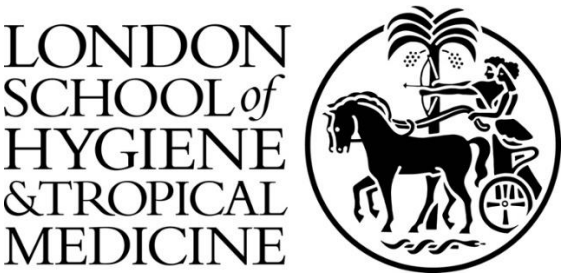

Immuno-epidemiological analysis of dengue to enhance surveillance

Joseph R. Biggs

Thesis submitted in accordance with the requirements for the degree of
Doctor of Philosophy of the University of London

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Department of Infection Biology
Faculty of Infectious and Tropical Diseases
London School of Hygiene and Tropical Medicine

Declaration

I, Joseph Biggs, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed 

October 2021

Abstract

As dengue continues to emerge globally, it is vital surveillance systems in endemic countries optimise routine case report data to accurately monitor dengue burden and target limited control interventions. Typical dengue surveillance practices, that often rely on case counts, are heavily distorted by underreporting. The WHO therefore promotes integrating additional surveillance practices to better describe dengue transmission. Across the Philippines, recently established laboratory surveillance routinely collects molecular and serological metrics from cross-sectional surveys of suspected dengue case reports. Research in this thesis aimed to investigate how analysis of laboratory surveillance data could be enhanced to better characterise dengue transmission dynamics across the country.

The variable clinical manifestations associated with dengue are influenced by successive serotype (DENV1-4) infections individuals experience and contribute to disease underreporting. Severe dengue disease is associated with a second DENV infection. However, distinguishing primary and secondary immune status remains challenging as molecular and serological kinetics change rapidly during disease and existing methods rely on paired sera collected from patients. Here, mixture modelling approaches were adopted to characterise DENV antibody dynamics and develop a dengue immune status algorithm that could determine primary and post-primary (secondary, tertiary or quaternary) status among acute-stage dengue case reports using single serum samples. This framework achieved 90.5% agreement with the WHO gold standard method using paired sera. Surveillance metrics from this algorithm were then investigated as potential surrogate indicators of the dengue force of infection (FOI) estimated using catalytic models of age-seroprevalence and compared using Pearson's R correlation coefficient. Across cities, the mean annual age of reporting primary infections strongly correlated (ρ: -0.85, p-value<0.001) with the FOI and highlighted prominent spatio-temporal heterogeneity in dengue burden. Notably, results also revealed reported dengue incidence was higher in cities with lower dengue FOI (ρ: -0.69, p-value:0.009) suggesting case reports represent inferior indicators of dengue burden.

Common dengue serological diagnostics detect flavivirus cross-reactive antibodies and growing evidence suggests prior Zika virus exposure exacerbates subsequent dengue disease. Therefore, serological evidence of Zika was explored among dengue case reports. Findings revealed historical Zika exposure was widespread across the Philippines and an estimated 5.7% (95%CI: 3.0–10.4%) of the population became infected annually. To enhance dengue surveillance practices in low resource settings where laboratory testing is unfeasible, logistic regression models were utilised to determine dengue immune status using point-of-care rapid diagnostic tests. On specific days of disease, certain combinations of rapid test outcomes gave rise to clear immune status classifications. Together, findings in this report demonstrate how characterising dengue immune status can enhance laboratory surveillance to accurately monitor dengue transmission intensity.

Acknowledgements

The completion of this PhD would not have been possible if it wasn't for the kind support of those around me. First and foremost, I would like to thank my supervisors Julius Hafalla and Martin Hibberd who provided me with the opportunity to complete this PhD. Throughout the degree, they have been a great source of encouragement and knowledge. Even during the challenging times of the COVID-19 pandemic, they ensured I had the correct equipment and kept in regular contact to help me maintain my momentum while working from home.

I would like to thank my other colleagues at LSHTM. To Oliver Brady, Adam Kucharski and Katharine Sherratt for their guidance on mathematical modelling and statistics. To William Jones-Warner and James Ashall for their laboratory work and general inputs. To Yalda Jafari, Stephane Hue and Sebastian Funk who I have the pressure of working with over the past 3 years. To Jackie Cook, Chris Drakeley and Immo Kleinschmidt for providing me with a good foundation in immuno-epidemiology prior to this PhD. The willingness of everyone at LSHTM to help, even during lockdown when many had to adapt to sudden changes, is something I will never forget and will always appreciate.

I am also extremely grateful to my collaborators overseas. To Ava Kristy Sy for her inputs, organising laboratory work and helping me while visiting the Philippines. To Joy and Mary and others in the virology lab at the RITM for conducting and managing the laboratory work. To Rose Capeding, Carmencita Padilla and Nemia Sucaldito for their expertise and inputs on dengue.

I also thank my family, friends and Dan. They have always supported and encouraged me to follow my passions. Without their love and kindness, pursuing a career in science may not have been achievable for me. For that I will always be grateful.

Statement of Contributions and Additional Publications

The work described in this thesis was conducted primarily at the London School of Hygiene and Tropical Medicine (LSHTM), London, UK. This research stems from collaborations between the LSHTM and the Research Institute for Tropical Medicine (RITM, Manila, Philippines), the University of the Philippines (UP, Manila, Philippines), the Philippine Epidemiology Bureau (EB, Manila, Philippines) the Pasteur Institute (Nha Trang, Vietnam) and the Eijkman Institute (Jakarta, Indonesia). Funding for this study was obtained from the Newton's Fund (grant number: 216416089), British Council, Commission on Higher Education, Royal Society (grant number: CHG/R1/170061) and ASTRA international. Funders had no role in study design, data analysis, or report preparation. Ethical approval for the use of surveillance and dengue case data was awarded to the RITM (ref: 2017-014), Pasteur Institute (ref: VN01057) and Eijkman Institute (ref: 136/2019) prior to this study. Ethical approval for the work presented in this thesis was awarded to me by the LSHTM ethical review committee (ref: 17965). I have detailed below my contribution to each of the chapters presented in this thesis.

Chapter 1

Chapter 1 is an introduction into the research topic and consists of original work.

Chapter 2

Chapter 2 outlines the thesis overall aim and objectives and consists of original work.

Chapter 3

Chapter 3 includes a published research article concerning methods to categorise the primary and post-primary immune status of dengue case reports using routinely collected laboratory epidemiological data from the Philippines. The algorithm was validated using laboratory data from confirmed dengue infections in Vietnam. Epidemiological surveillance data from the Philippines was provided by Ferchito L. Avelino and Nemia L. Sucaldito. Laboratory analysis of Philippine dengue case reports was conducted and overseen by Ava Kristy Sy, Mary Anne Joy Reyes, Mary Ann Quinones and Amado O. Tandoc, William Jones-Warner, Yun-Hung Tu and myself. Laboratory analysis of Vietnam dengue was conducted and overseen by Huynh Kim Mai, Le Thuy Lien, Hung Do Thai, Hien Anh Thi Nguyen, Dang Duc Anh, Chihiro Iwasaki, Noriko Kitamura and Lay-Myint Yoshida. Data analysis was led by me with assistance from Oliver J. Brady, Adam J. Kucharski, Sebastian Funk, Julius Clemence R. Hafalla and Martin L. Hibberd. The manuscript was authored by me and edited by Oliver J. Brady, Adam J. Kucharski, Julius Clemence R. Hafalla and Martin L. Hibberd. All co-authors approved the final version.

- ***Biggs, J.R****., Sy, A.K., Brady, O.J. et al. A serological framework to investigate acute primary and post-primary dengue cases reporting across the Philippines. (2020). BMC Medicine.* ***18****, 364.*

Chapter 4

Chapter 4 includes a published research article concerning methods to monitor the burden of dengue across the Philippines. Epidemiological surveillance data from the Philippines was provided by Ferchito L. Avelino and Nemia L. Sucaldito. Laboratory analysis of Philippine dengue case reports was conducted and overseen by Ava Kristy Sy, Mary Anne Joy Reyes, Mary Ann Quinones and Amado O. Tandoc and William Jones-Warner. Data analysis was led by me with assistance from Katharine Sherratt, Oliver J. Brady, Adam J. Kucharski, Sebastian Funk, Julius Clemence R. Hafalla and Martin L. Hibberd. The manuscript was authored by me and Ava Kristy Sy. The manuscript was edited by Oliver J. Brady, Adam J. Kucharski, Maria Rosario Z. Capeding, Julius Clemence R. Hafalla and Martin L. Hibberd. All co-authors approved the final version.

- ***Biggs, J.R.****, Sy, A.K., Sherratt, K. et al. Estimating the annual dengue force of infection from the age of reporting primary infections across urban centres in endemic countries. (2021). BMC Medicine.* ***19,*** *217.*

Chapter 5

Chapter 5 includes a published research article investigating evidence of widespread serological exposure to Zika across the Philippines. Epidemiological surveillance data from the Philippines was provided by Ferchito L. Avelino and Nemia L. Sucaldito. Laboratory analysis of Philippine dengue case reports was conducted and overseen by Yun-Hung Tu, Ava Kristy Sy, Mary Anne Joy Reyes, Mary Ann Quinones and Amado O. Tandoc & William Jones-Warner. Data analysis was led by me with assistance from Oliver J. Brady, Adam J. Kucharski, Sebastian Funk, Julius Clemence R. Hafalla and Martin L. Hibberd. The manuscript was authored by me and edited by Oliver J. Brady, Adam J. Kucharski, Maria Rosario Z. Capeding, Julius Clemence R. Hafalla and Martin L. Hibberd. All co-authors approved the final version.

- ***Biggs JR****, Sy AK, Brady OJ, et al. Serological Evidence of Widespread Zika Transmission across the Philippines. (2021). Viruses.* ***13****(8):1441.*

Chapter 6

Chapter 6 includes a drafted manuscript that investigates methods to categorise dengue primary and post-primary immune status using point-of-care rapid tests. Laboratory work was conducted and overseen by James Ashall, Ava Kristy Sy, Marsha S Santoso, Mary Anne Joy Reyes, Mary Ann Quinones, William Jones-Warner, Huynh Kim Mai, Le Thuy Lien, Hung Do Thai, Hien Anh Thi

Nguyen, Dang Duc Anh, Chihiro Iwasaki and Noriko Kitamura. Data analysis was led by me with assistance from Oliver J. Brady, Adam J. Kucharski, Sebastian Funk, Julius Clemence R. Hafalla and Martin L. Hibberd. The manuscript was authored by me and edited by Oliver J. Brady, Adam J. Kucharski, Maria Rosario Z. Capeding, Lay-Myint Yoshida, Tedjo R Sasmono, Julius Clemence R. Hafalla and Martin L. Hibberd. All co-authors approved the final version.

- ***Biggs JR**, Ashall J, Sy AK, et al. Combining rapid diagnostic tests to estimate primary and post-primary dengue immune status at the point-of-care. PLOS NTD (manuscript accepted pending format changes).*

Chapter 7

Chapter 7 includes the thesis discussion and conclusion and consists of original work.

Additional publications

I also contributed to the following manuscripts, which were not part of my PhD.

