Detection of Dengue, Chikungunya, and Zika Viruses Among Patients in Sarawak, Malaysia by a Novel Multiplexing Platform

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Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in the Duke Global Health Institute in the Graduate School of Duke University

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Abstract

Introduction: According to the World Health Organization (WHO), 500 million arbovirus cases are diagnosed around the world annually, with 2.7 million associated deaths [1]. The burden of disease caused by dengue, chikungunya, and Zika viruses is likely to be underestimated due to a lack of accurate diagnostic tools and knowledge gaps regarding their epidemiology [2, 3]. This thesis uses a subset of data from an on-going 24-month study to evaluate the potential etiology of dengue-like symptoms of patients recruited from medical facilities in Sarawak, Malaysia. A secondary aim is to assess the diagnostic clinical effectiveness of a new detection method, the novel T-Cor 8 Multiplexing Platform (Tetracore, Inc., USA), using qRT-PCR assays as the gold standard method for comparison. The prevalence of arboviral infections as determined by gold-standard qRT-PCR assays and potential risk factors in the study population were also analysed.

Methods: In this cross-sectional study, patients more than seven years of age with dengue-like symptoms were enrolled at medical facilities in the towns of Sibu and Kapit in Sarawak, Malaysia. Blood, urine, and gingival crevicular fluid samples, as well as risk factor data, were collected from participants at the time of enrolment. These samples were studied by qRT-PCR assays and the novel T-Cor 8 Multiplexing Platform.

Results: Seven (14%) of 51 participants’ serum RNA samples tested positive for arbovirus infection by gold-standard qRT-PCR assays. Two participants (4%) were positive for dengue subtype-1, four participants (8%) were positive for dengue subtype-2, and one participant (2%) was positive for dengue subtype-4.
No patient samples had molecular evidence of chikungunya or Zika viruses. The T-Cor 8 multiplexing platform demonstrated a 71% sensitivity (95% confidence interval 29-96%), 93% specificity (95% confidence interval 81-99%), and 90% accuracy (95% confidence interval 78-97%) compared to the gold-standard assays on serum RNA samples. From this subset of data, we failed to identify important risk factors for arboviral infection.

**Conclusion:** From this limited subset of data, we conclude that the T-Cor 8 platform’s simplicity and accuracy in detecting at least dengue virus infections has considerable potential for clinical usefulness in low-resource settings.
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1. Introduction

1.1 The global burden of dengue, chikungunya, and Zika

According to the World Health Organization (WHO), 500 million arbovirus cases are diagnosed around the world annually, with 2.7 million associated deaths [1]. Epidemiological studies of dengue, chikungunya, and Zika have pointed out the likelihood of underestimating the burden of disease caused by these viruses due to the lack of available diagnostic tools and current epidemiological knowledge gaps [2, 3]. These undetected burdens, in combination with the already considerable estimates, indicate that dengue, chikungunya, and Zika will continue to be important considerations for global public health as they spread and re-emerge around the world.

Dengue is a positive sense RNA flavivirus of the family Flaviviridae with four known subtypes (dengue 1-4). Syndromes caused by dengue range from febrile to a virulent form of infection called dengue hemorrhagic fever and/or dengue shock syndrome (DHF/DSS) [4]. Dengue is the most common arbovirus and an estimated four billion people across 128 countries at risk for infection [5]. In the year of 2013 alone, an estimated 1.14 million disability-adjusted life years (DALYs) were lost due to dengue infections worldwide [5].

Chikungunya, a small envelop positive sense RNA alphavirus of the family Togaviridae, was initially identified from human and mosquito biological samples during a viral fever outbreak in Tanzania in 1953 [6]. The name of the virus means “that which bends upward” and is referent for the intense pain felt at the joints by those who are infected [6]. Since its initial discovery, chikungunya
has been detected across Africa, Asia, and in parts of the Caribbean. In the Africa region, the transmission cycle of the virus has included non-human primates. Chikungunya virus in Asia historically has been sustained by human-to-human transmission [6].

Zika was first isolated from humans in Nigeria in 1954, but its initial entrance into Asia was documented in 1966 in Malaysia [7]. This single-stranded RNA flavivirus came to global recognition in the mid-2010s after global outbreaks of the virus lead to evidence of a connection to neurological sequelae in those infected and infants born to recently infected mothers [8]. The detection of Zika virus cases in areas where multiple arboviruses circulate has been challenging, as this virus and other arboviruses in the family Flaviridae cross-react serologically [9].

Dengue, chikungunya, and Zika viruses share a common primary vector, the female *Aedes* mosquito [10-12]. Though this vector was historically known to inhabit tropical and sub-tropical areas of the world, recent studies have shown the geographic footprint of the *Aedes* mosquito on every continent [13-15]. The expansion of this vector and the implications for future outbreaks in vulnerable populations have increased the need for robust global health systems with the capacities to accurately diagnose and respond to the burden of these viruses.

1.2 *The epidemiology of dengue, chikungunya, and Zika in Malaysia*

According to some estimates 55% of the Malaysia population is likely sero-positive for any one of the four dengue subtypes [16]. The virus has had a dynamic epidemiological impact on the population, with a dramatic increase of
dengue cases per year seen in the first decade of the 21st century [2]. On the island of Borneo, and particularly in the Malaysian state of Sarawak, there is well-established history of dengue in the population [17]. Phylogenetic analysis has indicated multiple potential introductions and/or importations of the four strains of the virus into Sarawak [18].

The first chikungunya outbreak in Malaysia was documented in 1998 [6]. The virus was absent from public health awareness for seven years following, until an outbreak of chikungunya with highly similar genetic traits to that of the original 1998 virus. This prompted the first consideration that chikungunya may be endemic to Malaysia [19]. In the three decades since its first emergence, Asian, West African, and Central/East African genotypes of the chikungunya have caused outbreaks across Malaysia, with molecular evidence of eventual entry into the state of Sarawak in 2008 [6]. A 2008-2010 outbreak of the virus affected more than 10,000 individuals and caused the first documented chikungunya-related death in Malaysia [20].

The first isolation of Zika virus in Asia was made in 1966 in Malaysia [7]. In the decades since, there have been multiple studies that found serological evidence of Zika infections both human and orangutan populations [21-25]. As Zika has a demonstrated history of re-emergence with pandemic potential in the Pacific and Latin America, areas like Southeast Asia, and specifically Malaysia, are at risk for similar future outbreaks [26].

Dengue has an approximate annual economic burden of 56 million USD on the national level in Malaysia, though this estimate does not include the cost
of control, prevention, surveillance, and long-term impact of infections [1]. Like
most countries, the Malaysian Ministry of Health relies on a passive surveillance
method for the detection of dengue, which inherently leads to an under-reporting
of the true burden of disease country-wide [2]. Notably, there are no routine
diagnostic or surveillance mechanisms currently in place for the detection of
chikungunya or Zika viruses, despite the likelihood of co-circulation with dengue
viruses due to the presence of the common vector and the favorable environment
for transmission.

1.3 Clinical presentations of dengue, chikungunya, and Zika viruses

The clinically apparent manifestation of infection with these three viruses
overlaps in presentation of fever (with varying severity), rash, conjunctivitis,
muscle and joint pain, malaise, and headache [27-29]. Dengue presents with
differential clinical symptoms based on the stage of infection. The infection can
progress from febrile to critical syndromes, and can mimic infections by other
pathogens at each stage (Appendix A). Clinical diagnosis of dengue is
complicated by asymptomatic manifestations in 20-60% of infections [3, 30].
Accurate diagnosis of dengue infection can be critical if the disease progresses to
severe dengue shock, as well as to prevent further transmission of the virus [28].
There is evidence to suggest that asymptomatic dengue-infected individuals can
continue to transmit the virus to mosquitos. This highlights the need for
diagnostic tools to aid clinicians in diagnosing dengue infections if the disease is
to be truly combatted [3].
Chikungunya mimics certain clinical presentations of febrile dengue, with typical cases featuring high fever and extreme joint pain [31]. In a study comparing symptomatic patterns between dengue and chikungunya patients in Malaysia, chikungunya patients had notably a longer duration of residual arthralgia [32]. While useful for retrospective risk-factor assessment, this post-infectious method of clinically differentiating the two arboviruses does nothing to aid in clinical management of febrile patients. Similar to dengue infections, most Zika infections do not manifest in clinically-recognizable symptoms [27]. Those that are clinically diagnosed are typically recognized by risk factor exposures combined with typical symptoms [27]. Tools and methods for active detection of all three viruses are necessary to understand the burden of disease, and to design intervention and prevention programs.

