12D11/7E8) at a 1:1500 dilution was added for both anti-ZIKV and -DENV IgM capture ELISA, and plates were incubated at 37 °C for 1 h. Finally, 5 mg of *o*-phenylenediamine hydrochloride (OPD) substrate (Sigma, St. Louis, USA) with 0.03% hydrogen peroxide and 0.05 M citrate phosphate buffer (pH 5.0) in 10 ml was added, and plates were kept in the dark at RT for 30–60 min. The reaction was stopped by adding 100 µl of 1 N sulphuric acid to each well, and the optical density (OD) was read at 492 nm (Multiscan JX, model no. 353, Thermolab System, Tokyo, Japan). The sample/negative OD ratio (mean sample OD ÷ mean NC OD) ≥ 2.0 was considered positive.


**Fig. 1.** Location of National Hospital Kandy (red circle), Negombo Distric General and Columbo North general Hospitals (blue circles) in Sri Lanka where serum samples collected from dengue suspected patients during 2017–2019.

*IgG indirect ELISA*

An in-house, indirect IgG ELISA was developed to detect ZIKV-specific IgG in serum samples using purified ZIKV as the assay antigen. The protocol was adapted from previous study [15]. Briefly, all wells of the 96-well microplates, excluding blanks (Nunc Maxisorp, Roskilde, Denmark), were coated with 250 ng/100 µl of ZIKV antigen in coating buffer overnight at 4 °C. Block ACE was added to all wells, except the blank wells, for 1 h at RT. Wells were then washed three times with PBS-T, and serum samples, NCs and PCs were prediluted 1:1000 in PBS-T, added in duplicate and incubated at 37 °C for 1 h. Plates were washed as previously described, and 100 µl HRP-conjugated goat anti-human IgG (American Qualex, San Clemente, USA) at 1:20000 dilution in PBS-T was added to the wells and incubated for 1 h at 37 °C. After washing, OPD substrate solution was added and incubated in the dark at RT for 30 – 60 min. Reactions were stopped with 1 N sulphuric acid and the plate was immediately read at 490 nm. IgG titers of serum samples were determined from the standard curve that was obtained from 2-fold serial dilutions of prediluted 1:1000 PC serum up to $1:2^{13}$. A cut off value for a positive sample was ≥ 1:3000.

*Focus reduction neutralization test*

To confirm ZIKV infection, serum samples were examined for the ability to neutralize ZIKV, JEV and the four serotypes of DENV with a 90% focus reduction neutralization test ($FRNT_{90}$) as described in previous study [15]. Prior to testing, samples were heat-inactivated on 56 °C heat block for 30 min. Due to the limitation of serum volume, all neutralization tests began at a 1:40 dilution.

The serum samples were 2-fold serially diluted from 1:80 in minimum essential medium (MEM) with 2% fetal calf serum (FCS), and equal volumes of each virus at 60 focus-forming units were added. The mixture was then incubated at 37 °C with 5% $CO_2$ for 1 h to allow for virus-antibody neutralization reactions. After incubation, the mixture was added in duplicate to a 96-well plate containing confluent Vero cell monolayers and incubated at 37 °C with 5% $CO_2$ for 1 h to allow for virus adsorption. After incubation, the infected cells were overlaid with 1.25% methylcellulose 4000 (WAKO Pure Chemical Industries, Japan) in 2% MEM. The plates were incubated again at 37 °C with 5% $CO_2$ for 72 h for DENV, 48 h for ZIKV and JEV. After incubation, the virus inoculum was aspirated gently from each well, and the cells were fixed with 4% paraformaldehyde. Next, the cells were permeabilized and blocked as described in previous study [24]. A pooled-human serum samples with high titre of anti-flavivirus IgG at 1:1500 were added to each well and the cells were incubated at 37 °C with 5% $CO_2$ for 1 h [15]. Then, HRP-conjugated anti-human IgG (American Qualex, San Clemente, USA) was added as the secondary antibody. Finally, viral foci were detected by immunostaining with substrate 3,3′diaminobenzidine tetrahydrochloride (DAB, Wako, Japan), containing hydrogen peroxide, for 10 min. The reciprocal of the endpoint serum dilution that provided 90% or greater reduction in the mean number of foci relative to the mean number of the control well was considered the $FRNT_{90}$ titer.