- *Bath D, Cook J, Govere J, Mathebula P, Morris N, Hlongwana K, Raman J, Seocharan I, Zitha A, Zitha M, Mabuza A, Mbokazi F, Machaba E, Mabunda E, Jamesboy E, **Biggs J**, Drakeley C, Moonasar D, Maharaj R, Coetzee M, Pitt C & Kleinschmidt I. (2021). Effectiveness and cost-effectiveness of reactive, targeted indoor residual spraying for malaria control in low-transmission settings: a cluster-randomised, non-inferiority trial in South Africa. Lancet.397 (10276). 816-827.*

- ***Biggs J**, Raman J, Cook J, et al. (2017). Serology reveals heterogeneity of Plasmodium falciparum transmission in northeastern South Africa: implications for malaria elimination. Malaria Journal. **16,** 48*

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Abbreviations

A1	Algorithm 1
A2	Algorithm 2
ADE	Antibody Dependent Enhancement
AIC	Akaike Information Criterion
AR	Attack Rate
AST	Aspartate Aminotransferase
CDC	Centres for Disease Control and Prevention
CI	Confidence Interval
Ct	Critical Threshold
DALY	Disability-Adjusted Life Year
DD	Disease Day
DDT	Dichlorodiphenyltrichloroethane
DENV	Dengue Virus
DOH	Department of Health
DR	Dependency Ratio
DRU	Disease Reporting Unit
EB	Epidemiology Bureau
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ESDI	Early Severe Dengue Identifier
FDA	The United States Food and Drug Administration
FOI	Force of Infection
GPS	Global Positioning System
HIA	Haemagglutination Inhibition Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRS	Indoor Residual Spraying
IVM	Integrated Vector Management
JEV	Japanese Encephalitis Virus
KAP	Knowledge Attitudes and Practises
LRT	Likelihood Ratio Test

MAC-ELISA	IgM Antibody-Capture Enzyme-linked Immunosorbent Assay
MIA	Multiplex Immunoassays
NAAT	Nucleic Acid Amplification Test
NS	Non-Structural Protein
NS1	Non-Structural Protein 1
OD	Optical Density
OR	Odds Ratio
PAGASA	Philippine Atmospheric, Geophysical and Astronomical Services Administration
PAHO	Pan American Health Organisation
PIDSR	Procedures for the Philippine Integrated Disease Surveillance and Response
PRNT	Plaque Reduction Neutralization Test
PSA	Philippines Statistics Authority
RBC	Red Blood Cells
RDT	Rapid Diagnostic Test
RITM	Research Institute for Tropical Medicine
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCR	Seroconversion Rate
SRR	Seroreversion Rate
VCAG	Vector Control Advisory Group
WHO	World Health Organisation
WNV	West Nile Virus
YFV	Yellow fever virus
ZIKV	Zika Virus

Chapter 1. Introduction

1.1 The Global Emergence and Distribution of Dengue

Dengue is the most important and rapidly spreading arboviral, infectious disease to burden the tropical and subtropical world [1]. The virus is primarily transmitted to humans through the bite of an infected female *Aedes* mosquito which thrive in warm, urban and peri-urban environments [2]. The disease causes a wide spectrum of clinical manifestations, with most experiencing asymptomatic infections and a minority who develop severe, life threatening, disease [3]. Despite the variability in clinical manifestations, the widespread distribution of the disease poses significant socio-economic challenges to affected countries [4,5]. Without specific therapeutics, disease management currently relies on supportive care [6]. Consequently, a huge focus has been placed on dengue prevention by combatting transmission through effective surveillance and vector control [7].

Despite dengue emergence, curbing the spread of this disease is not a recent endeavour. Historically, huge efforts have focused on *Aedes* eradication to stop the transmission of dengue and other *Aedes*-transmitted diseases including yellow fever virus. Soon after the Second World War, severe dengue outbreaks occurred in countries across the Americas and the Asia-Pacific regions. Then, following the introduction and widespread availability of the insecticide dichlorodiphenyltrichloroethane (DDT), the Pan American Health Organisation (PAHO) considered the notion of eradicating *Aedes* mosquitoes from the entire continent. Despite the enormity of the task, the PAHO received good government/public support and initiated the Continental Campaign for the Eradication of *A. aegypti* in 1947 [8]. The plan involved multiple annual rounds of DDT spraying inside the walls of properties, and for the first decade, enjoyed successes. 18 countries across the Americas declared *Aedes* eradication and only one circulating strain of the virus was detected in Brazil [9]. Soon after however, the political drive to pursue eradication in light of these accomplishments began to erode resulting in the withdrawal of vital funding. This coupled with a heavily centralised surveillance operations caused the programme to react too slowly to outbreaks or miss them completely [10]. In the 1970s, increased urbanisation, population growth and international travel prompted more and more dengue outbreaks and the programme dismantled. By 1995, further re-infestation of *Aedes* vectors caused dengue cases to surpass pre-campaign levels [11]. During the 21st century, global dengue case reporting increased 30 times higher than levels in the 1950s. However, it is acknowledged this is likely partly attributed to improved case report documentation as a consequence of countries recognising the huge economic burden of the disease [1]. Nonetheless, the sudden accelerated emergence is also thought to be due to recent global warming over the past 20 years expanding the geographical range of vector to transmit the virus [12,13].

In 2019, the World Health Organisation (WHO) reported a record 5.2 million global dengue case reports up from 2.4 million in 2010 with the majority from the Americas and Asian-Pacific region. [14]. This

increase caused numerous countries, such as Brazil, The Philippines, Vietnam, Colombia, Pakistan, Guatemala, Honduras and others to surpass their epidemic thresholds and send out outbreak alerts in an attempt to curb the spread of the virus [15,16]. During this year, dengue was also reported in Afghanistan for the first time and even across the temperate European continent, cases increased to 0.9 cases per 100,000. Of these however, approximately 65% were classified as imported cases signifying the intense global circulation of this disease [17]. Despite record numbers of case reports, notified cases only account for the minority who developed symptoms, sought health care, and were successfully documented by regional and centralised surveillance systems. Consequently, dengue is a heavily underreported disease. Indeed studies estimate 105-390 million annual infections occur globally and approximately only 25% develop clinical symptoms [3,18]. Due to this huge disease burden, recent focus has been to better understand the socio-economic impact of the disease. One recent study estimated a global loss of 2,922,630 (95%CI: 1,629,424-3,967,492) years of full health attributed to dengue in 2017, representing an 107% increase from 1990 [19]. Moreover, a separate study valued the global annual cost of dengue at US$8.9 billion (95%CI: US$3.7-19.7 billion) [20]. Interestingly, the burden of dengue is believed to be uneven across the tropics, with approximately 75% focused in Asia and 20% in the Americas [3]. If control strategies fail to combat dengue and the disease continues on its current trajectory, assisted by continued urbanisation and climate change, an estimated 6.1 (95%CI: 4.7-6.9) billion more will be at risk of dengue in 2080 compared to 2.25 (95%CI: 1.27-2.80) billion in 2015 [21].

To turn the tide on dengue, it is crucial lessons are learnt from the past. Sustaining effective surveillance and control programmes are essential to ensure any progress is not undermined to allow re-emergence of the disease. In 2012, the WHO published its ‘Global Strategy for dengue prevention and control 2012-2020’ where it laid out a roadmap to reverse the growing threat [7]. The programme recognised the importance of sustainable, multi-faceted approaches for combatting dengue and contained five key technical elements that were considered necessary to reduce dengue morbidity and mortality worldwide (Figure 1). These included: diagnosis and case management, integrated surveillance and outbreak preparedness, sustainable vector control, future vaccine implementation, and basic operational and implementation research. To reduce individual case mortality and morbidity, it is vital that suspected dengue infections are accurately diagnosed early during infection and appropriate prognostic markers are identified to help determine whether patients might progress to life-threatening severe disease [1,22]. At the population level, effective vector control strategies and vaccination programmes are important to combat transmission and reduce the risk of individuals becoming infected [23,24]. Moreover, continued scientific research is vital for the development of novel control strategies and ensuring current interventions remain effective [25,26]. Lastly, surveillance operations are crucial for detecting outbreaks, targeting limited control interventions to those most in need and evaluating the effectiveness of such strategies among populations [7,27]. Despite not achieving a 50% reduction in

mortality and a 25% reduction in morbidity globally in 2020 [28], striving for ambitious targets is commendable and strengthening these technical elements remains essential for reversing continued dengue emergence. The research in this thesis concerns all five of the WHO technical elements yet focuses on methods to strengthen integrated surveillance operations in dengue endemic countries.

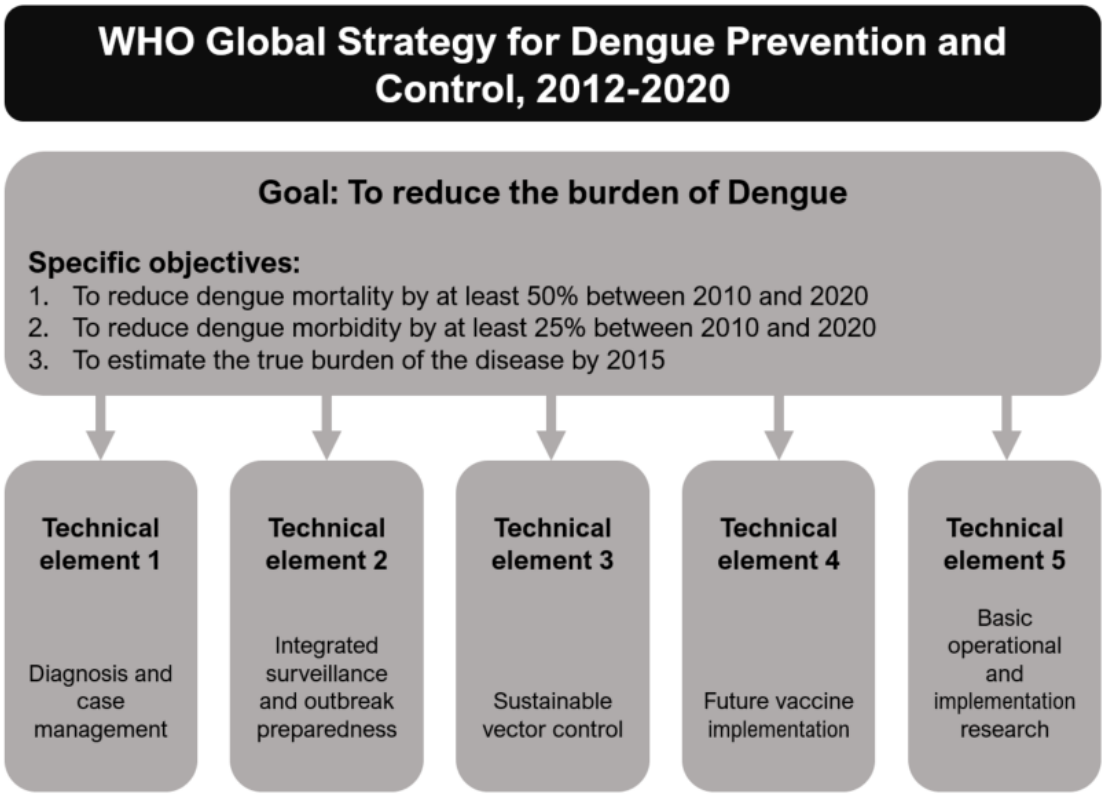

Figure 1. The WHO global strategy for dengue prevention and control, 2012-2020. To reduce the global burden of dengue, the WHO set out three main objectives: To reduce dengue mortality by at least 50% by 2020, to reduce dengue morbidity by at least 25% by 2020 and to estimate the true burden of disease by 2015. To achieve these goals, the WHO set out five technical elements that require strengthening: diagnosis and case management, integrated surveillance and outbreak preparedness, sustainable vector control, future vaccine implementation, and basic operational and implementation research. Adapted from [7].

1.2 The Clinical Manifestations and Complex Immunopathology of Dengue

Clinical Manifestations

Dengue is a disease that can give rise to a spectrum of clinical manifestations which are characterised according to renewed 2009 WHO criteria [1,7]. Asymptomatic cases account for approximately 75% of all infections and include those infected with, and able to transmit, the virus yet personally benefit from not displaying any clinical symptoms [3,29]. Symptomatic cases include those with acute febrile illness coupled with or without additional dengue-specific warning signs. Warning signs include abdominal pain, vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement, increased

haematocrit and decreased platelet counts. Symptomatic patients can also be classified as having severe dengue if their febrile illness is coupled with either severe plasma leakage, bleeding or organ impairment [14]. An overview of the dengue symptom criteria is shown in Figure 2. This new classification system was designed to improve patient prognosis by identifying those at risk of progressing to severe symptoms, however, recent studies have demonstrated that despite the classification system being an improvement on the 1997 WHO guidelines [30,31], it still has questionable sensitivities for capturing severe cases and might benefit from including additional criteria [32].