1.4 Associated risk factors of dengue, chikungunya, and Zika virus acquisition

Interaction with or exposure to the *Aedes* mosquito is a major risk factor for arboviral infection. In Sarawak, this vector has had a documented presence spanning almost half a century [33]. Increased *Aedes* mosquitos reproduction has been associated with instances and seasons of increased rainfall [34, 35]. In urban areas of Sarawak, the most common breeding habitats for *Aedes* mosquitos were found to be water-filled plastic cups and used tires in outdoor areas near homes [36]. Though Malaysia has had legislation in place for more than four decades that mandates the destruction of breeding sites, fidelity to these laws has waxed and waned over time [37].
Dengue infection has been associated with older age in both rural and urban areas of Malaysia, perhaps due to the greater exposure to Aedes-prone environments that comes with work and life habits of post-adolescent individuals [16]. Overall, research on occupational risk for arbovirus infection has found mixed associations. Researchers in Kenya found that occupation as a “farmer” was significantly associated with increased risk of alphavirus and flavivirus infection [38], while a study in Venezuela found that occupation as “domestic worker or housewife” was associated with living in a dengue “hot-spot” home [39]. Other studies suggested associations between chikungunya and spending more than eight hours per day out of doors, as well as associations with older age [32, 40]. Zika virus has been associated with the lack of access to municipal water system and the onset of rainy season [41].

1.5 Current diagnostic capabilities in Malaysia

Currently, dengue is the only pathogen among these three arboviruses with routine diagnostic methods in practice in Malaysia, commonly in the form of Dengue Rapid Combination Tests (RCTs). These RCTs assess for dengue non-structural protein 1 antigens (NS1 Ag), immunoglobulin M (IgM) and immunoglobulin G (IgG) [42]. The Dengue RCTs are relatively cheaper than other laboratory-based diagnostic methods, such as polymerase chain reaction (PCR), virus isolation, or antigen detection by immunofluorescence, but confirmatory testing is recommended in order to assure the accuracy of diagnosis [43]. Further diagnostic methods, such as polymerase chain reaction, are often not cost-effective for routine testing [43].
In consideration of Malaysia’s current surveillance methods for arboviruses, there is a significant gap in the health system’s capacity to accurately differentiate between dengue, chikungunya, and Zika viruses in clinically ill patients. Furthermore, previous studies have indicated that collection of alternative biological samples, such as urine and saliva, may serve as less invasive modes for detection of arboviruses [44, 45]. A diagnostic tool, with the potential to diagnose these three arboviruses of interest using multiple available biological samples, may meet this need.

1.6 T-Cor 8 Multiplexing Platform

Figure 1: T-Cor 8 Multiplexing Platform (Tetracore Inc., Rockville, MD)

Image: Tetracore Inc.

Based in Rockville, Maryland, USA, Tetracore Industries is a research & development-focused biotechnology company that has generated diagnostic products for the detection of infectious diseases, as well as pathogens and toxins with bio-terrorism potential. The T-Cor 8™ Real-Time PCR Thermocycler (Tetracore Inc, USA) (Figure 1) that has been developed as a high-efficiency diagnostic machine for low-resource settings. Physically fit for use in low-resource settings, it weighs less than 10 pounds and can run off of a rechargeable
battery for up to four hours. With the aim of finding a role in low-resource contexts, it is largely “technician proof”, requiring a brief training for medical personnel before use and delivers the results of experiments within 90 minutes in a simple, comprehensible format. This platform has yet to be validated for its arbovirus diagnostic field use the diagnosis of dengue, chikungunya, and Zika viruses.

1.7 Study aims

In June of 2018, our cross-sectional study titled “Detection of Dengue, Chikungunya, and Zika Viruses by a Novel Multiplexing Platform Among Patients in Sarawak, Malaysia” was initiated in Sarawak, Malaysia. This study is being conducted via a partnership between Duke University, Sibu Hospital Clinical Research Center, Kapit Hospital, and Tetracore Inc. The aims of this study included:

Aim 1: Estimate the prevalence of dengue, chikungunya, and Zika viruses in patients with dengue-like symptoms who present to recruitment sites at Sarawak medical facilities, as well as determine associated risk factors for arboviral infection

Aim 2: Determine the clinical effectiveness of using the T-Cor 8 Multiplexing Platform to detect dengue, chikungunya, and Zika viruses in biological samples from these patients as shown by T-Cor 8’s sensitivity and specificity of detected arboviral RNA in comparison to gold standard qRT-PCR assays.
This study will conclude in June of 2020, with an enrollment cap of 600 participants. This thesis will serve as a feasibility assessment of the study, using data collected from the first 52 enrolled individuals. The likelihood of this study meeting the stated aims within the expected timeframe and in consideration of the limitations found during the initial period of the study will be assessed.

The data used for this thesis was collected between June 16th and December 31st of 2018. For the purposes of the on-going study (n=600), it was hypothesized that dengue infection would be detected among 45-54% of participants, and chikungunya and Zika virus infections would be detected among 3-7% of participants.
2. Methods

2.1 Setting

The participants included in this analysis were enrolled between June 19th and December 31st, 2018 in the state of Sarawak. Sarawak is the largest state in Malaysia, spanning 12.4 million hectares \[46, 47\]. Located on the northwest side of the island of Borneo, the state has annual average temperatures between 26 and 27.5 degrees Celsius (°C), and average rainfall of about 3.5 meters \[35\]. Increases in rainfall and temperatures occur in the monsoon season between November and February, which often coincides with peak mosquito season and associated increases in incidence of arboviral cases \[35\].

Considered to be an icon of biodiversity, 65% of the state is covered by forest \[46\]. Many globally-recognized animals, such as the orangutan, continue to thrive in the wild in these forested areas \[46\]. Logging and other agricultural activities provide the livelihood for much of the rural-dwelling population \[46\]. Recent anthropogenic activity in and destruction of the forests have been associated with emergence and re-emergence of zoonotic infectious diseases, including the relatively recently discovered 5th human malarial parasite \textit{Plasmodium knowlesi} \[48\].

With a population of more than two million, Sarawak hosts more than 26 unique ethnic groups. The largest of the ethnic groups are Malay, Chinese, and indigenous communities which can be further divided into the Iban, Bidayuh, and Melanau ethnic groups, among others \[49\]. The city of Sibu hosts a large
Chinese population, while the town of Kapit serves rural communities of the largely Iban ethnic group.

Recruitment for this study occurred at medical facilities in the city of Sibu and the town of Kapit in Sarawak. In Sibu, enrollments took place at Sibu Hospital, the main referral hospital for central Sarawak serving a population of more than 700,000 residents, and Polyclinic Lanang, one of multiple primary care facilities that exist in Sibu. Accessible only by 3-hour boat ride up the Rajang River from Sibu and serving a population of about 130,000 local residents, Kapit Hospital was our recruitment and enrollment point for the Kapit Division.

2.2 Participants

The inclusion and exclusion criteria for this study were adapted from WHO guidelines for diagnosing dengue infections and refined by Dr. Teck-Hock Toh and Dr. Gregory Gray (Appendix A).

All patients seven years of age and older who presented to Sibu or Kapit medical facilities with dengue-like symptoms were eligible to be screened by a medical officer who was involved in the study. Dengue-like symptoms were defined as a high fever (identified by self-reported subjective elevation in temperature or clinically documented temporal or oral temperature greater than 38°C), and at least two of the following symptoms: severe headache, eye pain, joint pain, muscle or bone pain, rash, nausea and/or vomiting, mild bleeding manifestations or low white blood cell count, fit the inclusion criteria for enrolment. Patients who received a diagnosis other than dengue, chikungunya, or Zika, were recently hospitalized (<7 days for immunocompetent children and
<90 for immunosuppressed children, <28 days for immunocompetent adults and <90 days for immunosuppressed adults), or received antiviral treatments within four weeks prior to screening were excluded. Children below the age of seven were excluded from this study.