*ZIKV case definition and interpretation of laboratory tests*

Considering the World Health Organization standards [25] and published study [15], a case was defined as confirmed ZIKV infection if laboratory tests demonstrated at least one of the following: (a) positive qRT-PCR for ZIKV, (b) positive for ZIKV IgM and positive for neutralization test (NT) only against ZIKV but not with other flaviviruses, or (c) positive for ZIKV IgM and positive for NT against ZIKV with a NT of ≥ 4 times compared with titers against other flaviviruses. If the clinical sample was positive for ZIKV IgM and had a positive NT against ZIKV with a NT < 4 times compared with titers against other flaviviruses, the case was considered a probable ZIKV infection.

## Results

*Molecular findings*

We conducted qRT-PCR to detect ZIKV RNA in patient serum from the two study areas in Sri Lanka. Of the 295 serum samples collected from the Kandy National Hospital, 4 (1.4%) were positive by ZIKV qRT-PCR. Of the 300 serum samples from Negombo District General Hospital, two (0.6%) was positive by ZIKV qRT-PCR in this study. However, detection of their ZIKV RNAs by conventional RT-PCR showed negative results.

*Serological findings*

There was positive serological evidence of ZIKV infection from the two study regions in Sri Lanka. Of the 295 serum samples collected from the Kandy National Hospital, 11 (3.7%) were positive ZIKV IgM which included 7 (2.4%) for IgM against both ZIKV and DENV, 4 (1.4%) for ZIKV only, and 102 (34.5%) were positive for IgG against ZIKV (Table 1). Of the 300 serum samples from Negombo District General Hospital, 96 (32%) were positive ZIKV IgM which included 69 (23%) for IgM against both ZIKV and DENV, 27 (9%) for ZIKV only, and 128 (42.6%) were positive for IgG against ZIKV (Table 1).

*Neutralizing antibody (Nab) levels*

To confirm ZIKV infection status and characterize it further, 295 and 300 serum samples from Kandy and Negombo study sites were analysed with 90% FRNTs. In Kandy samples, 3 (1%) samples were confirmed ZIKV cases since they were ZIKV IgM positive and had neutralization titres against ZIKV at least four times higher than those against all DENV serotypes and JEV (Table 2). For Negombo samples, 10 (3.3%) samples were confirmed ZIKV cases since they were ZIKV IgM positive and had neutralization titres against ZIKV at least four times higher than those against all DENV serotypes and JEV. One case (0.3%) was a probable ZIKV cases since it was ZIKV IgM positive and had neutralization titres against ZIKV at four times less than those against all DENV serotypes and JEV (Table 3).

**Table 1**
Antibody profiles of dengue suspected patients who enrolled in two hospitals in Sri Lanka during 2017–2019.

| Sample collection year | Sample collection site | Number of tested samples | ZIKV IgM (%) | ELISA positive samples | | |
|---|---|---|---|---|---|---|
| | | | | ZIKV IgM only (%) | DENV & ZIKV both IgM (%) | ZIKV IgG (%) |
| 2017–2018 | National Hospital Kandy | 295 | 11 (3.7) | 4 (1.4) | 7 (2.4) | 102 (34.5) |
| 2018–2019 | Negombo District General Hospital | 300 | 96 (32.0) | 27 (9.0) | 69 (23.0) | 128 (42.6) |

DENV=Dengue virus, ZIKV=Zika Virus, IgM= Immunoglobulin M, IgG=Immunoglobulin G.

**Table 2**
Confirmed ZIKV infection among dengue suspected patients in National Hospital Kandy during 2017–2018 dengue outbreak in Sri Lanka.

| sample ID | Collection date | Clinical Severity | Age | Sex | IgM (P/N) ratio | | ZIKV IgG titer | Neutralization titre ($FRNT_{90}$) | | | | | | ZIKV real time RT-PCR (copies/ml) | Diagnosis Interpretation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ZIKV IgM | DENV IgM | | ZIKV | DENV-1 | DENV-2 | DENV-3 | DENV-4 | JEV | | |
| S-1 | Jul-2017 | DWoWS | 19 | M | **8.6** | 1.3 | 2387 | **320** | < 40 | 40 | < 40 | < 40 | < 40 | 15,903 | confirmed ZIKV |
| S-2 | Jul-2017 | SD | 32 | M | 0.9 | 0.6 | 175 | **< 40** | < 40 | < 40 | < 40 | < 40 | < 40 | 55,970 | confirmed ZIKV |
| S-3 | Aug-2017 | SD | 55 | M | **2.7** | 0.6 | 214 | **< 40** | < 40 | < 40 | < 40 | < 40 | < 40 | 27,661 | confirmed ZIKV |
| S-4 | Oct-2017 | DWoWS | 38 | F | **9.9** | 5.9 | 6066 | **320** | 40 | < 40 | < 40 | < 40 | < 40 | ND | confirmed ZIKV |
| S-5 | Oct-2017 | SD | 45 | M | **6.9** | 1.9 | 7851 | **320** | 40 | < 40 | < 40 | < 40 | < 40 | ND | confirmed ZIKV |
| S-6 | Jan-2018 | SD | 22 | F | 1.4 | 0.7 | 9898 | **< 40** | < 40 | < 40 | < 40 | 80 | < 40 | 8094 | confirmed ZIKV |