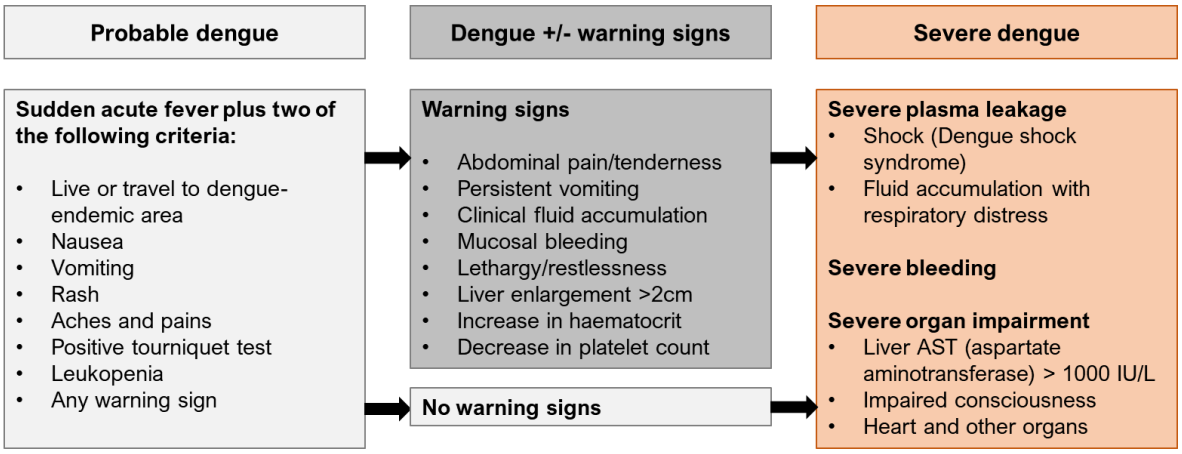

Figure 2. The symptom classification criteria for dengue disease according to 2009 WHO guidelines. Probable dengue infections presenting with non-specific fever can experience additional/no additional warning signs before progressing to severe disease. Adapted from [33].

Dengue symptoms are heavily influenced by the rapid disease progression. Following virus inoculation during a mosquito bloodmeal, the incubation period begins and lasts approximately 4-7 days [34]. Symptomatic patients progress to the febrile stage of the disease, which is often characterised by non-specific fever that typically lasts 3-4 days [35]. At this phase of infection, the disease is difficult to differentiate from other febrile illnesses but fortunately most make a full recovery [34]. Nonetheless, a minority of patients can develop additional warning signs (Figure 2) towards the end of the febrile period which is indicative of further deterioration. Although patients can still progress to severe disease without any warning signs [33]. During the subsequent critical phase, patients can rapidly develop severe, life-threatening forms of the disease characterised by increased vascular permeability (Figure 2). This is the more distinguishable phase of dengue disease yet requires careful monitoring to facilitate effective case management [1].

Severe dengue disease is a rare outcome that occurs in less than 5% of all cases [3]. Recent research has focused heavily on identifying risk factors of severe disease, yet few studies have identified reliable prognostic markers of severe disease development [22,36]. Despite this, some biomarkers have been identified as potential predictors of subsequent severe disease including serum chymase levels [37] and

the persistence of non-structural protein 1 (NS1) in the blood [38]. Moreover, studies have investigated whether multiple predictors can be incorporated into an early severe dengue identifier (ESDI). These included a history of vomiting, low platelet count, elevated aspartate aminotransferase (AST), and a positive NS1 test [39]. This ESDI showed promising sensitivity and specificity for subsequent severe disease although suffered a low positive predictive value as only a minority (<10%) of patients predicted to develop adverse outcomes experienced severe disease. Consequently, using this procedure, a high proportion of patients expected to develop severe disease would simply recover resulting in a waste of limited health care resources. Moreover, the ESDI relies on a variety of laboratory techniques that may be difficult to conduct in low resource settings and/or delay patient prognosis.

A number or risk factors have been identified with severe dengue disease [40,41]. Of these, most notable is age and prior exposure to DENV. The age at which individuals are most at risk of severe dengue is heavily influenced by dengue transmission intensity – the amount of transmission that occurs in the community. In hyperendemic settings, the burden of disease falls on younger individuals as the chance of experiencing an infection early in life is high. Consequently, children and young adults are at greater risk of severe disease. In low transmission settings however, the disease burden is believed to shift to older age groups as individuals are more likely to live for an extended period without ever being exposed to the virus. Therefore, older individuals in these settings are at greater risk of developing severe disease [42–44]. Lastly and counter-intuitively, a major risk factor for developing severe disease, is prior dengue virus (DENV) exposure [45,46]. However, to understand how a DENV infection primes individuals for subsequent severe disease requires a detailed appreciation of its complex biology.

Dengue Virus Structure and Replication

Dengue virus along with Zika virus (ZIKV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV) and West Nile virus (WNV) are all members of the *Flaviviridae* family [47]. They all consist of single-stranded positive-sense RNA genomes encapsulated by capsid (C) envelope (E) and membrane (prM) structural proteins [48]. Flaviviruses also encode non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) which are required for viral replication and assembly [49]. DENV consists of four antigenically similar, yet serologically distinct, serotypes (DENV1-4) which are all capable of infecting humans [1].

DENV enters host cells through receptor-mediated endocytosis and are internalised within cell endosomes. Inside these vesicles, endosomal acidification alters the configuration of viral E proteins resulting in fusion between the viral envelope and the endosome cell membrane [50]. This allows the viral genome to enter the host cell cytoplasm where the positive-sense RNA is encoded into a negative-sense RNA. This acts a template for host cell machinery to synthesize multiple rounds of viral proteins. Following rounds of viral protein translation, the virus switches to asymmetric replication of positive sense-RNA to be packaged within newly synthesised virions [51]. Virion assembly and maturation then

occur in the endoplasmic reticulum (ER) and Golgi apparatus of the cell. The newly synthesised viral RNA encapsulates within viral C proteins to form nucleocapsids. These immature virions subsequently pass through the ER and become enveloped and encased in M and E viral proteins. Maturation of virions occurs in the Golgi apparatus after which they are released from the host cell ready to infect other cells [48,52]. During replication, newly synthesised NS1 is secreted from host cells and becomes detectable in the blood for a few days post-viremia [33,34].

Immunopathology

In response to a DENV infection, the host counters the virus through both innate and adaptive immunity. At the site of mosquito inoculation, DENV invades host lymphoid and non-lymphoid skin cells wherein pattern recognition receptors detect viral material and trigger localised innate immunity [53]. Detection induces a host anti-viral state characterised by a pro-inflammatory type 1 interferon response and a complement cascade which impedes viral replication and recruits additional immune cells to the site of infection to promote viral clearance [54]. DENV however evades innate immunity by inhibiting the production of interferon and replicating inside recruited immune cells, including dendritic cells and macrophages [55]. Moreover, DENV utilises dendritic cells as transport vehicles into the lymphatic system to cause a systemic infection within the host [56].

In addition to acting as delivery cells, infected dendritic cells present viral antigen to both CD4+ and CD8+ T cells which triggers the adaptive immune response against surging viremia [46]. CD4+ T cells are instrumental in promoting the development of plasma and memory B-cells which secrete anti-DENV IgM. IgM antibodies surge in host serum approximately 6-8 days post-infection and persist for months offering short-term protection from all DENV serotypes (Figure 3) [52,57]. Days after the IgM antibody response, hosts elicit a general IgG response which subsequently wane following affinity maturation, mediated by CD4+ T cells, resulting in the long-term persistence of serotype-specific IgG in host serum (Figure 3) [58,59]. Both anti-DENV IgM and IgG act by neutralising DENV and prevent further cell entry and replication [46]. In addition to orchestrating humoral immunity, T cells are also vital for cell-mediated adaptive immunity against DENV. Activated CD4+ T cells produce cytokines that both promote/suppress the inflammatory responses while activated cytotoxic CD8+ T cells destroy DENV infected host cells by secreting granzyme B and perforin. The extent to which cell-mediated or humoral adaptive immunity contributes to viral clearance however remains unclear [46,55,60,61].

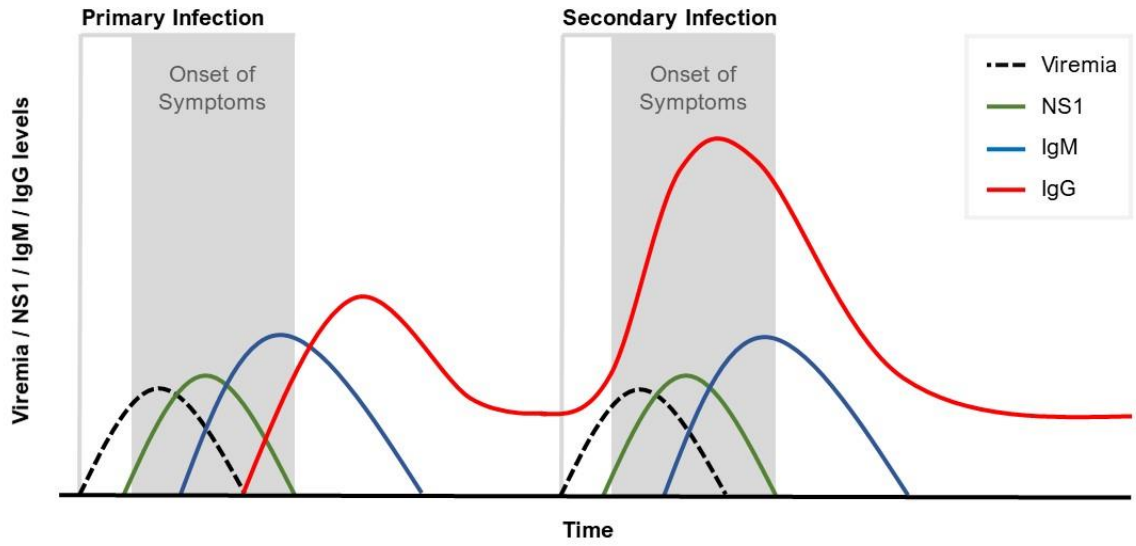

Figure 3. A schematic representation of viral RNA, NS1 antigen and virus specific antibody kinetics during a primary and secondary DENV infection over time. During the intrinsic incubation period of primary and secondary infections, viremia begins to surge which in turn leads to the secretion of detectable NS1 into the bloodstream. Approximately four-six days later, IgM is secreted and persists for months after infection. During a primary infection IgG is seven-ten days into infection yet persists long-term. Upon a secondary infection, pre-existing, non-neutralising IgG surges to high levels during the early stages of infection. Adapted from [62].

Following the resolution of a primary DENV infection, serotype-specific B cells are retained during immune memory formation. Consequently, a second DENV infection with a homologous serotype evokes a memory recall response which quickly and effectively clears infection [46]. Upon a subsequent secondary infection with a heterologous serotype however, the memory recall fails to combat infection and host DENV IgG, elicited from a previous infection, surges during the viraemic stage, and exceeds IgM levels (Figure 3). The contrasting levels of IgG and IgM represents a key distinguishable feature between primary and secondary DENV infections [46,52,62]. The increase in non-neutralising IgG is thought to exacerbate viral replication through an antibody-mediated enhancement (ADE) mechanism. Low levels of sub-neutralising IgG titres cross-react and bind to heterologous serotypes and facilitate enhanced receptor-mediated endocytosis. Fc receptors situated on host target cells bind to virus-bound IgG which enhances virus uptake and helps the virus to evade host immune responses [45,63]. Consequently, ADE during a secondary infection boosts virus replication in host cells which induces a cytokine storm that leads to immune-modulated vascular leakage – the hallmark characteristic of severe dengue disease. Following a secondary dengue infection, non-specific IgG wanes over time leaving neutralising IgG that prevent subsequent homologous serotype infections [64]. Furthermore, as there are four known DENV serotypes in global circulation, individuals can suffer two further post-secondary (tertiary/quaternary) infections. These types of infections are less characterised than primary and secondary infections, yet they are thought to be mild and not associated with ADE mechanisms [65,66].

Although post-secondary infections have been shown to heavily contribute to community transmission [67].