No previous seroepidemiological data were available for informing sample size calculations. We postulated that dengue viruses would be the most prevalent among our ill patients and that 50% of patients would have molecular evidence of infection. As chikungunya and Zika viruses were seldom studied, we based sample size estimates upon dengue. If the true prevalence figure of 50% was used for dengue, the overall sample size of 600 participants would have allowed this study to estimate the prevalence within 4%.

2.3 Procedures

2.3.1 Ethical review

This study was independently reviewed and approved by the Institutional Review Boards (IRB) at Duke University, the National Medical Research Register of Malaysia (NMRR), and the Malaysian Research and Ethics Committee (MREC) prior to being granted approved status by all three of these bodies. Administrative permission was provided by the hospital directors at Sibu and Kapit Hospital prior to beginning enrollment at these locations.

2.3.2 Informed consent

Study materials, including the Patient Information Sheet, Consent Form, and Risk Factor Questionnaire were made available in English, Malay, and Mandarin, per recommendations of Sibu and Kapit Hospital collaborators. In
accordance with the MREC regulations, assent forms, and associated adult guardian consent were completed for all enrolled children between ages seven and eighteen years.

Licensed medical officers (MOs) trained in Good Clinical Practice and who were registered with the NMRR/MREC as co-investigators in this study guided willing and eligible patients through the informed consent process. Five MOs at Sibu Hospital, four MOs from Kapit Hospital, and two MOs at Polyclinic Lanang were trained in the informed consent, enrollment, and sample collection processes by the study team. MOs were provided with study-related instruments and information for their own use, including standardized operating procedures (SOPs) for the enrollment procedure and sample collection, as well as the inclusion and exclusion criteria. During the first several enrollments, MOs were observed by the study team to ensure SOP adherence. We used a convenience sampling method, largely due to the dependence upon the availability of MOs for participant enrollment.

![Recruitment by Site per Month](image)

**Figure 2:** Recruitment of study participants at Sibu and Kapit medical facilities
The details of the study were explained to eligible patients by MOs, at which time eligible patients and/or their guardians were handed the Patient Information Sheet and Informed Consent Sheet by the MOs, which described the details of the study, the risks involved, and the breadth of participation. The patients and/or patients’ guardians were given sufficient time to read through the documents. Illiterate individuals were read the informed consent sheet by a medical officer. For children under the age of 18, the consent of the parents or guardian was sought, as well as the assent of the child to participate in the study. All patients were assured by the MOs that enrollment or denial of enrollment would have no impact on the care delivered by the MOs. Patients were not offered compensation to enroll in the study. Further study procedures did not occur until informed consent was gained from eligible patients.

2.3.3 Sample collection & sample processing

After participants consented to participation, the MO explained the procedures involved in biological sample collection. This included the collection of blood, urine, and gingival crevicular fluid (GCF) from all participants. Study questionnaires [Appendix C] were completed by each study participant, with the assistance of an MO if necessary.

For children ages 7 to 12, no more than 3 mL of blood was collected by trained medical officers using proper phlebotomy techniques. For all participants older than 12, no more than 5 mL of blood was collected. All participants were provided a midstream specimen collection cup, in which they produced a urine
sample of at least 2 mL. GCF was collected by swabbing the gum line of each participant using the Gumline Swab (Tetracore, Inc., Rockville, MD). The MO had the participant open their mouth with a slightly relaxed jaw, at which point the swab was gently swiped along the lower gum line between the gum and the cheek. The swab was then returned to its housing, where it was submerged in a buffer fluid. Biological samples were stored immediately at 4°C until they could be transported for processing in Sibu Clinical Research Center (CRC) or Kapit Hospital laboratories. Samples were delivered to Sibu CRC or Kapit Hospital Laboratory to be processed within 12 hours of sample collection and were transported from storage sites to the laboratories in coolers with ice packs to maintain a cold-chain until processing could occur.

Blood samples were centrifuged at 1,500 x g for 10 minutes at room temperature, after which the sera were aliquoted out into microcentrifuge tubes and stored immediately at -20 or -80°C. Urine samples were gently swirled in their collection cups just prior to aliquoting into 1.7 mL microcentrifuge tubes, after which they were stored at -20°C or -80°C. After submerging the swab in buffer fluid within Gumline Swab (Tetracore Inc., USA), this fluid was aliquoted for storage into 1.7 mL microcentrifuge tubes at -20°C or -80°C.

2.3.4 Gold-standard molecular analysis of serum samples

The gold-standard molecular assays used as reference tests in this study were published by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) and Centers for Disease Control & Prevention (CDC) in the United States. The assays for dengue virus 1-4 have a reported limit
of detection of between $1 \times 10^2$ – $1 \times 10^3$ Genome Copy Equivalents per millilitre of sample (GCE/mL) [50]. Extraction of viral ribonucleic acid (RNA) from biological samples was completed using the Viral RNA Isolation kit (Macherey-Nagel, USA), following the manufacturers’ instructions. Extractions (75 µL) were preserved at -80°C in microcentrifuge tubes. Superscript® III Platinum One-Step qRT-PCR System with Platinum® Taq DNA Polymerase (Thermo Fisher Scientific, Inc., Waltham, MA) was used in real-time reverse-transcriptase qPCR single-plex assays for dengue virus subtypes 1, 2, 3, & 4 [50], chikungunya virus[51], and Zika virus[52]. Concentrations for forward and reverse primers were 1µM for dengue-1 and dengue-3, and 500µM for dengue-2 and dengue-4. Final concentration for each Taqman probe was 180 nM for all dengue assays. Assays for both chikungunya and Zika used 900µM concentration for forward and reverse primers, with 200µM concentrations for the probe.

All gold-standard molecular analyses were conducted on a BIO-RAD CFX 96 platform (Hercules, CA, USA). Samples were analysed against positive controls for dengue (BEI NR-82, chikungunya, and Zika viruses, with nuclease-free water used as negative control. The following reagents were obtained through BEI Resources, NIAID, NIH, as part of the WRCEVA program: Dengue Virus Type 1, Hawaii, NR-82, Dengue Virus Type 2 (DEN-2), New Guinea C (NGC), NR-84, Dengue Virus Type 3, Philippines/H87/1956, NR-80, Dengue Virus Type 4, H241 (Tissue Culture Adapted), NR-86, Genomic RNA from Chikungunya Virus, S-27, NR-50129, and Zika Virus, MR 766, NR-50065. Specific primers and probes used are described in Table 1.
Dengue assay cycling conditions were as follows: a 30 minute denaturing stage at 50°C, followed by a 2 minute annealing stage at 95°C, and then an extending stage of 45 repetitions of: 95°C for 15 seconds followed by 55°C for 30 seconds. Zika and Chikungunya assays were denatured at 50°C for 15 minutes, held at the annealing stage at 95°C for 5 minutes, at which point the extending stage occurred through cycles of 95°C for 5 seconds and 55°C for 20 seconds, which was repeated 45 times. The conditions concluded with 30 seconds of 40°C cycling.

Included in each experiment were positive synthetic controls for the virus of interest as well as a negative controls. In instances in which either the positive or negative controls failed, the experiments were repeated. Ct values <38 were
deemed to be “positive” for the virus of interest, values 38-40 were considered “suspect positive”, and all Ct values >40 were considered “negative”.

2.3.5 T-Cor 8 molecular analysis on serum, urine, and GCF

Both RNA-extracted and unextracted (direct) forms of biological samples were tested by a multiplex assay on the T-Cor 8 (Tetracore Inc., Rockville, MD). Primers and probes for this assay were provided in lyophilized form in reaction vessels that were then directly utilized in testing on the T-Cor 8 platform. The multiplexing assay used has a reported limit of detection of approximately 400 GCE/mL.

Sample preparation for testing on the T-Cor 8 involved dissolving the lyophilized primers and probes in 20 uL of T-Cor 8 Buffer Solution (Tetracore Inc.) within the reaction vessel. Five uL of direct or extracted sample was added to the solution in the reaction vessel, and then cycled through the T-Cor 8’s internal cycling conditions. These cycling conditions were as follows: 15 minutes at 15°C, 2 minutes at 95°C, followed by 95°C for 15 seconds, then 58°C for 30 seconds. Then, for a repeat series of 10, 95°C was held for 15 seconds, followed by 72°C for 30 seconds. Finally, 15 seconds at 95°C followed by 30 seconds at 58°C was repeated 32 times.