DENV-1–4 =dengue virus serotype 1–4. ZIKV= Zika virus. JEV= Japanese encephalitis virus. DWoWS=dengue without warning signs. SD=severe dengue. M= male. F= female. FRNT90 = 90% focus reduction neutralization test. Bold font indicates highest IgM and neutralization value in Table.

**Table 3**
Confirmed and probable ZIKV infection among dengue suspected patients in Negombo District General Hospital during 2018–2019 dengue outbreak in Sri Lanka.

| sample ID | Collection date | Clinical Severity | IgM (P/N) ratio | | ZIKV IgG titer | Neutralization titer ($FRNT_{90}$) | | | | | | ZIKV real time RT-PCR (copies/ml) | Diagnosis Interpretation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ZIKV IgM | DENV IgM | | ZIKV | DENV-1 | DENV-2 | DENV-3 | DENV-4 | JEV | | |
| S-1 | Jun-2018 | DWoWS | 1.1 | 0.9 | 10,632 | < 40 | 40 | 80 | 40 | 80 | < 40 | 24,956 | confirmed ZIKV |
| S-2 | Oct-2018 | DWoWS | **2.9** | 2.1 | 5154 | **320** | < 40 | 80 | < 40 | < 40 | 40 | ND | confirmed ZIKV |
| S-3 | Nov-2018 | DWoWS | **3.3** | 0.4 | 35,960 | < 40 | 80 | 160 | 40 | 320 | 160 | 8294 | confirmed ZIKV |
| S-4 | Nov-2018 | DWoWS | **2.6** | 1.8 | 15,939 | **320** | 40 | < 40 | 40 | < 40 | < 40 | ND | confirmed ZIKV |
| S-5 | Nov-2018 | DWoWS | **5.0** | 2.9 | 13,982 | **1280** | < 40 | 160 | 160 | 40 | 160 | ND | confirmed ZIKV |
| S-6 | Apr-2019 | DWoWS | **2.7** | 1.8 | 11,113 | **320** | < 40 | < 40 | 40 | 40 | 40 | ND | confirmed ZIKV |
| S-7 | Sep-2019 | DWoWS | **12.0** | 5.7 | 87,723 | **1280** | 320 | 160 | 40 | 320 | 80 | ND | confirmed ZIKV |
| S-8 | Oct-2019 | DWWS | **3.5** | 2.3 | 27,136 | **5120** | 80 | 1280 | 40 | 160 | 40 | ND | confirmed ZIKV |
| S-9 | Oct-2019 | DWoWS | **10.1** | 4.8 | 56,023 | **10,240** | 160 | 320 | 40 | 320 | 160 | ND | confirmed ZIKV |
| S-10 | Oct-2019 | DWWS | **5.8** | 3.5 | 21,891 | **1280** | < 40 | 320 | < 40 | 40 | < 40 | ND | confirmed ZIKV |
| S-11 | Nov-2019 | DWoWS | **7.5** | 4.8 | 125,919 | **1280** | 40 | < 40 | < 40 | 40 | 40 | ND | confirmed ZIKV |
| S-12 | May-2019 | DWoWS | **5.4** | 1.8 | 5982 | **640** | 320 | < 40 | 40 | < 40 | < 40 | ND | probable ZIKV |

DENV-1–4 =dengue virus serotype 1–4. ZIKV= Zika virus. JEV= Japanese encephalitis virus. AUFI= acute undifferentiated febrile illness. DWoWS=dengue without warning signs. DWWS=dengue with warning signs. FRNT90 = 90% focus reduction neutralization test. Bold font indicates highest IgM and neutralization value in Table.