In addition to ADE mechanisms, recent studies have demonstrated the importance of non-structural protein 1 (NS1) in dengue pathogenesis [68]. NS1 is a highly conserved glycoprotein among flaviviruses and is essential for viral replication [69]. During intracellular DENV replication, NS1 is continuously secreted from host cells and is detectable in serum during, and after, the viraemic stage of infection (Figure 3) [70]. Interestingly, compared to those with asymptomatic or mild dengue disease, serum NS1 levels have shown to be higher among those with severe dengue disease [71–73]. Although one animal study identified no such trend in mice [74]. Several proposed mechanisms in which NS1 contributes to pathogenesis have been identified [68]. Firstly, NS1 is believed to exacerbate vascular leakage by interacting with the host endothelium and disrupting cellular structural integrity through glycocalyx degradation [70]. Secondly, NS1 is thought to promote immune evasion by interrupting the host complement cascade inhibiting cell lysis thus enabling further viral replication [68]. Lastly, anti-DENV NS1 antibodies, despite been shown to offer protection from DENV, have recently been shown to intensify dengue pathogenesis due to host cross-reactivity. Anti-DENV NS1 antibodies have been shown to bind to host molecules and promote haemorrhage, thrombocytopenia and liver damage [75–77]. The exact mechanisms underlying severe dengue disease are not fully characterised, yet it likely involves a combination of viral and host factors [75].

1.3 The Challenge of Diagnosing Primary and Secondary Dengue

Diagnosing Dengue Infection

Dengue can be diagnosed according to a variety of methods and is crucial for both surveillance and case management. Prompt dengue diagnosis has two major benefits for individuals as it guides supportive treatment and eliminates the need for further investigative testing [78]. For surveillance purposes, accurate diagnosis can ensure more accurate estimates of disease burden are generated across populations which can assist in allocating limited control interventions [79]. Typically in endemic settings, DENV infections are diagnosed according to clinical presentation (Figure 1) then can be later confirmed according to a host of laboratory or non-laboratory methods [1,33]. However, given the expense and labour intensiveness of laboratory procedures, additional confirmatory testing is often not conducted, particularly in low resource settings. According to the PAHO estimates in 2019, only 44% (1,415,771/3,190,771) of all reported cases across the Americas received a laboratory test demonstrating the need for more affordable and easy to use diagnostics [80]. Current laboratory dengue diagnostics consist of direct molecular and indirect serological methods that are appropriate at different stages of disease (Figure 4) [81,82].

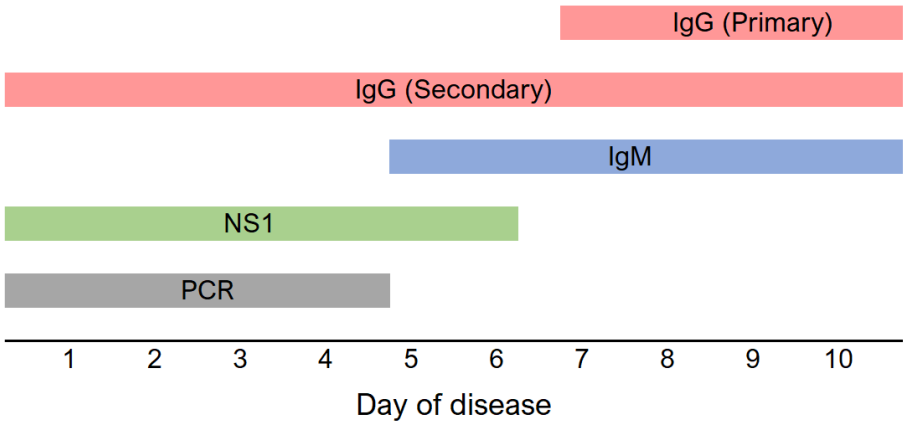

Figure 4. Suitability of dengue-specific diagnostic markers during infection. During the acute stage of disease, RT-PCR tests can detect viremia and NS1 ELISAs/rapid tests can be used to detect secreted NS1. During the later stages of disease, serological diagnostics can be utilised to detect IgM and IgG. During a primary infection, IgG is detectable later compared to a secondary infection. Adapted from [82].

Molecular DENV diagnostics

Molecular nucleic acid amplification tests (NAAT), such as reverse transcriptase polymerase chain reaction assays (RT-PCR), can be used to diagnosis an early-stage DENV infection. DENV-specific primers are utilised to detect and amplify viral RNA in host serum and can quantify the amount of virus according to critical threshold (Ct) cycle outputs [83]. RT-PCR tests benefit from typically having high (>80%) sensitivity/specificity for detecting DENV, only rely on single serum samples and can provide results within one or two days [84,85]. Moreover, the recent FDA-approved CDC DENV1-4 RT-PCR assay was developed to accurately diagnosis specific serotype DENV infections as various serotype-specific primers have been designed [86,87]. However, one of the major caveats of nucleic acid testing is the short window of detection. Given individuals with dengue disease report during the symptomatic stage of infection, many of those tested for dengue may have surpassed the viraemic stage or have viral loads below detectable levels [78]. Consequently, it remains crucial PCR testing is conducted early during disease and ideally in combination with other, later-stage, laboratory methods.

Serological DENV diagnostics

During the later immunogenic stage, serological diagnostics can be utilised to identify a DENV infection. Currently, a range of serological tests can be adopted to detect anti-DENV antibodies in patients including Haemagglutination inhibition assays (HIAs), plaque reduction neutralisation tests (PRNTs) and enzyme-linked immunosorbent assays (ELISAs) [33]. In HIAs, the presence anti-DENV IgG antibodies in serum inhibits the haemagglutination of red blood cells (RBC) by dengue antigen to form observable pellets at the bottom of microtitre plates. Through serial dilutions of host serum, DENV antibody titres can be calculated [88]. According to WHO criteria, a four-fold increase in DENV

antibody titres between the acute and convalescent (approximately 7 days apart) stage of disease is indicative of current DENV infection. Moreover, using paired sera, HIAs can be utilised to determine primary or secondary DENV infection. An increase in DENV antibody titres above and below 1:1280 titre value at convalescence is indicative of a secondary and primary infection, respectively. However, the reliance on paired sera for this assay makes it unsuitable for large-scale epidemiological studies and has limited value for individual case management.

Another serological method for detecting DENV IgG antibodies includes the PRNT assay. The assay involves exposing cells cultures, infected with DENV, to serial dilutions of host serum. In the absence of host DENV antibody, cells infected with DENV form detectable plaques where the virus has spread across the media. DENV IgG antibody PRNT titres are therefore obtained from serial dilutions of sera that achieve ≥50% reduction in plaque counts [89]. Additionally, by infecting cell cultures with specific serotype DENV strains, PRNTs can also be used to determine whether patients have been exposed to single or multiple serotype(s) indicating primary or post-primary DENV exposure, respectively [90]. Both PRNTs and HIAs however are associated with major practical shortcomings. They are both labour intensive and require trained laboratory staff in well-equipped facilities. As such, these diagnostics are impractical in low resource settings. Moreover, both PRNTs and HIA lack standardisation between laboratories which makes inferences about results challenging [78,90–92].

Of all the available serological techniques, the anti-DENV IgM and IgG capture ELISAs remain the mostly commonly used for dengue diagnosis [78]. Unlike HIAs and PRNTs, they are more high-throughput and a range of standardised commercial assays are available [33,93]. IgM antibody capture ELISAs, otherwise known as MAC-ELISAs, detect IgM in patients soon after the development of symptoms. Microtitre plates, coated in anti-μ chain specific antibodies, capture host anti-DENV IgM specific to all serotypes. The presence of host IgM antibodies are bound by monoclonal antibodies conjugated with an enzyme which alters the colour of the assay substrate. The amount of host antibody corresponds to the degree of colour change measured by optical density [92]. In addition, IgG capture ELISAs detect host IgG to any DENV serotype indicating a recent or past infection. The method is similar to the MAC-ELISA except microtitre plates are coated with DENV E/M protein-specific antibodies which capture DENV IgG instead of IgM. Using paired sera, a four-fold increase in IgG between the acute and convalescent stage of disease indicates an active infection [94,95]. However, given individuals with primary infections elicit IgG much later during the course of infection, IgG capture ELISAs have higher sensitivities for diagnosing secondary dengue infections [78]. Overall however, despite serological techniques offering larger windows of detection compared to molecular diagnostics, cross-reactivity with other flaviviruses remains a major caveat. The structural homology between the E-proteins of flaviviruses including DENV and ZIKV, JEV mean elicited antibody responses cross-react making it challenging to determine the true causative agent of infection [12]. Furthermore, although commercial ELISA kits have higher throughput than HIAs and PRNTs, they still

require trained staff in adequate laboratory facilities. Consequently, this limits the value of these assays in low resource settings (Table 1) [81].

Table 1. **The suitability of contrasting dengue diagnostics in primary, district and reference health care facilities**. In centralised, reference centres a wider array of dengue diagnostics are utilisable compared to smaller, primary health care facilities. Adapted from [7].

DENV Diagnostic Test	Primary Health Facility	District Health Facility	Reference Centre
Virus Detection			
PCR			+
Antigen Detection			
NS1 ELISA		+	+
NS1 RDT	+	+	+
Antibody Detection			
IgM ELISA		+	+
IgM RDT	+	+	+
IgG ELISA		+	+
IgG RDT	+	+	+

DENV rapid tests

Recently, the WHO has advocated the use of new diagnostic rapid tests (RDTs) for dengue, particularly in low resource settings due to their ease of use (Table 1) [7]. To date, numerous, inexpensive RDTs are commercially available which provide results within 20-30 minutes. These immunochromatographic tests consist of cellulose strips impregnated with monoclonal anti-antibodies which detect either DENV antigen or antibodies in patient samples through capillary action. The presence of DENV antigen/antibody is revealed by a distinct maroon line on the test strip and can only be utilised to provide either a positive or negative result [96]. Different types of RDTs, including NS1 IgM and IgG, can be used to determine dengue at different stages of infection [97,98].

The DENV NS1 RDT detects secreted NS1 that is present in the bloodstream during, and just after, the viraemic stage (Figure 4). This gives it a slightly longer window of detection than PCR tests yet suffers slightly lower sensitivity that traditional molecular testing [99]. In addition, IgM and IgG RDTs can be used to detect antibodies during the immunogenic stage of infection [100,101]. As kinetics change rapidly during a dengue infection [46], combining different types of DENV rapid tests has been shown to improve sensitivity, although this is dependent on the type of commercial test used [101,102]. NS1 RDTs have been shown to be highly specific for DENV when tested against ZIKV and YFV [103], yet the risk of IgG and IgM RDTs providing false-positive results when patients experience other flaviviral infections is still concerning [78,104]. Lastly, despite the array of available diagnostic tests, they are

primarily concerned with diagnosing active dengue infections and determining whether patients are experiencing primary or secondary infections using such tests remains poorly characterised.

Distinguishing Primary and Secondary Dengue Immune Status

Differentiating primary and secondary immune status among reporting patients, particularly at the early stage of infection, could be useful for dengue control. Categorising those with secondary infections prior to onset of severe symptoms has the potential to be a useful prognostic marker health care workers could use to inform patient clinical care. At the population level, determining the immune status of those reporting with dengue disease could identify groups who might benefit from post-exposure vaccination [66,105] and those at risk of severe disease in the future [45,46]. The current 'gold standard' method for determining primary and secondary immune status retrospectively remains the 1997 WHO serological technique [92]. Paired sera, collected from patients during the acute and convalescent stage of infection (approximately 7 days apart), are assayed for both DENV IgM and IgG. During the sampling interim, an increase in IgM, coupled with high and low IgM to IgG ratio at convalescence, are indicative of secondary or primary dengue infections, respectively. The necessity of paired sera however, makes this method unsuitable for large scale surveillance and patient case management.