“Negative”, “Positive”, and “Invalid” results were determined by the T-Cor 8’s internal computer. Experiments that were “Invalid” by the T-Cor 8’s standards were repeated if sufficient sample was available. Urine samples that were found to be “Invalid” were diluted 1:5 and retested, per the recommendation of the Tetracore technicians. T-Cor 8 Positive Controls (Tetracore Inc.,) were run
within 24 hours of sample testing. Negative controls were run intermittently at the discretion of the laboratorian, per instruction from Tetracore technicians.

2.3.6 Risk factors assessed via questionnaire

Medical data, including presenting symptoms, were gathered by the MO at the time of enrollment and recorded on the risk factor questionnaire. Basic demographic information, such as information regarding location of enrollment, date of birth, gender, ethnicity, and neighbourhood of residence was gathered. Sibu Hospital and Polyclinic Lanang were grouped as “Sibu Medical Facilities” in analysis, as these two medical facilities serve the same base population.

Other medical data of interest included pregnancy status for women and expected due date if pregnant, dengue vaccination status, previous positive tests for dengue, chikungunya, or Zika, and on-site NS1 Antigen Rapid Combination Test results, if performed. The Dengue RCT was performed per protocol of the Malaysian Ministry of Health, outside the scope and design of this study. For this reason, it was only performed on a subset of participants per the discretion of the treating MO.

To assess behavioural, occupational, and environmental risk factors for arboviral infection, patients were asked to assign themselves to categories regarding their work place, mosquito control methods they use (if any), number of days in a week they venture out of doors at dawn or dusk, the number of mosquito bites they remember receiving in the last week, whether fogging took place in their place of living in the two weeks prior to enrollment, and whether they check for mosquito breeding sites in their place of living. Within 3 months of
patient contact, the enrolling medical officer attempted to contacted enrolled participants to deliver the results of their gold-standard tests, and to ask questions regarding their medical outcome.

2.4 Analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at Duke University [53]. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources. Statistical analyses were completed using R-Studio Version 1.1.456 software.

Descriptive statistics were used to generate the prevalence of arbovirus infection among the study population based the results of the gold-standard molecular testing. Frequency values were tabulated for both dichotomous and categorical risk factors comparing across sites of recruitment. Significance of risk factor exposures on gold-standard assay results were assessed using Fisher’s Exact test, due to the small sample size and the binary nature of the outcome. Results of these tests were considered statistically significant with a p-value of less than .05 and if the confidence interval for the resultant odds ratio did not include the null value of 1. For the calculations involving gold-standard or T-Cor 8 test results, invalid results were eliminated for each individual calculation so as to preserve as much of the data set as possible. Missing data from risk factor
variables were excluded from analysis for individual statistical tests for the same reason.

The performance characteristics of the T-Cor 8 for detecting arboviral infection was assessed by comparing the results of gold-standard qRT-PCR and T-Cor 8 testing on participants’ serum RNA samples. The sensitivity, specificity, and accuracy were calculated. Agreement between the results of T-Cor 8 testing on serum RNA was compared to the results of T-Cor 8 testing on the five other types of biological samples (urine in direct and extracted forms, gingival crevicular fluid in direct and extracted forms, and direct serum). Sensitivities and specificities were calculated to compare these biological sample types. Cohen’s kappa was used to determine the interrater reliability for participant diagnosis by different samples, with McNemar’s test used to discern the statistical significance of the kappa score.
3. Results

3.1 Demographic description of enrolled participants

In approximately seven months of recruitment, 52 participants were enrolled at Sibu and Kapit medical facilities (Table 2). The majority of this population was male (60%) and identified as part of the Iban ethnic group (67%). The average age of enrollment was 35.3 years. One participant was pregnant. No participant had previously received vaccination for dengue or had previously tested positive for dengue, chikungunya, or Zika during the three years prior to enrollment.

<table>
<thead>
<tr>
<th>Factor</th>
<th>All N (%)</th>
<th>Sibu N (%)</th>
<th>Kapit N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>52 (100%)</td>
<td>31 (60%)</td>
<td>21 (40%)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31 (60%)</td>
<td>19 (61%)</td>
<td>12 (39%)</td>
</tr>
<tr>
<td>Female</td>
<td>21 (40%)</td>
<td>12 (57%)</td>
<td>9 (43%)</td>
</tr>
<tr>
<td><strong>Age Groups</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;= 17</td>
<td>7 (14%)</td>
<td>4 (57%)</td>
<td>3 (43%)</td>
</tr>
<tr>
<td>&gt;17 &amp; &lt;=45</td>
<td>30 (60%)</td>
<td>20 (67%)</td>
<td>10 (33%)</td>
</tr>
<tr>
<td>&gt;45</td>
<td>13 (26%)</td>
<td>6 (46%)</td>
<td>7 (54%)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bidayuh</td>
<td>1 (2%)</td>
<td>1 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Chinese</td>
<td>5 (10%)</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Iban</td>
<td>35 (67%)</td>
<td>21 (60%)</td>
<td>14 (40%)</td>
</tr>
<tr>
<td>Malay</td>
<td>3 (6%)</td>
<td>1 (33%)</td>
<td>2 (67%)</td>
</tr>
<tr>
<td>Melanau</td>
<td>4 (8%)</td>
<td>4 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Other</td>
<td>4 (8%)</td>
<td>0 (0%)</td>
<td>4 (100%)</td>
</tr>
<tr>
<td><strong>Workplace</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mostly Outdoor</td>
<td>29 (52%)</td>
<td>20 (69%)</td>
<td>9 (31%)</td>
</tr>
<tr>
<td>Mix Indoor/Outdoor</td>
<td>9 (19%)</td>
<td>5 (56%)</td>
<td>4 (44%)</td>
</tr>
<tr>
<td>Mostly Indoor</td>
<td>8 (14%)</td>
<td>4 (50%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td><strong>Mosquito Barriers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical Repellent</td>
<td>18 (35%)</td>
<td>10 (62%)</td>
<td>8 (38%)</td>
</tr>
<tr>
<td>Electric Repellent</td>
<td>3 (6%)</td>
<td>3 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Bed net</td>
<td>10 (19%)</td>
<td>4 (40%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>Mosquito Coil</td>
<td>21 (40%)</td>
<td>13 (62%)</td>
<td>8 (38%)</td>
</tr>
<tr>
<td>Window Net</td>
<td>10 (15%)</td>
<td>8 (80%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Fogging</td>
<td>11 (21%)</td>
<td>10 (91%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>Check for Breeding sites</td>
<td>17 (33%)</td>
<td>11 (65%)</td>
<td>6 (35%)</td>
</tr>
</tbody>
</table>
One participant’s gold-standard test could not be completed due to inappropriate storage of the serum sample, and the data related to this participant was removed from prevalence calculation and the analysis of risk factors for the outcome of dengue virus detection.