*Clinical features of ZIKV infection cases*

Through ZIKV qRT-PCR and neutralization test in ZIKV IgM positive samples, 6 (2.0%) and 11 (3.7%) were confirmed as ZIKV infection in Kandy (Table 2) and Negombo (Table 3) regions, respectively. Six ZIKV infected patients in the Kandy area were comprised two patients with dengue without warning signs (DWoWS) and four patients with severe dengue (SD). Of the 6 patients, 1 patient with positive qRT-PCR, IgM, NT, 3 patients with positive qRT-PCR and 2 patients with positive IgM, NT were noted. Eleven ZIKV confirmed patients in Negombo area comprised 9 patients with DWoWS and 2 patients with dengue with warning signs (DWWS). Of the 11 patients, 2 patients with positive qRT-PCR and 9 patients with positive IgM, NT were detected.

*Monthly distribution and age distribution of ZIKV infection cases*

Six confirmed ZIKV infection cases, which included two cases in July, one case in August, two cases in October in 2017 and one case in January in 2018, occurred in Kandy during the 2017–2018 dengue outbreak (Table 2). The 11 (3.7%) confirmed ZIKV infection cases in Negombo included one case each in June and October and three cases in November (2018), one case each in April, September and November (2019) and three cases in October (2019) (Table 3). The age and sex of the study population was unavailable for Negombo, while age and sex information were available for all patients admitted to the Kandy Hospital. Six (2.0%) samples were confirmed ZIKV infections, comprising four males ages 19-, 32-, 45- and 55-years-old and two females ages 22- and 38-years-old.

## Discussion

This study is the first to demonstrate the existence of ZIKV infection in Sri Lanka using molecular and serological analysis. The prevalence of confirmed ZIKV 2.0% and 3.7% in Kandy and Negombo among clinically suspected dengue patients, respectively in the study population are consistent with other studies which have confirmed ZIKV infections in other Asian countries including India [20], Thailand, Myanmar [26] and Vietnam [27]. Despite mosquito-borne infections being abundant in Asia, only a few accounts regarding ZIKV infections have been reported in this region. This could be due to a high likelihood of underdiagnosis or misdiagnosis [28], which represents a major public health issue [29]. The limited amount of data regarding ZIKV infections from these areas warrants the need for further research.

ZIKV infections are commonly associated with acute febrile illness, haemorrhagic fever, congenital abnormalities and neurological complications [30]. Determining the onset of ZIKV fever based on clinical symptoms is difficult as opposed to dengue fever [31]. In this study, the most common clinical feature of ZIKV-suspected cases was fever, which ranged in duration from 1 – 5 days. In the 2017 massive dengue outbreak in Sri Lanka, many unusual and severe clinical manifestations, such as Guillain-Barre syndrome, multi-organ failure in 44 patients with 11 deaths, myocarditis and encephalitis, were observed among 300 dengue-suspected patients in the Kandy Hospital from our previous study [11]. Interestingly from this study population, a patient (45-year-old male) admitted to Kandy Hospital with SD symptoms and died later (Table 2) was infected with Cosmopolitan genotype of DENV-2 and had serological evidence of acute or recent ZIKV. Another patient (22-year-old male) admitted to Kandy Hospital with SD symptoms and encephalitis and recovered finally was infected with Cosmopolitan genotype of DENV-2 and had positive for ZIKV real time PCR. These findings might highlight that the combination of ZIKV and DENV exposure is associated with high risks to patients and other adverse outcomes during infection in adults. However, most of the ZIKV-infected patients in Negombo region were DwoWS during 2018–2019 dengue outbreak. It has been reported that an estimated 80% of the ZIKV infected persons are asymptomatic [22].

Although detection of ZIKV RNAs by qRT-PCR showed 1% in two areas of study population, the nucleotide sequences were not obtained from all samples. Molecular detection of ZIKV is difficult due to a very short viraemic phase after disease onset. However, a negative result does not exclude a positive diagnosis for ZIKV infection [32]. This could be because most patients sought medical care for a rash, likely after the viraemic stage. Another possibility is that an only ZIKV infection might not cause severe diseases. The prevalence of IgM (18.0%) antibodies against ZIKV observed in this study is likely due to the serological evidence of recent ZIKV infections, which have been currently reported around India [33]. Additionally, this could be attributed to cross-reactions of the tested antibodies against ZIKV, DENV and JEV, all of which belong to the same *Flaviviridae* family. These highlight the importance of differential diagnosis of these viruses, especially during arboviral outbreaks. Hence, effective diagnosis of these viruses can result in reduced number of hospitalizations, and the number of patients developing complications can be reduced.