Recently, focus has been on whether primary and secondary immune status can be determined using a single serum sample at the early, acute stage of disease. To date, numerous serological algorithms have been developed that state specific IgG titre thresholds or IgG:IgM titre ratios can be used to differentiate immune status, although variation between estimates exist. One study suggested a 1:29,000 titre of DENV IgG represented a suitable threshold for determining secondary dengue as IgG is believed to be absent during the acute stage of a primary infection [106]. Immune status distinguishing IgG:IgM ratios proposed by separate studies varied between 1.10 [107], 1.14 [108] and 1.70 [109]. The variability between these estimates is likely a consequence of contrasting methodologies, specific serological assays used and rapidly changing infections kinetic during infection. For each of these studies, investigators assessed the optimal IgG:IgM sensitivity/specificity that best distinguished immune status. However, the gold standard method for initially determining the reference immune status was based on contrasting methods including RDTs/ PRNT using single serum samples and HIAs using paired sera. Furthermore, investigators in these studies used different serological assays to determine immune status, which with varying sensitivities, gives rise to contrasting titre and ratio estimates. Lastly, during both primary and secondary dengue infections, titre differences between IgG and IgM change rapidly (Figure 3) [45,46], therefore a single IgG cut off or IgG:IgM ratio threshold used to distinguish immune status would likely result in immune status misclassification. Investigating whether functional, disease-day specific thresholds better characterise primary and secondary immune status, therefore warrants further investigation.

Combining IgG thresholds and IgG:IgM ratios has been recently proposed to improve immune status classification. One study demonstrated a combination of IgG cut offs at the very early stages of infection and IgG:IgM ratios at the later stages of infection, was optimal for categorising primary and secondary immune status. This was likely due to the fact IgG was detectable at the later stages of a primary infection yet absent during the early stages of a primary infection [110]. Another challenge associated with categorising dengue immune status is the sole reliance on serological diagnostics. Using just serology, very early-stage non-immunogenic primary infections, who would present with undetectable levels of IgM and IgG [46], would be unclassifiable according to such algorithms. A diagnostic algorithm that can determine DENV immune status using a combination of molecular and serological techniques is therefore required to capture all reporting primary and secondary infections.

1.4 The Limited Strategies for Combatting Dengue

Currently, no specific therapeutics against dengue exist and case management relies solely on supportive care [1]. For those experiencing dengue fever, which can often be accompanied with agonising body discomfort, a regular course of paracetamol is recommended for pain management [14]. Studies have shown however, there is no convincing evidence paracetamol has an analgesic benefit and even has potential safety issues [111]. For patients experiencing severe dengue, rapid intravenous hydration therapy can counter the loss of fluids caused by vasculopathy. However, this requires trained health care workers in well-equipped clinical settings to diligently monitor patients. This is necessary for up to three days to prevent any complications until the severe symptoms subside [1].

Dengvaxia® (CYD-TDV, Sanofi Pasteur) is a live-attenuated, tetravalent vaccine that can prevent DENV infections and is now fully licensed in several countries. The recombinant vaccine consists of a YFV backbone with structural components of all four DENV serotypes [23]. In 2016, the WHO recommended vaccine rollout and the Philippines was one of the first countries to launch it among children and adults in several highly endemic settings [112]. In 2017 however, an increased risk of hospitalisation due to severe dengue-like disease was identified among dengue-naïve children around the time of vaccination. Public outcry halted the roll out programme and lead to suspicion towards vaccines in general across the country [113]. In 2018, long-term findings released from Sanofi Pasteur concurred with discoveries in the Philippines and revealed that after 13 months post vaccination, dengue-seronegative individuals aged 2-16 years were at a higher risk of hospitalisation than seropositive recipients (Hazard ratio: 1.75 (95% CI: 1.14-2.70)) [114]. It should be noted however no vaccine is 100% effective and overall the vaccine would likely reduce incidence in the population [112]. Today the vaccine is still recommended by the WHO, but to only those with one prior exposure to DENV in endemic areas aged between 9-45 years [14]. Whether this age range is suitable for administering the vaccine in all ‘high’ endemic areas remains unknown and warrants further investigation. If children experience their first infection sooner in certain areas, monitoring the age of

reporting primary infections may better help inform which age ranges should be considered for pre-vaccination screening.

With limited therapeutics and controversy surrounding dengue vaccination, vector control interventions remain the predominant method for combatting dengue transmission. In addition, the emergence of ZIKV and other arboviruses, that are transmitted by the same vector, has renewed WHO focus on integrated vector management (IVM) [7]. In March 2016, the WHO Vector control advisory group (VCAG) recommended a series of vector strategies against *Aedes* mosquitoes. This included targeted indoor residual spraying (IRS) of insecticide to combat resting adult mosquitoes within properties. Secondly, indoor and outdoor space spraying (fogging) of insecticide. This targets adult mosquitoes in the vicinity yet has no residual effect. Thirdly, larval control through source reduction and larviciding to minimise the propagation of mosquitoes. Lastly, individual protection using repellents and clothing at night [115]. Despite being shown to combat *Aedes* populations and being widely advocated, the impact of these interventions on dengue burden remains poorly characterised [24,116]. Moreover, vector control strategies are highly intrusive, need good community engagement, require adequate coverage and are expensive. One study in Malaysia estimated the total annual cost of vector control against dengue at $73.5 million [117]. Despite this, considering the economic burden of dengue on impacted countries, the high cost of vector control is still considered cost-effective particularly when combined with other preventative strategies [118]. Consequently, huge focus has been on how best to target these interventions to ensure they have the greatest impact and vital resources are not wasted [7].

Despite limited strategies against dengue, there are novel dengue therapeutics and control interventions in the research pipeline [1,119]. Drug therapeutic treatments such as the NS4B inhibitor which targets viral entry into host cells, developed by Janssen Pharmaceuticals, has shown promise and is about to enter clinical trials [120]. By combatting dengue viremia, the drug has potential to both prevent adverse clinical symptoms and even onward transmission, although this requires further investigation. In addition, the release of genetically modified, dengue resistance *Aedes* vectors has been proposed as a method to minimise transmission. The introduction of Wolbachia-infected *Aedes* mosquitoes has recently shown promise in Indonesia where a cluster randomised trial reported an intervention protective efficacy of 77.1% [95% CI: 65.3 – 84.9%] against virologically confirmed DENV infections [121].

1.5 The Sudden Recognition of the Threat of Zika

Zika virus (ZIKV) is flavivirus that shares 55-56% structural homology and a common mosquito vector with DENV [122]. Like DENV, ZIKV can cause a febrile illness that often results in mild, self-limited non-specific fever. Unlike DEMV however, it is composed of one serotype [123]. During the 20th and early 21st century, Zika spread from Africa, to Asia, to the pacific island region causing small sporadic,

poorly documented, outbreaks and was of limited public health concern [124]. In 2015/16 however, Zika suddenly gained global prominence. A major outbreak in north-eastern Brazil coincided with a sudden spike in severe birth abnormalities including microcephaly, a condition which impedes brain development and growth in neonates [125]. The outbreak caused global concern and continued to spread rapidly across the country. During the outbreak, there was no conclusive evidence that microcephaly was caused by Zika infection, however there was convincing spatio-temporal overlap between reported birth abnormalities and Zika case reporting that prompted additional research [126]. Studies later provided further evidence that microcephaly was associated with ZIKV infection, particularly during the early stages of pregnancy [127,128]. Today, the clinical manifestations of Zika in adults are more defined and symptoms according to WHO criteria include fever, rash, conjunctivitis, muscle and joint pain, malaise and headache [129]. Symptoms which mirror those of other flaviviruses including dengue, except that Zika has also been to be associated with Guillain-Barre syndrome in adults [130]. As of 2019, heightened global disease recognition and surveillance operations revealed evidence of autochthonous transmission in over 87 countries worldwide with numerous others at risk of Zika [129].

It is well known that prior DENV exposure is a risk factor for progressing to severe disease during a subsequent, heterologous DENV serotype infection [45,46]. However, given the structural homology between ZIKV and DENV, particularly in the antibody-binding structural E-protein [131], many have speculated that exposure to one flavivirus might elicit cross-reactive IgG that triggers ADE mechanisms during a subsequent heterologous flavivirus infection. Yet the evidence as to whether ZIKV can prime individuals for a more severe, subsequent DENV infection, and vice versa, remains ambiguous as studies are often based on animal models or conducted *in vitro* [132]. In humans however, there is growing evidence that prior DENV exposure is not associated with elevated viremia during a Zika infection [133,134] or congenital abnormalities in pregnant women infected with ZIKV [135]. In contrast, there is mounting evidence that prior ZIKV exposure is associated with severe disease in subsequent DENV infections. Despite an epidemiological study reporting a reduction in the number of dengue cases following a Zika outbreak [136], a recent cohort study conducted in Nicaragua found those with prior ZIKV exposure were more likely to develop severe disease upon a DENV-2 infection compared to those without ZIKV exposure [132]. This poses a threat to future vaccination programmes and highlights the potential risks of ZIKV and DENV co-endemicity. Therefore, characterising ZIKV transmission dynamics, at sub-national levels, is important to better identify the dangers posed by these viruses circulating together.

A major obstacle for large scale Zika epidemiological studies is differential diagnosis from dengue. Commonly used serological diagnostics used in serosurveys detect cross-reactive antibodies which makes it challenging to determine to true causative agent of infection [123,131]. Several serological studies have investigated Zika transmission patterns across Laos [137], Taiwan [138], and Brazil [139] and revealed evidence of widespread transmission yet cross-reactivity sheds doubt on their findings.

Recently in Thailand however, a large scale population-based survey which utilised more specific molecular assays revealed evidence of widespread, persistent, transmission across the country [140]. Yet whether this is the case in other dengue-endemic countries, with environmentally suitable conditions for Zika, remains poorly defined and warrants further investigation to determine if transmission is well established or just routinely imported.

1.6 Harnessing Flavivirus Immune Responses for Surveillance

Surveillance Practices

Flavivirus surveillance operations are critical for monitoring the burden of disease and outbreak preparedness. Effective programs can ensure costly and limited control interventions are targeted to populations most in need. Typical passive surveillance operations in endemic countries rely on case reporting to generate incidence estimates. Incidence being a measure of disease risk according to the number of new cases in a population over a specified time period [7]. These attainable metrics, which account for the underlying population size, can be used to inform dengue control strategies. However, generated estimates can be inaccurate. Firstly, counts included depend on variable case definitions that can differ between and within countries [141]. Although renewed WHO case classification criteria generated in 2009 attempted to improve and standardise case reporting [7], not all health facilities in endemic countries adhere to WHO guidelines [142]. Secondly, as most flavivirus cases are thought to be asymptomatic or mildly symptomatic, few likely seek care and are detected by any passive surveillance system [3]. Moreover, even if cases are symptomatic and are prompted to seek treatment, variable health care infrastructure in endemic countries mean not all those who report are successfully documented. In fact, regions/countries with more reliable surveillance practises can appear to have more disease burden than those with incomplete case reporting [79].

To enhance surveillance operations, it is recommended additional practises should accompany routine case reporting to better describe the burden of dengue [7,27,143]. According to the 2016 WHO 'technical handbook for dengue surveillance, dengue outbreak prediction/detection and outbreak response', further strategies could include: epidemiological sub-analysis of routinely reported data, syndromic surveillance, laboratory-based dengue reporting and active surveillance operations [144]. Epidemiological sub-analysis involves analysing, not just collating, data from case reports. For instance, characterising the age distribution of reported cases can be used to reveal trends in transmission intensity [79]. Syndromic surveillance includes utilising rapid, electronically based systems to monitor specific events that are indicative of dengue burden. Such examples include school absentee numbers, hospital patient volume and new serotypes in the population [145]. Laboratory-based dengue reporting can be employed to improve the specificity of confirmed cases identified according to clinical symptoms, enhance syndromic surveillance practises and monitor national trends in circulating serotypes

[144,146]. Lastly, active surveillance is a technique in which cases are detected in the population, regardless of symptom development or reporting. The latter being a resource-intensive approach that involves outreach to laboratories/hospitals and population-based surveys, yet has a higher sensitivity for capturing cases than passive surveillance operations [147,148].