Table 3: Participant Risk Factors for qRT-PCR Detection of Dengue

<table>
<thead>
<tr>
<th>Factor**</th>
<th>All N (%)</th>
<th>qRT-PCR Positive</th>
<th>Odds Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>51 (100%)</td>
<td>7 (14%)</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Enrollment Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibu</td>
<td>30 (59%)</td>
<td>4 (13%)</td>
<td>.92 (.14, 7.09)</td>
<td></td>
</tr>
<tr>
<td>Kapit</td>
<td>22 (41%)</td>
<td>3 (14%)</td>
<td>REF</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td>.6902</td>
</tr>
<tr>
<td>Male</td>
<td>31 (61%)</td>
<td>5 (16%)</td>
<td>1.71 (.25, 19.87)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>20 (39%)</td>
<td>2 (10%)</td>
<td>REF</td>
<td></td>
</tr>
<tr>
<td>Age Groups</td>
<td></td>
<td></td>
<td></td>
<td>.224</td>
</tr>
<tr>
<td>18&lt;age &amp; age&lt;=45</td>
<td>30 (59%)</td>
<td>6 (20%)</td>
<td>4.39 (.47, 218.04)</td>
<td></td>
</tr>
<tr>
<td>17&gt;=age &amp; age&gt;45</td>
<td>19 (37%)</td>
<td>1 (5%)</td>
<td>REF</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td>.6731</td>
</tr>
<tr>
<td>Iban</td>
<td>34 (67%)</td>
<td>4 (12%)</td>
<td>.628 (.09, 4.87)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>17 (33%)</td>
<td>3 (17%)</td>
<td>REF</td>
<td></td>
</tr>
<tr>
<td>Workplace</td>
<td></td>
<td></td>
<td></td>
<td>.6501</td>
</tr>
<tr>
<td>Mostly Outdoor</td>
<td>29 (57%)</td>
<td>3 (10%)</td>
<td>.51 (.059, 4.33)</td>
<td></td>
</tr>
<tr>
<td>Indoor/Outdoor &amp; Mostly Indoor</td>
<td>16 (16%)</td>
<td>3 (23%)</td>
<td>REF</td>
<td></td>
</tr>
<tr>
<td>Mosquito Barriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical Repellent</td>
<td>18 (35%)</td>
<td>2 (12%)</td>
<td>.78 (.07, 5.45)</td>
<td>1.000</td>
</tr>
<tr>
<td>Electric Repellent</td>
<td>3 (6%)</td>
<td>1 (33%)</td>
<td>3.38 (.05, 74.76)</td>
<td>.364</td>
</tr>
<tr>
<td>Bed net</td>
<td>10 (20%)</td>
<td>3 (30%)</td>
<td>3.82 (.46, 28.76)</td>
<td>.2614</td>
</tr>
<tr>
<td>Mosquito Coil</td>
<td>21 (41%)</td>
<td>2 (10%)</td>
<td>.53 (.05, 3.70)</td>
<td>.6852</td>
</tr>
<tr>
<td>Window Net</td>
<td>10 (20%)</td>
<td>2 (20%)</td>
<td>1.77 (.14, 13.60)</td>
<td>.6116</td>
</tr>
<tr>
<td>Fogging</td>
<td>11 (22%)</td>
<td>1 (9%)</td>
<td>.52 (.01, 5.20)</td>
<td>1.000</td>
</tr>
<tr>
<td>Checking for</td>
<td>17 (33%)</td>
<td>4 (24%)</td>
<td>2.80 (.41, 22.02)</td>
<td>.2256</td>
</tr>
<tr>
<td>Breeding sites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days Out at Dawn/Evening</td>
<td></td>
<td></td>
<td></td>
<td>.8687</td>
</tr>
<tr>
<td>0-5</td>
<td>25 (49%)</td>
<td>3 (12%)</td>
<td>.69 (.09, 4.62)</td>
<td></td>
</tr>
<tr>
<td>5-7</td>
<td>24 (47%)</td>
<td>4 (17%)</td>
<td>REF</td>
<td></td>
</tr>
<tr>
<td>Mosquito Bites in Last Week</td>
<td></td>
<td></td>
<td></td>
<td>.1153</td>
</tr>
<tr>
<td>0-6</td>
<td>29 (57%)</td>
<td>2 (7%)</td>
<td>.24 (.02, 1.71)</td>
<td></td>
</tr>
<tr>
<td>&gt;=6</td>
<td>21 (41%)</td>
<td>5 (31%)</td>
<td>REF</td>
<td></td>
</tr>
</tbody>
</table>

**Missing data for each variable was removed for individual calculations so as to preserve as much of the data set as possible.
3.2 Behavioral risk factors of recruited participants from Sibu and Kapit

Bivariate analysis of potentially significant risk factors against arboviral infection status was performed (Table 3). Categorical variables with sparse data were collapsed into fewer categories to permit the study of potential associations between exposures and arboviral infection outcomes. Due to the high number of Iban people in the participant pool, the “Ethnicity” variable levels were collapsed into “Iban” and all other ethnicities for the purpose of comparison across viral infection status. “Workplace” levels were combined into respondents who work completely outdoors versus respondents who work completely or partially indoors. Age of participants were collapsed into levels between ages 18 and 45, or outside of this age range. The number of days in a week with time spent outside at dawn or dusk was divided into those who spent the majority of the week outside at dawn or dusk versus those who spent almost every day of the week outside at dawn or dusk. Those participants who remembered receiving six or less mosquito bites were combined to compare against those who remember receiving more than six mosquito bites in the last week.

There were no statistically significant differences in the potential risk factors by arboviral infection status using bivariate analysis.

3.3 Detection of dengue, chikungunya, and Zika among study participants

Dengue was the only arbovirus detected in the enrolled participant pool. Seven of the tested participants (13%) were positive by gold-standard qRT-PCR for dengue virus detected in the participants’ serum RNA samples. Two participants (4%) were positive for dengue subtype-1, four participants (8%) were
positive for dengue subtype-2, and one participant (2%) was positive for dengue subtype-4.

Serum RNA was tested by the gold standard qRT-PCR assays and the T-Cor 8 for a direct comparison point across the two methods. Serum RNA testing by the T-Cor 8 novel multiplexing platform demonstrated a 71% sensitivity (95% confidence interval 29-96%), 93% specificity (95% confidence interval 81-99%), and 90% accuracy (95% confidence interval 78-96%) compared to the gold-standard qRT-PCR assays.

The T-Cor 8’s abilities to detect pathogen RNA among blood, urine, and oral fluid samples were assessed by calculating sensitivities, specificities, and Cohen’s kappa test for inter-rater reliability, using the T-Cor 8 serum RNA as the reference and comparing results from the five other forms of biological sample: direct serum, urine RNA, direct urine, GCF RNA, and direct GCF (Table 4).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum RNA</td>
<td>REF</td>
<td>REF</td>
<td>REF</td>
<td>REF</td>
</tr>
<tr>
<td>Direct Serum</td>
<td>100%</td>
<td>94%</td>
<td>95%</td>
<td>.83*</td>
</tr>
<tr>
<td>GCF RNA</td>
<td>50%</td>
<td>85%</td>
<td>80%</td>
<td>.15</td>
</tr>
<tr>
<td>Direct GCF</td>
<td>0</td>
<td>83%</td>
<td>84%</td>
<td>-.04</td>
</tr>
<tr>
<td>Urine RNA</td>
<td>0</td>
<td>83%</td>
<td>84%</td>
<td>-.07</td>
</tr>
<tr>
<td>Direct Urine</td>
<td>NA</td>
<td>84%</td>
<td>81%</td>
<td>0</td>
</tr>
</tbody>
</table>

* p-value for McNemar's test was less than .05
** Serum RNA served as reference for comparison to all other biological sample types

Compared to all other biological samples examined by T-Cor 8 testing, direct serum had the highest sensitivity and specificity, as well as a kappa value that indicated “strong” agreement between the two testing methods with statistical significance [54].
As a part of patient care outside the scope of this study, 26 (50%) participants were selected by MOs to receive Dengue RCTs. Of this subset of participants, 14 (54%) tested positive for NS1 Ag presence, indicating acute dengue infection. The Dengue RCT demonstrated 36% sensitivity and 83% specificity to both the gold-standard test and T-Cor 8 serum within this subset.

3.4 Symptoms in Association with Recruitment & Arboviral Infection

There was no statistically significant association between the symptoms of dengue-positive versus dengue-negative participants. (Table 5). Notably, two participants were recruited that demonstrated “No Fever”, but the recruiting MO strongly suspected arboviral infection. One of these non-febrile patients tested positive for dengue infection by gold-standard testing.
Table 5: Participant symptoms in association with gold standard (GS) test results

<table>
<thead>
<tr>
<th>Symptom</th>
<th>GS Positive</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fever</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever (n=49)</td>
<td>7 (14%)</td>
<td>.18</td>
<td>(.00, 14.94)</td>
<td>.2918</td>
</tr>
<tr>
<td>No Fever (n=2)</td>
<td>1 (50%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rash</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rash (n=6)</td>
<td>1 (17%)</td>
<td>1.08</td>
<td>(.02, 12.18)</td>
<td>1.000</td>
</tr>
<tr>
<td>No Rash (n=45)</td>
<td>7 (16%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Headache</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache (n=44)</td>
<td>6 (14%)</td>
<td>.40</td>
<td>(.05, 5.15)</td>
<td>.3004</td>
</tr>
<tr>
<td>No Headache (n=7)</td>
<td>2 (29%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Joint pain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Joint pain (n=23)</td>
<td>3 (13%)</td>
<td>.69</td>
<td>(.10, 4.10)</td>
<td>.7153</td>
</tr>
<tr>
<td>No Joint Pain (n=28)</td>
<td>5 (18%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Conjunctivitis/Red Eyes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red Eyes (n=5)</td>
<td>1 (20%)</td>
<td>1.38</td>
<td>(.02, 17.13)</td>
<td>1.000</td>
</tr>
<tr>
<td>No Red Eyes (n=46)</td>
<td>7 (15%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Muscle Pain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle Pain (n=34)</td>
<td>7 (21%)</td>
<td>4.06</td>
<td>(.45, 198.24)</td>
<td>.242</td>
</tr>
<tr>
<td>No Muscle Pain (n=17)</td>
<td>1 (6%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nausea/Vomiting</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea/Vomiting Present (n=28)</td>
<td>4 (19%)</td>
<td>.80</td>
<td>(.13, 4.87)</td>
<td>1.000</td>
</tr>
<tr>
<td>No Nausea/Vomiting (n=23)</td>
<td>4 (9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fatigue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatigue Present (n=38)</td>
<td>5 (13%)</td>
<td>.51</td>
<td>(.08, 3.88)</td>
<td>.4042</td>
</tr>
<tr>
<td>No Fatigue (n=13)</td>
<td>3 (23%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Discussion