Consistent with other studies [34], high IgM and IgG seropositivity among the younger adults aged 15–30 years in Kandy was observed. This could be due to the increased number of being bitten by *Aedes* mosquitoes, as getting older. The low level of ZIKV infections in old adults might be owing to the past ZIKV infection. The high IgG antibody titres in young adults may suggest that Zika virus infection is not endemic in Sri Lanka, and rather, the virus has been introduced to an immunologically-naïve human population in recent past. As endemicity is achieved, ZIKV infections in old adults decrease as the infection in the young population increases [35]. Valencia et al. reported evidence of past ZIKV infection by using anti-DENV Envelope and ZIKV NS1 IgG ELISA kits in Colombo, Sri Lanka and showed that combined prior ZIKV and DENV exposure is associated outcomes during a subsequent DENV infection in adults [36]. We diagnosed acute ZIKV infection by RT-PCR or serological (IgM, IgG, neutralization tests) investigations in the present study.

To discriminate against infections caused by different flaviviruses, neutralization assays [26] were used in this study. We identified 13 positive ZIKV-neutralizing antibodies among the 595 patient samples from Kandy and Negombo during 2017 – 2019. These results indicate that DENV and ZIKV were co-circulating during the dengue outbreaks in Sri Lanka. Furthermore, this study determined that ZIKV infections in Sri Lanka are higher in Negombo compared to Kandy. This phenomenon could be attributed to the abundance of *Aedes* mosquito vectors; Negombo have much mosquitoes than as Kandy has [37]. Moreover, it has been shown that DENV, which is an antigenically-related mosquito-borne flavivirus to ZIKV, historically causes seasonal epidemics that affect urban areas, especially the Gampaha district, which is densely populated with 11% of the country's population [38]. There seemed to be a seasonal trend of ZIKV infections with increased cases during the rainy period, which coincides with favourable environmental conditions for *Aedes aegyptus* breeding [39]. ZIKV transmission increases during monsoon season in Sri Lanka due to increased mosquito breeding locations. Other factors that could increase ZIKV transmission include urbanization [40], climate change [41] and poor vector control and disease prevention [17]. Therefore, vector control measures should be enacted preceding the rainy season to prevent ZIKV outbreaks. This will simultaneously solve the problem of other mosquito-borne diseases like dengue, chikungunya, Japanese encephalitis, malaria and filaria. To estimate the extent of the ZIKV impact in Sri Lanka, a long-term, rigorous surveillance network is needed coupled with the active participation of the concerned public and health authorities at the local, regional, state and country levels.

In conclusion, this study is the first to demonstrate the possible co-circulation of acute ZIKV and DENV utilizing molecular and

serological analysis during the 2017 – 2019 dengue outbreaks in Sri-Lanka. Data presented here reveal that co-circulation of ZIKV and DENV potentially increased severity of dengue infections during 2017 outbreak and unveiled a high prevalence of ZIKV infection with mild symptoms during the 2018–2019 dengue outbreak. The detection of ZIKV infected cases during the largest 2017 dengue outbreak in Sri Lanka indicates that multiple arboviral circulations are possible and could easily go undetected, especially during outbreak situations. In light of the high morbidity rate and to reduce the disease burden, it is important to provide accurate differential diagnoses for these viruses, especially during inter-epidemic periods. Furthermore, effective diagnosis can provide basic understanding for supporting clinical care, pathogenesis studies, vaccine research and epidemiological surveillance programs. Our findings will help preparedness in preventing future outbreaks and guide national governments in forming public-health policies and boost the efficiency of an early warning system for circulation of ZIKV in Sri Lanka.

## Funding statement

This research work was supported by AMED (Japan Agency of Medical Research and Development) under SCARDA (JP223fa627004h0001, JP233fa627004h0002), e-Asia (JP22jm0210079h003, JP23jm0210079h004), JP22wm0125006, 22fk018123h1103, JP22fk0108123, JP20wm0225017, 22wm0125006h0003, 23wm0125006h0004, 22fm0208101j0006, 23fk0108656h1201, 22ab0123456h0001, JSPS (Japan Society for the Promotion of Science) under Grant Number 21K07059, 22KK0115, The Naito Foundation, Takeda Science Foundation and National Science Foundation, Sri Lanka (Grant number RPHS/2016/D/03).

## Ethical statement

Ethical approvals for this study were provided by the Institutional Ethical Review Committees on Faculty of Medicine, University of Kelaniya, Sri Lanka P/101/02/2017; Medical Research and Review, National Hospital Kandy, Sri Lanka THK/ERC/73/2017, and the Institute of Tropical Medicine Ethical Committee, Japan (180608200, 201223250, 230209288). Written informed consent and assent from the parents and legally authorized representatives were collected from participants for collection of blood specimens.

## Declaration of Competing Interest

No interest to declare.

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