Immuno-epidemiological Surveillance

Following a flavivirus infection, hosts elicit long-lived antibody responses, irrespective of symptom development, that act as serological markers of exposure [46]. These antibody markers have considerably larger windows of detection compared to other infection kinetics, including viremia and NS1 (Figure 3), and can be easily detected using affordable, high-throughput ELISAs [149,150]. Elicited DENV/ZIKV IgM and IgG have been reported to persist in the host serum for months and decades after infection, respectively [46,123,151]. Immuno-epidemiological surveys are primarily concerned with detecting serological markers in populations and have been utilised for various infectious diseases that elicit stable antibody responses including malaria [152–154], measles [155] and polio [156]. One of the major benefits of conducting population-based immuno-epidemiological studies is they can be used to estimate the force of infection (FOI) – the intensity of transmission in the community. By stratifying IgG seroprevalence (long-term marker of exposure) by age, mathematical catalytic models can be utilised to estimate the rate of accumulating IgG exposure [157–159]. Assuming dengue naïve individuals transition solely from an IgG seronegative to seropositive state upon infection, and refrain from reverting to seronegative status again, simple, opposed to reversible, catalytic models can estimate the annual average rate that the study population seroconvert (Figure 5A). This rate, otherwise known as the seroconversion rate, is analogous to the FOI. The faster the accumulation of IgG exposure with age, the larger the FOI as this indicates that individuals are experiencing dengue infection(s) at a young age. In contrast, the slower the accumulation of IgG exposure with age, the lower the force of infection, as individuals are experiencing dengue infections later in life (Figure 5B). By stratifying age seroprevalence across geographical areas, spatial patterns in the force of infection can also be determined, which can be useful for understanding the spatial heterogeneity in the burden of dengue [160].

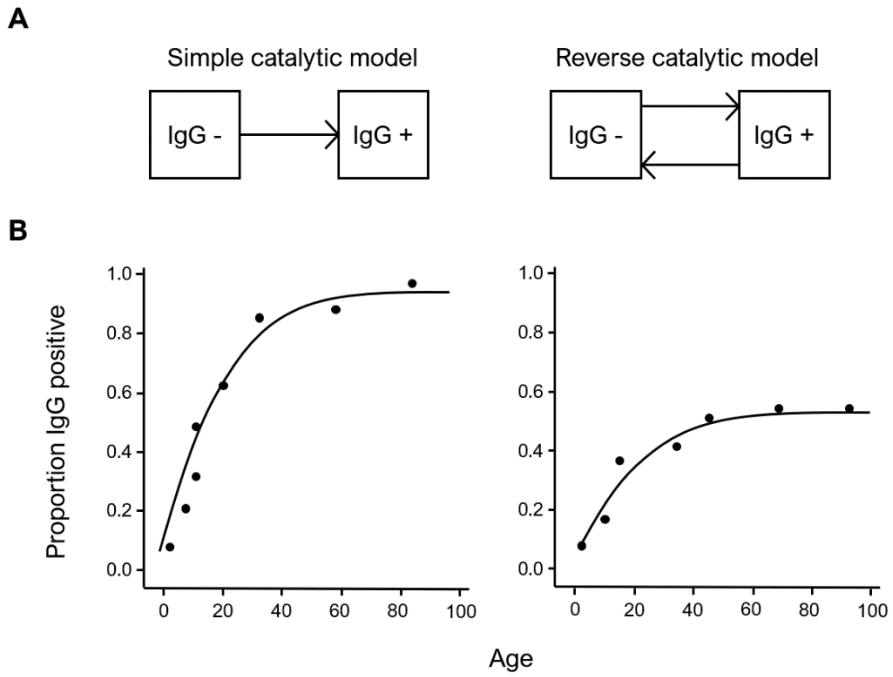

Figure 5. A schematic representation of catalytic models used to estimate the dengue force of infection (FOI). A. Simple catalytic models assume seronegative (IgG-) individuals seroconvert to seropositive status (IgG+) upon infection. Reverse catalytic models assume seronegative (IgG-) individuals seroconvert to seropositive status (IgG+) upon infection yet can serorevert back to seronegative (IgG-) status with time. **B.** Age IgG-seroprevalence curves fitted with catalytic models used to estimate the dengue seroconversion rate (FOI) in a sample population. Black dots: Observed age-seroprevalence. Black line: Predicted age-seroprevalence. Adapted from [152,160–162]

There are challenges associated with estimating the FOI from immuno-epidemiological studies. Firstly, determining who is 'seropositive' and 'seronegative' for dengue antibody markers. Widely available dengue commercial ELISA kits often include pre-defined antibody cut offs, individuals with titres above and below these thresholds can be considered seropositive and negative, respectively. Previous studies in Indonesia have utilised these pre-existing thresholds to estimate the FOI [160,162]. However, commercial kits often refrain from explaining how pre-defined seropositivity cuts were derived and many kits are concerned with determining those current dengue infections, and not those previously exposed to dengue [163]. Consequently, IgG thresholds can be elevated, meaning those with prior dengue exposure are considered seronegative. Fortunately, there are other methods for determining antibody seropositivity. Some antibody seropositivity cut offs correspond to assay signals that significantly exceed signals from known negative controls. For instance, in Taiwan, a study classified IgG seropositivity according to a ratio of the sample IgG optical density (OD) reading compared to the negative control IgG optical density reading greater than 2.0 [105]. Despite the simplicity of this approach, arbitrarily defining a significant difference that constitutes a seropositive antibody response likely leads to individuals with slightly elevated responses being misclassified. Finite mixture models can also be utilised to determine seropositivity and have become common practise in numerous

infectious disease immuno-epidemiological studies [153,154,164,165]. Mixture modelling is deemed an appropriate approach to characterise seropositivity when the sample size is large enough to reveal distinct antibody distributions in the data [166,167]. The model assumes the sample population antibody distribution data consists of two subpopulations: 'seronegatives' with low antibody responses and 'seropositives' with elevated antibody responses (Figure 6A). Probabilistic mixture models can characterise these subpopulations and estimate the probability of being seronegative and seropositive for any given antibody titre. From these probabilities, thresholds that distinguish the seropositive and seronegative population can be derived (Figure 6B) [168]. The major benefit of this approach is that it only relies on antibody response data from the study population, and not the data from confirmed negative controls.

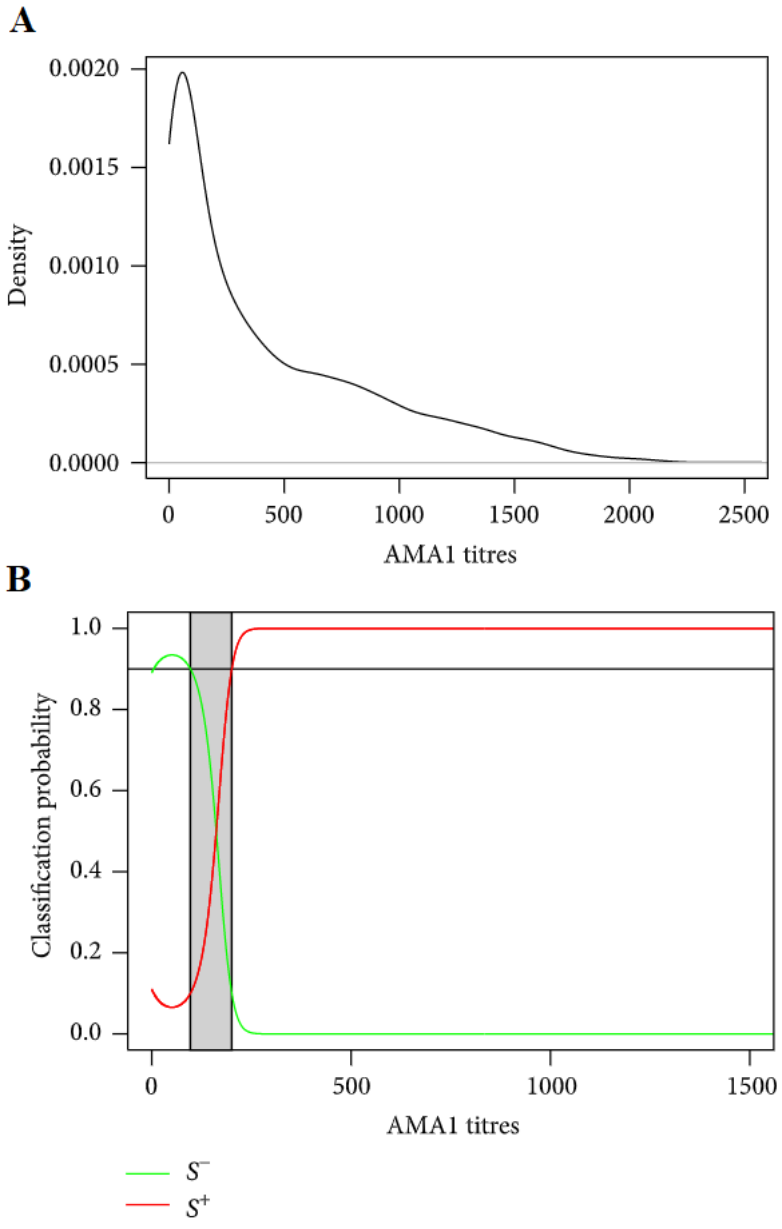

Figure 6. Utilising mixture models to determine antibody seropositivity. A. Distribution of IgG antibody responses to *Plasmodium falciparum* antigen AMA-1 in a study population from Bioko, Equatorial Guinea, 2004. Bimodal distribution of seronegative individuals with low antibody responses and seropositive individuals with elevated antibody responses. **B.** Classification probability of being seronegative (green) or seropositive (red) according to 2 component gaussian mixture model. Seropositivity cut off refers to >90% classification probability of being seropositive. Adapted from [168].

A separate challenge associated with determining flavivirus FOI is the potential for elicited IgG responses to wane to very low levels, or completely, over time. It is widely presumed that dengue-elicited IgG remains detectable in hosts for decades if not life [46]. Consequently, fitting simple catalytic models (which assume individuals can only transition from a seronegative to seropositive state (Figure 5A)) to age IgG seroprevalence data has become common practise [13,44,158–160]. Nonetheless, there have been recent studies that contradict this assumption and highlight individuals can serorevert to seronegative status after being seropositive for DENV IgG. In India, a seroprevalence study conducted among children sampled in 2014 and 2016 revealed 4.3% [95%CI: 3.1-5.9%] reverted from being IgG positive to IgG negative two years later [149]. Similarly in China, a seroprevalence survey of individuals three years after an outbreak, who had confirmed dengue infections, revealed 34.6% (37/107) became IgG negative, implying IgG waning [150]. Interestingly authors also showed those asymptomatic during the outbreak, who likely experienced primary dengue infections, were more likely to be IgG negative three years after the outbreak compared to those who experienced symptomatic dengue infections, who are more likely secondary infections. It could therefore be speculated experiencing dengue once may not be enough to sustain IgG for life and that multiple dengue infections are needed to elicit life-long responses. Lastly, a previous modelling study identified age-IgG seroprevalence sometimes increased then decreased with age suggesting a protection decay in DENV IgG [161]. Therefore, authors proposed altering their catalytic models to account for IgG waning by incorporating an additional seroreversion parameter. This is otherwise known as a reverse catalytic model (Figure 5A) and is commonly used to characterise the FOI in malaria studies where IgG responses are known to wane with time [152–154]. For ZIKV, studies have also demonstrated evidence of IgG waning years after experiencing an infection [169,170].

Another challenge associated with generating flavivirus FOI estimates is that they represent long-term estimates of transmission intensity and must be calculated from a representative sample of the general population. Conducting serosurveys over large geographical scales is logistically difficult and would be expensive for routine surveillance operations. Therefore, determining whether the FOI can be estimated from regularly collected case report data could be a cost-effect alternative. A recent study demonstrated how FOI estimates, derived from sero-surveys, correlated with FOI estimates obtained from passively collected, age-stratified incidence data [79]. Yet whether alternate, simpler, surveillance

metrics can be calculated to determine the force of infection routinely over space and time remains unknown.