4.1 Feasibility of recruitment of participants for arboviral detection

We expected to have low recruitment at the start of this study, with steep increases in participant enrollment throughout the rainy season. This timeline for this subset of data includes half of the expected peak season for arbovirus infection in Sarawak, without any marked increase in enrollment or arboviral infections. The highest recruitment in any month thus far was in June, the first month of study enrollment (Figure 2). This month is in the middle of the dry season in Sarawak, when mosquitos are typically less active. The recruitment in subsequent months doesn’t follow an identifiable pattern. This indicates that the recruitment of participants for this study may be contingent upon other factors, such as the travel of Sarawakians at the end of Ramadan in the month of June and the availability of enrolling MOs to devote time to screening potential participants.

This study is not on-track to recruit the target number of 600 participants within 24 months. If the rate of recruitment for the first six months of the study is predictive of the full two years, we will recruit approximately 200 participants by May 2020.

We overestimated the prevalence of arboviral-positive participants that we would recruit for this study. The prevalence of arboviral infection among participants recruited for this study to date is lower than that from similar studies in other contexts [55, 56]. It is possible that the low recruitment and prevalence is
due to the timeframe’s center in the dry season, but could be also due to reasons outside the scope and design of this study.

News reports at the end of 2017 and 2018 both reported dengue prevalence between the months of January to November to have decreased by more than 60% (2017) and 30% (2018) compared to each previous year [57, 58]. The number of dengue-associated deaths also decreased each year [57, 58]. These reports indicate that the low prevalence in this study may reflect a true decrease in the prevalence of arboviruses in the base population.

4.2 T-Cor 8 validation for field-use

As indicated in previous research, the need for more objective diagnostic tools for detecting arboviruses in Sarawakian medical facilities is reinforced by the issues we experienced in this study while relying on clinical symptoms for the purposes of recruitment. The complicated patterns of symptoms presented by dengue-positive patients detected in this sub-set of data, as well as the limitations in recruiting by symptoms for three different arboviruses, reinforces the inaccuracies of clinical judgment in determining the etiology of arboviral infections.

The T-Cor 8 is still in the process of being validated for use in clinical settings. While this platform will ideally perform as a “technician-proof” tool, the trouble-shooting process for this machine to-date requires the expertise of the Tetracore technicians who manufactured the platform and assays. In the month of June, the primary method of sample preparation was found to be problematic due to repeated invalid test results. A secondary method of sample preparation was created by Tetracore, which required the use of an isolated biomedical
cabinet to prevent contamination of the reaction set up. As this is not ideal for a tool that is meant to be used in low-resource settings, experts from Tetracore are currently engaged in the trouble-shooting process to return the T-Cor 8’s methodology to the “technician proof” format.

By projecting the anticipated distribution of gold-standard positive and negative participants compared to T-Cor 8 positive and negative participants, we were able to estimate that, at n=600, the T-Cor 8’s will have a sensitivity of 71% (95% confidence interval of 61-81%) and specificity of 93% (95% confidence interval of 90-95%). These small confidence intervals indicate that a sample of size of n=600 would allow us to reference the T-Cor 8’s diagnostic capabilities with fairly high precision.

A platform that is designed for implementation in low- and middle-income countries should have an affordable cost of use for public health and medical practitioners in these settings. The cost of reagents alone needed to analyze a single sample for a single virus by the gold-standard qRT-PCR used in this study was USD 5.49 (USD 3.57 per extraction plus USD 1.71 per reaction). Testing a given sample for all six viruses of interest by the single-plex assays cost USD 13.83. This price is not inclusive of the consumables and energy resources necessary to perform the associated laboratory work involved, or the cost of running controls.

Although the T-Cor 8 is not yet available on an open retail market, estimates provided to us by Tetracore Inc. indicate an expected price of about USD 12 per multiplex reaction that analyses for pan-dengue, pan-chikungunya, pan-Zika, and internal controls, not including associated consumables or control
runs. Adding the cost of extraction for a single biological sample, the cost of running serum RNA on the multiplexing assays was USD 15.57. This cost comparison indicates that the T-Cor 8 may serve as a slightly more cost-effective tool if direct forms of samples are used. Direct serum samples had 100% sensitivity and 96% specificity compared to serum RNA samples on the T-Cor 8, though further research would be needed to validate its accuracy to a gold-standard. The T-Cor 8 multiplexing platform has the added benefit of requiring relatively few consumables in addition to the materials provided in each reaction kit.

Though the validation process is still on-going, we are optimistic that this study will demonstrate the T-Cor 8’s usability in the field. The preliminary sensitivity and specificity in field-testing of this platform are promising. It is ideal for use in low-resource settings due to its form and potential functionality, particularly in that it can be used by laboratorians after a brief training, and can be powered by a car-battery in energy-deficient contexts.

4.3 Implications for policy and practice

The introduction of increased diagnostic power carries with it ethical implications for medical providers and public health authorities. As noted by Hofmann and Welch [59], the rise and implementation of novel diagnostic tests will likely increase the numbers of false positives, overdiagnosis, and subsequent overtreatment. According to these authors, the onus of responsibility is on clinicians and robust medical systems to prevent harm to patients that is potentially introduced by novel diagnostic tools.
Our primary consideration in this study was that patient involvement in the study would not impact patient treatment. MOs treated patients per their training and protocols put in place by the Malaysian Ministry of Health without regard to the outcome of the qRT-PCR gold standard test. Only the gold-standard test result was shared with the treating MO of a given patient after laboratory testing was complete. The results from the T-Cor 8 were not shared.

We believe that this study and associated activities will extend the reach of the existing surveillance network for arboviruses in place in Sarawak. We designed a system of reporting at the start of recruitment which empowered the MO in charge of the patient to be the determinant of diagnoses delivered to the patient and reported to the Ministry of Health, given the experimental nature of this study and the diagnostic methods used. Future research involving the T-Cor 8 and other diagnostic tools will hopefully continue in Malaysia following the completion of this study. As these diagnostic capabilities continue to increase in use, mechanisms of support for patients with diagnoses of novel or emerging diseases with complex biopsychosocial outcomes, such as Zika virus, should be in consideration.

The decreases in arbovirus infection detected during the course of this study are attributed in part to the public health efforts that include increased community engagement in mosquito breeding habitat destruction [57, 58]. We found that participants that were recruited from medical facilities in Sibu reported use of most mosquito barrier methods in higher numbers than those recruited from Kapit. Though these differences were not statistically significant,
these findings may be useful from a public health perspective to direct future interventions and efforts involving vector barrier methods.

4.4 Implications for further research

This study demonstrates the complicated nature of diagnosing arboviral infection based on clinical presentation alone. This small study sample supports existing literature stating that arboviral infections present with multiple collections of signs, symptoms, and routine test results, further reinforcing the need for objective, molecular methods of diagnosis.

There were three false-positives by the T-Cor 8 for serum RNA samples, two of which were also positive for NS1 Ag by the Dengue RCT. In a comparison study for diagnostic potential for RNA and NS1 detection potential, investigators in China found that viral RNA was a highly sensitive indicator of infection between the first and fourth day of infection, after which sensitivity would drop [60]. The diagnostic sensitivity of NS1 increased during the first week of infection, with highest sensitivity of detection on the seventh day. Samples that are positive by both T-Cor 8 and NS1 Ag testing, but negative by the gold-standard assays, could indicate that the T-Cor 8’s assays are more sensitive than the gold standard in detecting viral RNA presence later in the infection period. The current study provides insufficient evidence to support this theory of T-Cor 8 sensitivity, as any associations based on this subset of data are complicated by the incomplete allocation of Dengue RCTs, which only 50% of participants received. However, further research into the T-Cor 8’s ability to detect dengue infection in paired sera samples as dengue infection progresses in individuals would provide insight into this process.
Moving forward in this line of research should include an adjustment to structure of recruitment that accommodates the challenges inherent in screening and enrolling participants for three different arbovirus infections. A study conducted in 2017 suggested that the detection of Zika viruses may not be possible in concert with other arboviruses, given that rash and conjunctival hyperemia are the strongest clinical predictor for Zika infection in areas in with high arboviral transmission, with other arboviruses are best clinically identified by high fever and other associated symptoms [61]. With this in mind, future cross-sectional studies may benefit from highly specified recruitment criteria that differentiates between traditionally high-fever arbovirus infections like dengue and chikungunya, compared to typically low-fever arbovirus infections like Zika.

4.5 Limitations

A major limitation in the structure of this study is that only T-Cor 8 testing of serum RNA was verified by gold-standard molecular testing. This restricts the validation of the T-Cor 8’s capabilities to detect arboviruses using other clinical sample types. This study was able to report the accuracy of each biological samples (direct serum, urine RNA, direct urine, GCF RNA, direct GCF) compared to the serum RNA, but this validation by association is limited by the sensitivity and specificity of the serum RNA tested by the T-Cor 8 in comparison to the gold-standard. Including all biological samples in the gold standard testing in future study designs of this nature would allow for increased instrument reliability assessment.

This study has several limitations in regards to recruitment. Participants with low-grade febrile dengue infections and typical Zika infections may have
been missed by our recruitment system due to the primary inclusion criterion of high fever, objectively or subjectively assessed. Previous research has suggested that majority of Zika infections are associated with mild symptoms, such as low-grade fever [27] and that up to 60% of dengue infections are asymptomatic [3]. Future studies exploring the epidemiology of all three of these arboviruses at once may benefit from targeting high-risk groups for arbovirus infections using study methodology such as a prospective cohort design.

Enrollment for this study used a convenience method of sampling, as this study relied heavily upon the availability of trained MOs to screen and enroll eligible participants. Patients with dengue-like symptoms who presented to Sibu and Kapit medical facilities were recruited and enrolled by the MOs if the MOs were either charged with the care of the patient, or if another MO who was not involved in the study directly was able to inform an involved MO about the patient with dengue-like symptoms. This is likely to have led to an unknown number of patients with dengue-like symptoms moving through the Sibu and Kapit medical facilities without being screened by our collaborators.

Previous research has demonstrated that, while PCR is the gold-standard for many diagnostic tests, assays that detect dengue RNA begin to decrease in sensitivity after about four days of infection in primary infections, and after about two days in patients with secondary dengue infections [62]. It is possible that some infections were missed by our gold-standard qRT-PCR assays.

Other limitations of this study include the self-reported nature of the data regarding risk factor exposure. Participants’ inexact estimation or memory of
their recent environmental or behavioral exposures may have led to a misclassification bias.
5. Conclusion

In conclusion, this thesis reports a 13% prevalence in patients with dengue-like symptoms that were recruited thus far from an on-going 24-month study in medical facilities in Sibu and Kapit in Sarawak, Malaysia. The novel T-Cor 8 diagnostic platform demonstrated a 71% sensitivity and 93% specificity to the reference diagnostic assays for dengue viruses. As the study continues to enroll patients for diagnostic testing, the precision with which we can determine the sensitivity and specificity of this novel platform will increase. Further research into the causes of the low prevalence of arboviral infections in the recruited participant pool may be warranted. This baseline of arboviral infection pattern may serve future researchers in understanding the manifestations of dengue, chikungunya, and Zika virus infections in Sarawak over time.
# Appendix A

Table 6: Infections with Comparable Dengue Symptoms by Dengue Stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>Symptoms</th>
<th>Similar Illnesses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flu-like</td>
<td>Influenza, measles, Chikungunya, infectious mononucleosis, HIV seroconversion illness</td>
</tr>
<tr>
<td></td>
<td>Rashes</td>
<td>Rubella, measles, scarlet fever, meningococcal infection, Chikungunya, drug reaction</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>Rotavirus, enteric infections</td>
</tr>
<tr>
<td></td>
<td>Neurological Manifestations</td>
<td>Meningo/encephalitis, febrile seizures</td>
</tr>
<tr>
<td>Febrile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Critical Phase</td>
<td>Infectious</td>
<td>Acute gastroenteritis, malaria, leptospirosis, typhoid, typhus, viral hepatitis, acute HIV seroconversion illness, bacterial sepsis, septic shock</td>
</tr>
<tr>
<td></td>
<td>Malignancies</td>
<td>Acute leukemia and other malignancies</td>
</tr>
<tr>
<td></td>
<td>Other clinical pictures</td>
<td>Acute abdomen (appendicitis, cholecystitis, perforated viscus) Diabetic ketoacidosis Lactic acidosis Leukopenia and thrombocytopenia (+/- bleeding) Platelet disorders Renal failure Respiratory distress Systemic Lupus Erythmatosus</td>
</tr>
</tbody>
</table>

[Adapted from WHO 2009 [28]]
Appendix B
Table 7: Inclusion and Exclusion Criteria

<table>
<thead>
<tr>
<th>Inclusion Criteria – Children ( &gt;7 to 18 years)</th>
<th>Inclusion Criteria – Adults (18 years &amp; older)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• They seek medical care at Sibu or Kapit medical facilities;</td>
<td>• They seek medical care at Sibu or Kapit medical facilities on the basis of a clinical assessment by the treating clinician;</td>
</tr>
<tr>
<td>• Patient shows symptoms of dengue-like infection including a high fever (defined by self-reported subjective elevation in temperature or clinically documented temperature greater than 38 degrees Celsius or equivalent) and at least two of the following: severe headache, eye pain, joint pain, muscle or bone pain, rash, nausea/vomiting, mild bleeding manifestations or low white blood cell count.</td>
<td>• Have evidence of dengue-like infection with symptoms including a high fever (defined by self-reported subjective elevation in temperature or clinically documented temperature greater than 38 degrees Celsius or equivalent) and at least two of the following: severe headache, eye pain, joint pain, muscle or bone pain, rash, nausea/vomiting, mild bleeding manifestations or low white blood cell count.</td>
</tr>
<tr>
<td>• A parent or legal guardian provides written informed consent. In addition to parental consent, signed assent document will be sought from children 7 to 17 years of age.</td>
<td>• Agree to sign written informed consent form (and assent form if 7 to 17 years of age)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exclusion Criteria – Children ( &gt;7 to 18 years)</th>
<th>Exclusion Criteria – Adults (18 years &amp; older)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• If they had been hospitalized recently (&lt;7 days for immunocompetent children and &lt;90 days for immunosuppressed children)</td>
<td>• If they had been hospitalized recently (&lt;28 days for immunocompetent patients and &lt;90 days for immunosuppressed patients),</td>
</tr>
<tr>
<td>• If they had already been enrolled in this study within the previous 28 days</td>
<td>• If they have already been enrolled in this study within the previous 28 days</td>
</tr>
<tr>
<td>• If they resided in an extended-care facility or under the care of child protection service</td>
<td>• If they were functionally dependent nursing home residents, or children under the care of child protection service</td>
</tr>
<tr>
<td>• If they had an alternative diagnosis other than dengue, chikungunya or Zika or viral fever (e.g. leukemia, metastasis, autoimmune diseases, rheumatological disorders, etc.)</td>
<td>• If they have an alternative diagnosis other than dengue, chikungunya or Zika or viral fever (e.g. leukemia, metastasis, autoimmune diseases, rheumatological disorders, etc.)</td>
</tr>
<tr>
<td>• If they have a tracheostomy tube</td>
<td>• If they have tracheotomy</td>
</tr>
<tr>
<td>• If they have cancer with neutropenia</td>
<td>• If they have cancer with neutropenia</td>
</tr>
<tr>
<td>• If they have received a solid-organ or hematopoietic stem-cell transplant within the previous 90 days</td>
<td>• If they have received a solid-organ or hematopoietic stem-cell transplant within the previous 90 days</td>
</tr>
<tr>
<td>• If they have active graft-versus-host disease or bronchiolitis obliterans</td>
<td>• If they have active graft-versus-host disease</td>
</tr>
<tr>
<td>• If they have human immunodeficiency virus infection with a CD4 cell count of less than 200 per cubic millimeter (or a percentage of CD4 cells &lt;14%).</td>
<td>• If they have human immunodeficiency virus infection with a CD4 cell count of less than 200 per cubic millimeter.</td>
</tr>
<tr>
<td>• They are less than 7 years of age</td>
<td>• Participant received invasive mechanical ventilation or non-invasive respiratory support (i.e.,</td>
</tr>
</tbody>
</table>
- Participant received invasive mechanical ventilation or non-invasive respiratory support (i.e., continuous or bilevel positive airway pressure) in the 4 weeks prior to screening.
- Participant has received one or more doses of antiviral or treatment or prophylaxis with any antiviral compound (e.g., ribavirin, intravenous immunoglobulin, or vaccine for dengue or other viruses at any time one month prior to screening.
- Participants given any oral or intravenous steroid at any time two weeks prior to screening. Participant on a maintenance therapy of inhaled / intranasal corticosteroids and/or topical corticosteroids for skin disorders are permitted.
Appendix C
Enrollment Questionnaire 参加调查表格 [Soalan penyertaan]