1.7 Enhancing Flavivirus Laboratory Surveillance in the Philippines

Dengue and Zika in the Philippines

The Philippines is an archipelago of approximately 7,640 islands located in the WHO Western Pacific region. The country consists of three major island groups: Luzon in the north, Visayas in the centre and Mindanao in the south. At lower administrative levels, the country is further divided into 17 regions, 81 provinces, 1,488 municipalities and 42,046 local barangays. According to Philippine census data, the countries population grew from 100,981,437 in 2015 to 109,035,343 in 2020, corresponding to a population growth rate of 1.63% (Philippine Statistics Authority). Of the 146 cities across the Philippines, 33 (22.6%) are considered highly urbanised, as they consist of >200,000 inhabitants and have average annual income of > ₱50 million (Pesos) (approximately £727,000). Most cities are situated in low-lying areas and 60% are positioned on the coast [171]. The capital region, Metropolitan Manila, has an average population density of 20,785 individuals per square kilometre according to 2020 census data (Philippine Statistics Authority), making it one of the most densely populated urban centres in the world. Heavy rains occur across the country during the main rainy season which lies between June and November (PAGASA: Philippine Atmospheric, Geophysical and Astronomical services administration). The unique physical and human geography of the Philippines provide the perfect conditions for Flaviviral infections, including DENV and ZIKV, to thrive.

Like other dengue-endemic countries, the Philippines has experienced a huge increase in the dengue burden over the past 70 years and it is now one of the leading causes of morbidity and mortality [115]. According to the Philippine Department of Health (DOH), all four DENV serotypes co-circulate across the country and 414,532 cases were reported including 1,546 deaths, in 2019, an increase from 200,415 cases and 598 deaths in 2015 [172]. Despite this increase, dengue remains heavily underreported revealed by one study in the country that estimated only 21% of those with symptomatic infections reported to health facilities in Cebu city (Visayas) in 2017 [173]. Moreover, a recent study revealed the burden of dengue was markedly higher in the Philippines compared to many other dengue-endemic countries. They projected an age-standardised DALY rate per 100,000 population in the Philippines during 2017 at 219.53 [CI: 108.83-307.08], a rate much higher than Brazil (32.32 [CI: 15.98-50.49]), Vietnam (26.44 [CI: 16.14-42.35]) and Colombia (33.26 [CI:13.21-48.02]) [19].

In contrast to dengue, the burden of Zika remains poorly characterised across the Philippines. This is likely a consequence passive surveillance system not detecting cases as ZIKV typically causes mild infections and serological differential diagnosis is challenging [124,131]. Prior to 2016 however, there

were isolated cases of non-travelling individuals with PCR-confirmed ZIKV infections in Quezon City in 2010 [174] and Cebu City in 2012 [175]. This alluded to autochthonous, not imported, Zika transmission across the Philippines. This notion was later confirmed in 2016 when 47 non-travelling, PCR-confirmed, ZIKV cases were identified after incorporating Zika symptoms into updated surveillance operations [176]. Yet today, it remains unknown whether Zika is widespread or focal across the entire country.

Philippine Dengue and Zika Surveillance Operations

Centralised Flavivirus surveillance in the Philippines is conducted by the Philippine Epidemiology Bureau (EB) - a division of the Department of Health (DOH). EB provides weekly case reports on dengue which are collected in line with PIDSR (Procedures for the Philippine Integrated Disease Surveillance and Response) and WHO criteria [7,177]. The PIDSR was initiated in 2008 to strengthen and standardise infectious disease surveillance across the country, consequently, dengue is now notifiable across all disease reporting units (DRUs) of the Philippines, ranging from major regional hospitals to rural health facilities. Epidemiological data collected from patients include: age, sex, date of symptom onset, date of reporting, symptoms, disease outcome, DRU/home location (Region, province, municipality, barangay) and DRU position (GPS) (Appendix A). Zika is also notifiable in the Philippines. Suspected Zika patients include those with fever, conjunctivitis, skin rash and either of the following: joint pain, myalgia, headache, malaise, retro-orbital pain. Infants/Foetuses with microcephaly are also considered suspected Zika infections. Cases are documented and asked to provide a urine/serum sample for further laboratory testing (Figure 7). Samples are assayed for Zika PCR using CDC methods described in [178] and Zika IgM/IgG using a commercial Euroimmune™ ELISA kits (Cat No: El 2668–9601 M and El 2668–9601 G, Lübeck, Germany).

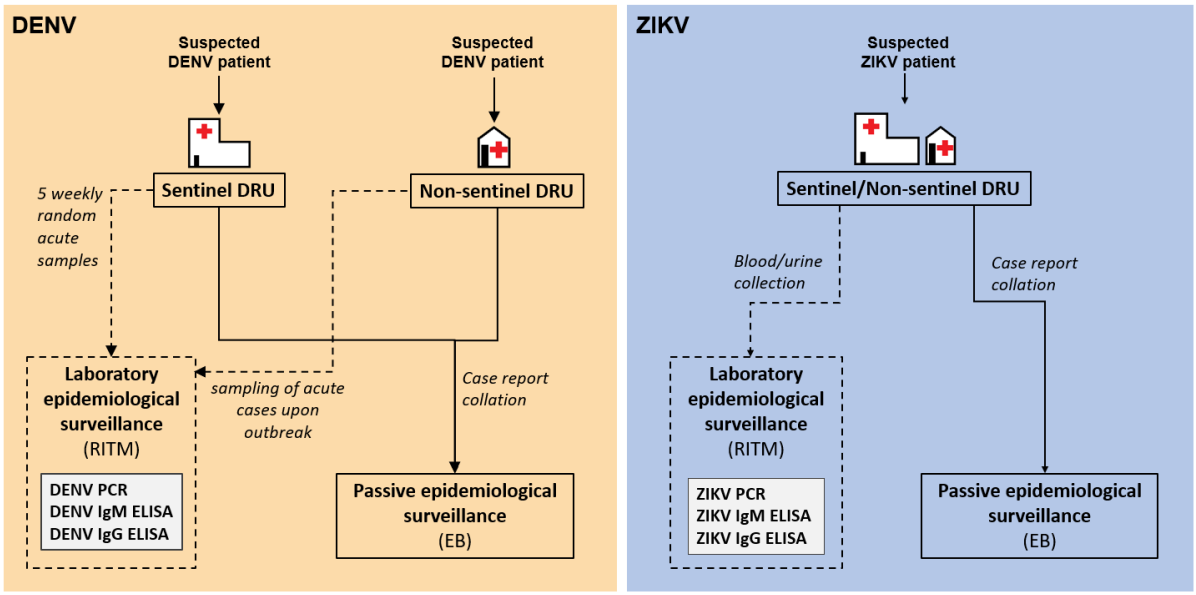

Figure 7. **Dengue and Zika epidemiological surveillance operations in the Philippines.** All dengue and Zika case reports who visit disease reporting units (DRUs) are collated by the Philippine Epidemiological Bureau (EB). For dengue, a regular random sample of case reports who visit sentinel DRUs, and all those who visit non-sentinel DRUs during an outbreak period, are asked to provide serum for subsequent laboratory testing. All suspected Zika cases who visit DRUs are asked to provide serum/urine samples for further laboratory testing [177].

In addition to typical dengue case reporting, the Philippine DOH also performs routine dengue laboratory surveillance (Figure 7). The Research Institute for Tropical Medicine (RITM - the research arm of the DOH) orchestrate annual cross-sectional surveys of suspected dengue case reports across the Philippines and collect single serum samples and basic epidemiological data (according to PIDSR criteria) from consenting patients. Patients are sampled in both sentinel and non-sentinel DRUs located across the Philippines (Figure 8). Sentinel DRUs include major regional hospitals which collect random serum samples from five dengue cases per week. Non-sentinel DRUs include any health facility that experiences a sudden rise in dengue cases then proceeds to collect serum samples according to PIDSR criteria [177]. At the RITM, stored samples are then assayed for serotype specific DENV RNA using the CDC DENV1-4 PCR assay [87]. This enables spatio-temporal monitoring of circulating serotypes across the country which is a key recommendation of the WHO global dengue strategy for control [7]. Serum samples are also assayed for the presence of DENV IgM and IgG antibodies using Panbio® capture ELISA kits (Cat: 01PE20/01PE21, Abbott, Brisbane, Australia) according to manufacturers' specifications.

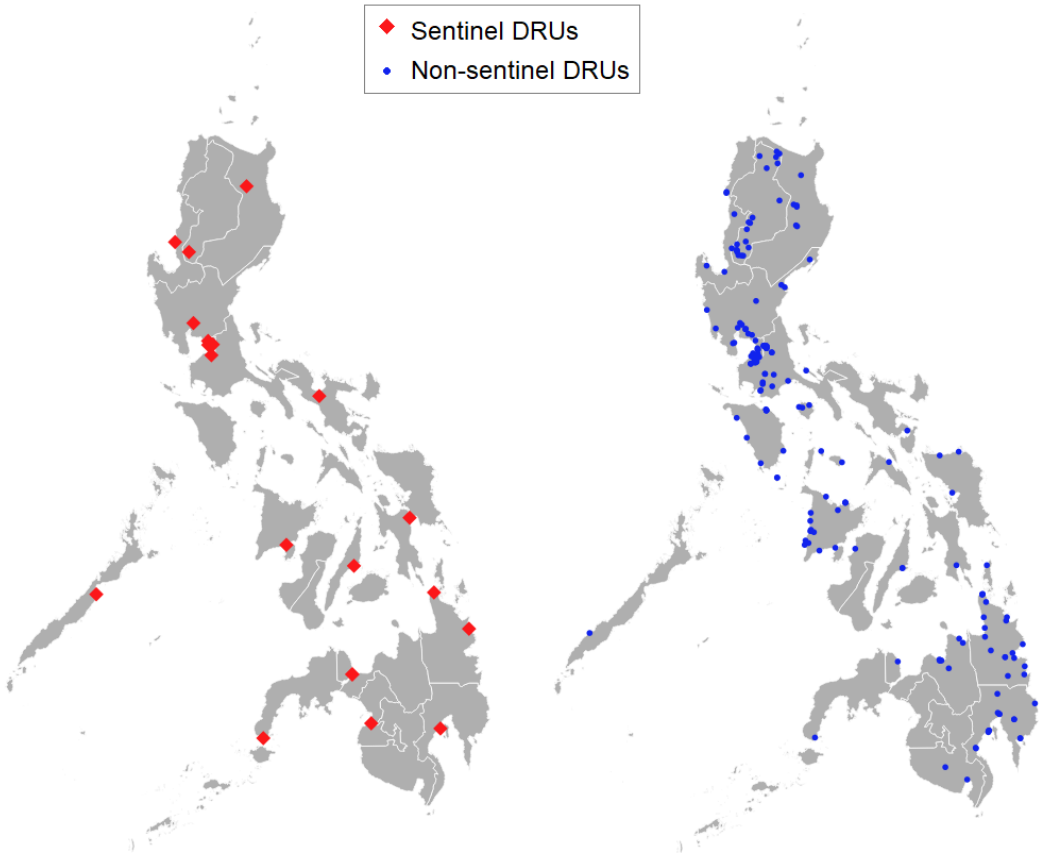

Figure 8. Location of Sentinel and Non-sentinel DRUs (Disease reporting units) across the regions of the Philippines. Dengue and Zika are notifiable across all DRUs [177].

Philippine Integrated Vector Management (IVM)

To counter the spread of vector-borne diseases, including Dengue and Zika, across the Philippines, the Department of Health conducts integrated vector management (IVM) [179]. The aim of this programme is to prevent and control vector-borne diseases by integrating various vector control strategies coordinated at different administrative levels. IVM strategies include environmental management (source reduction), house improvements, community engagement and the use of chemical and biological insecticides. Insecticides are used for larviciding, space spraying (fogging), and indoor residual spraying IRS. Vector control strategies are formulated centrally by the Department of Health while implementation is coordinated by regional/provincial local health authorities.

Given the cost and labour intensiveness of vector control strategies [115], it remains crucial IVM is deployed at the right time and to appropriate locations to achieve maximum impact. In the Philippines, different IVM strategies are deployed at different stages of the year. Home improvements and community awareness programmes are implemented all year round and help prevent local dengue outbreaks from occurring. Environmental management, larviciding and IRS strategies are deployed prior to the onset of the rainy season when breeding sites are limited and most susceptible to control interventions (although often continue into the rainy season). Lastly, given the lack of residual impact,

space spraying programmes occur during rainy seasons to minimise local vector populations and spread of disease [179]. Yet, determining where within regions IVM should be deployed remains challenging. Interventions are currently deployed according to the expertise of local health authorities who typically target areas with higher case reporting. However, it remains unknown whether areas with increased case reporting represent areas of higher transmission intensity. Determining accurate surrogate indicators of the dengue force of infection could therefore assist in appropriately deploying IVM within regions.