Title of Study: Detection of Dengue, Chikungunya and Zika Viruses by A New Multiplexing Platform among Patients in Sarawak, Malaysia
研究主题: 使用多路复用平台新技术检测马来西亚砂拉越患者的骨痛热症、基孔肯雅和兹卡病毒

Tajuk Kajian: Pengesanan Virus Denggi, Chikungunya dan Zika dengan menggunakan Platform Multiplexing Baru di Kalangan Pesakit Sarawak, Malaysia

Medical Facility (check one): Hospital Sibu Hospital Kapit

Today's Date今日日期: ____________________ (dd 日 hari / mm月 bulan / yy年 tahun)

Please check all that apply by marking an “X” in the box given 请在所有相关的格子内画“X” [Sila tandakan semua yang berkenaan dengan tanda “X” di dalam kotak yang disediakan]

Patient’s Data and Demographics 患者个人资料 [Data dan Demografik Pesakit]

1. What is your (the patient’s) gender 您（患者）的性别 [Apakah jantina anda (pesakit)]?
   □ Male 男性 [Lelaki] □ Female 女性 [Perempuan]

2. What is your (the patient's) date of birth 您（患者）的出生日期 [Bilakah tarikh lahir anda (pesakit)]?
   ____________________ (dd 日 hari / mm月 bulan / yy年 tahun)

3. What is your (the patient’s) ethnicity 您（患者）的种族 [Apakah bangsa anda (pesakit)]?
   □ Iban 伊班 [Iban] □ Chinese 华裔 [Cina] □ Melanau 马兰诺 [Melanau]
   □ Malay巫裔 [Melayu] □ Bidayuh比达友 [Bidayuh] □ Other 其他 [Lain-lain]:________

4. For the purposes of classifying your place of stay, what is your address/neighborhood 为了归类您的居住地，请问您的地址/小区在哪 
   [Bagi tujuan pengkelaan tempat kediaman, apakah alamat/kawasan kejiranan anda]?

_____________________________________________________________

Medical Data 健康资料 Maklumat Perubatan
5. Are you pregnant (check one)? [Adakah anda hamil (pilih satu)?]
   - No (or does not apply) [Tidak (atau tidak berkenaan)]
   - If yes, the expected due date is [Jika ya, jangkaan tarikh kelahiran adalah] ________________ (dd hari / mm bulan / yy tahun)

6. Have you received the Dengue vaccine? [Adakah anda telah menerima vaksin Denggi?]
   - No [Tidak]
   - If yes, date of last dose of vaccination [Jika ya, nyatakan tarikh terakhir vaksinasi] __________________

7. Have you tested positively for Dengue, Chikungunya, and/or Zika in the last 3 years? [Pernahkah anda disahkan positif untuk Denggi, Chikungunya dan/atau Zika dalam 3 tahun lepas?]
   - No [Tidak]
   - IF yes, which virus? [Jika ya, virus yang mana?] __________________

8. Are you experiencing any of the following symptoms? (If yes, list duration and indicate days/weeks) [Adakah anda mengalami sebarang simptom berikut? (Jika ya, senaraikan tempoh dan nyatakan hari/minggu)]

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Duration</th>
<th>Days哈利</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rash</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Joint Pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctivitis/red eyes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle Pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea/Vomiting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatigue/General malaise</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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9. On-site Rapid Combo test result: 诊所内快速病毒检测结果: [Keputusan Ujian On-Site Rapid Combo]:

- Positive 阳性 [Positif]
- Negative 阴性 [Negatif]

---

**Behavioral, Occupational and Environmental Risk Factors 行为、职业及环境风险因素 [Faktor Risiko Tingkah Laku, Pekerjaan, dan Alam Sekitar]**

10. For patients older than 18 years of age 针对18岁以上的患者[Untuk pesakit 18 tahun ke atas]:
Out of the following options, which best describes the place in which you work 以下哪一项描述最符合您工作的地方?
Daripada pilihan di bawah, yang manakah paling tepat menggambarkan tempat kerja anda?

- Mostly outdoors 主要在户外 [Kebanyakan di luar rumah]
- A mix of outdoors and indoors 户外及室内都有 [Campuran di dalam dan luar rumah]
- Mostly indoors 主要在室内 [Kebanyakan di luar rumah]

11. Select which of the following mosquito control methods you have used in your home in the past week, if any: 如果有，请选出在过去的一个星期内您家里所使用的防蚊方法：
[Pilih kaedah pengawalan nyamuk berikut yang anda gunakan di rumah dalam minggu lepas, sekiranya ada:]

- Chemical mosquito repellent 防蚊液 [Penghalang nyamuk kimia]
- Bed net 蚊帐 [Kelambu]
- Mosquito coil 蚊香 [Lingkaran ubat nyamuk]
- Electronic mosquito coil/repellent 电子蚊香或驱蚊器 [Lingkaran nyamuk elektronik/penghalang]
- Window net 纱窗 [Jaring di tingkap]
- Others 其他 [Lain-lain]_________________________

12. During a typical week, how many days a week do you go out of your home at dawn or evening time, for work or leisure? 在一个星期里，您有几天会因为工作或休闲活动的缘故在黎明或傍晚时离开家外出?
[Kebiasannya dalam seminggu, berapa hari anda keluar rumah pada waktu subuh atau lewat petang untuk kerja atau santai]

- 0-1
- 1-3
- 3-5
- 5-7
13. How many mosquito bites do you remember receiving in the last week?
   在过去的一个星期内您记得被蚊子叮咬的次数是多少？
   [Mengikut ingatan, berapa banyak kaligigitan nyamuk yang anda terima dalam minggu lepas?]
   □ None 没有 [tiada]
   □ 1-5
   □ 6-10
   □ 11-20
   □ 21 or more 21或以上 [21 atau lebih]

14. In the last two weeks, was there any fogging in your place of living?
   在过去的两个星期内，您的住家是否有进行防蚊喷雾？
   [Dalam dua minggu lepas, adakah terdapat penyemburan (fogging) di kawasan kediaman anda?]
   □ Yes 有 [Ya]
   □ No 没有 [Tidak]

15. In the last one week, do you or your family check your place of living and the surrounding for mosquitoes breeding (e.g. standing water, containers, tyres, clogged gutters, etc.)?
   在过去的一个星期内，您和您家人是否有检查住家或周围环境的蚊子滋生（例如积水，废弃容器及轮台，堵塞的排水沟等等）?
   [Dalam satu minggu lepas, adakah anda atau keluarga anda memeriksa kawasan kediaman dan sekeliling bagi tempat pembiakan nyamuk (cth. air bertakung, bekas-bekas, tayar, longkang tersumbat dan lain-lain)]
   □ Yes 有 [Ya]
   □ No 没有 [Tidak]
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44. Andries, A.C., Value of Routine Dengue Diagnostic Tests in Urine and Saliva Specimens. 2015. 9(9).