Research Justification

As dengue is such an underreported disease in the Philippines [173], it remains unknown whether current case reporting strategies, conducted by routine epidemiological surveillance, accurately represents dengue transmission dynamics. In the Philippines, routine laboratory surveillance practises have recently been incorporated into existing programmes and have been instrumental in revealing spatio-temporal patterns in circulating serotypes across the country. Yet, given a range of laboratory markers, both molecular and serological, that are obtained from suspected dengue patients who reported across the Philippines, further investigations are necessary to determine how best this data can be used to inform control efforts.

Combining molecular and serological techniques for surveillance purposes has previously been conducted in Brazil [180], Burkina Faso [181], Argentina [182] and India [183] to better characterise case reports with true dengue infections. This enabled investigators to characterise outbreaks, identify risk factors associated with dengue and explore patterns in imported and local transmission. Further laboratory characterisation of the reporting dengue population is therefore warranted in the Philippines. Accurately determining the primary and post-primary (secondary, tertiary and quaternary) immune status of dengue case reports could assist in identifying individuals/populations at risk of severe disease upon a subsequent DENV infection [45,46] and be used to target post-exposure vaccinations [23,114]. Moreover, additional laboratory characterisation of case reports could be useful in routinely estimating the dengue force of infection across the Philippines. This could help target limited vector control interventions to populations most in need – a key WHO strategy for sustainably combatting dengue transmission [7].

As routinely used dengue serological diagnostics detect cross-reactive antibodies to other flavivirus infections which can cause non-specific fevers [123,131,136], it is crucial dengue case reports are assayed for other infections including ZIKV. Furthermore, as mounting evidence suggests prior exposure to ZIKV can cause severe disease in subsequent DENV infections [132], characterising sub-national dengue and Zika transmission patterns is important to stratify severe disease risk within the population. Lastly, as regional surveillance systems are often less equipped than centralised operations [7], it is essential enhanced surveillance practises are adapted to suit low resource settings. Investigating whether the immune status of dengue patients can be accurately determined using cheap, easy to use

rapid tests warrants further investigation. Accurately characterising those with primary and post-primary at the point of care using RDTs could assist regional surveillance and has the potential to assist in dengue disease prognosis [39].

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Chapter 2. Overall Aim and Objectives

The overall aim of this research was to investigate how analysing laboratory data from dengue case reports in the Philippines can be optimised to enhance surveillance operations. The scheme of work is divided into the following general and specific objectives:

Objective 1. To develop and validate a serological framework capable of characterising the DENV immune status of suspected dengue patients (Chapter 3).

i. To develop a novel molecular and serological algorithm that can distinguish primary from post-primary DENV immune status.

ii. To validate the generated algorithm according to the WHO gold standard methods.

Objective 2. To investigate which routinely collected surveillance metrics represent suitable surrogate indicators of the dengue FOI and can be utilised to monitor variations in the burden of disease (Chapter 4).

i. To describe the long-term spatial patterns in the force of infection across the Philippines according to age IgG-seroprevalence.

ii. To investigate which, easily computed, laboratory and non-laboratory surveillance metrics correlate with the FOI according to age-stratified IgG seroprevalence.

Objective 3. To determine whether there is evidence of Zika transmission across the Philippines (Chapter 5).

i. To investigate whether there is evidence of short and/or long-term exposure to ZIKV among those reporting with suspected dengue across the Philippines.

ii. To characterise the serological cross-reactivity between ZIKV and DENV.

Objective 4. To investigate utilising point-of-care diagnostics for determining DENV immune status (Chapter 6).

i. To determine whether combining different types of dengue rapid tests accurately captures primary and post-primary DENV infections.

ii. To estimate the probability of being primary or post-primary according to combinations of RDT result by specific day of infection.

Chapter 3. A Serological Framework to Investigate Acute Primary and Post-primary Dengue cases reporting across the Philippines

An online, full text version of chapter 3 is available at:
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London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646
F: +44 (0)20 7299 4656
www.lshtm.ac.uk

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Student ID Number	1402440	**Title**	Mr
First Name(s)	Joseph Robert		
Surname/Family Name	Biggs		
Thesis Title	Immuno-epidemiological analysis of dengue to enhance surveillance		
Primary Supervisor	Dr Julius Clemence R. Hafalla		

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A serological framework to investigate acute primary and post-primary dengue cases reporting across the Philippines

Joseph R. Biggs[1*], Ava Kristy Sy[2,3], Oliver J. Brady[4,5], Adam J. Kucharski[4,5], Sebastian Funk[4,5], Mary Anne Joy Reyes[2,3], Mary Ann Quinones[2,3], William Jones-Warner[1], Yun-Hung Tu[1], Ferchito L. Avelino[6], Nemia L. Sucaldito[6], Huynh Kim Mai[7], Le Thuy Lien[7], Hung Do Thai[7], Hien Anh Thi Nguyen[8], Dang Duc Anh[8], Chihiro Iwasaki[9], Noriko Kitamura[9], Lay-Myint Yoshida[9], Amado O. Tandoc[2], Eva Cutiongco-de la Paz[10,11], Maria Rosario Z. Capeding[3,10], Carmencita D. Padilla[10,11], Julius Clemence R. Hafalla[1] and Martin L. Hibberd[1,10,11]

Abstract

Background: In dengue-endemic countries, targeting limited control interventions to populations at risk of severe disease could enable increased efficiency. Individuals who have had their first (primary) dengue infection are at risk of developing more severe secondary disease, thus could be targeted for disease prevention. Currently, there is no reliable algorithm for determining primary and post-primary (infection with more than one flavivirus) status from a single serum sample. In this study, we developed and validated an immune status algorithm using single acute serum samples from reporting patients and investigated dengue immuno-epidemiological patterns across the Philippines.

Methods: During 2015/2016, a cross-sectional sample of 10,137 dengue case reports provided serum for molecular (anti-DENV PCR) and serological (anti-DENV IgM/G capture ELISA) assay. Using mixture modelling, we re-assessed IgM/G seroprevalence and estimated functional, disease day-specific, IgG:IgM ratios that categorised the reporting population as negative, historical, primary and post-primary for dengue. We validated our algorithm against WHO gold standard criteria and investigated cross-reactivity with Zika by assaying a random subset for anti-ZIKV IgM and IgG. Lastly, using our algorithm, we explored immuno-epidemiological patterns of dengue across the Philippines.

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* Correspondence: Joseph.Biggs1@lshtm.ac.uk
[1]Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK
Full list of author information is available at the end of the article

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Results: Our modelled IgM and IgG seroprevalence thresholds were lower than kit-provided thresholds. Individuals anti-DENV PCR+ or IgM+ were classified as active dengue infections (83.1%, 6998/8425). IgG− and IgG+ active dengue infections on disease days 1 and 2 were categorised as primary and post-primary, respectively, while those on disease days 3 to 5 with IgG:IgM ratios below and above 0.45 were classified as primary and post-primary, respectively. A significant proportion of post-primary dengue infections had elevated anti-ZIKV IgG inferring previous Zika exposure. Our algorithm achieved 90.5% serological agreement with WHO standard practice. Post-primary dengue infections were more likely to be older and present with severe symptoms. Finally, we identified a spatio-temporal cluster of primary dengue case reporting in northern Luzon during 2016.

Conclusions: Our dengue immune status algorithm can equip surveillance operations with the means to target dengue control efforts. The algorithm accurately identified primary dengue infections who are at risk of future severe disease.

Keywords: Dengue, Flavivirus, Primary, Post-primary, Immuno-epidemiology, Surveillance, Serology, Philippines

Background

Dengue has become the most significant disease-causing arbovirus in the tropical and subtropical world. According to World Health Organization (WHO) global figures, notified cases of dengue have increased 30-fold in the past 5 decades [1], and a further 247,000 suspected dengue cases in the Western Pacific region were reported in 2014 compared to 2008 [2]. This reporting likely grossly underestimated true numbers given the range of dengue clinical manifestations and variable healthcare infrastructures in endemic countries. Instead, modelled estimates approximate 390 million annual dengue cases occur globally, of which 75% are asymptomatic [3]. Dengue emergence is believed to be attributed to rapid population growth, urbanisation, human migration, climate change, and is unhampered by costly control interventions [1].

Infection with one of the four known immunologically distinct dengue virus serotypes (DENV1–4) causes a delayed increase in viremia, combined with potential fever, which decreases within days. This is followed by an increase in immunoglobulin M (IgM) that wanes over months [4]. During a primary infection, immunoglobulin G (IgG) increases during the convalescent stage of disease and persists for life, rendering individuals immune to homologous but not heterologous dengue virus serotypes. Upon a post-primary (secondary, tertiary, quaternary) infection with a contrasting serotype, IgM resurgence is subdued while pre-circulating, non-neutralising IgG increases rapidly with viremia [5]. This enhanced level of non-protective IgG is believed to facilitate rapid viral replication in hosts through an antibody-mediated enhancement (ADE) process [6, 7]. Dengue symptoms range from asymptomatic to severe [3, 8]. According to WHO guidelines, severe symptoms include critical plasma leakage, haemorrhage and organ impairment [9]. These symptoms are thought to arise from host-mediated cytokine storms that occur in response to viral replication [10, 11], and as a consequence, post-primary dengue infections are major risk factors for developing severe disease [12–14].

The gold standard serological method for determining a dengue infection, including whether it is primary or post-primary dengue, remains the WHO haemagglutination inhibition assay (HIA) using acute and convalescent paired sera. A fourfold increase in IgG titre that exceeds or falls below 1:2560 during convalescence is indicative of secondary and primary infections, respectively [15]. Despite the high-throughput nature of this technique, the need for paired sera, collected at least 7 days apart, makes it undesirable for large-scale epidemiological surveillance. To overcome this, commercial IgM and IgG capture ELISAs, used concurrently, can distinguish primary and secondary dengue using a single acute-stage serum sample. For instance, Panbio® capture ELISAs (Alere, Brisbane, Australia, Cat. No.: 01PE10/01PE20) state their IgG seroprevalence threshold of 22 panbio units corresponds to a HAI 1:2560 IgG titre. Therefore, individuals assayed using these kits who are IgM+/IgG− and IgM+/IgG+ supposedly represent primary and secondary dengue infections, respectively.

For dengue surveillance purposes, IgM and IgG capture ELISAs are an affordable and logistically simple way to investigate epidemiological patterns in primary and post-primary dengue. However, this technique is not without caveats. First, given the delay in eliciting anti-DENV IgM following infection, it remains unknown whether early stage, non-immunogenic (IgM− and IgG −), primary dengue cases are detectable using this solely serological diagnostic. Second, given a recent study highlighted primary dengue infections can elicit high IgG levels during the febrile period [16], commercially provided IgG thresholds may misclassify acute primary and post-primary dengue infections. The incorporation of commonly used molecular (PCR) tools may improve the diagnostic capability of this algorithm. In addition, IgG:IgM ratios have been proposed as useful metrics for

categorising dengue immune status given that major differences between IgG and IgM occur during post-primary, compared to primary, infections [17, 18]. However, the practical application of these thresholds during the febrile stage of infection is limited [16], suggesting that further studies investigating the stage of infection at which ratios become appropriate, if at all, are warranted.

In dengue-endemic countries including the Philippines, optimising the use of passively collected dengue case report data could strengthen disease surveillance and control. In the Philippines, laboratory-based surveillance efforts currently include routine molecular characterisation of dengue, using sera collected from a representative sample of all case reports. This allows surveillance operations to monitor spatio-temporal dengue serotype patterns across the Philippines. However, molecular characterisation alone does not indicate whether case reports experienced primary or post-primary dengue infections, information that may prove useful in identifying populations at risk of severe symptoms. The aim of this study was to develop a novel dengue immune status algorithm using routinely collected serological and molecular metrics and compare its performance with commercial and WHO-approved practice. The co-circulation of other arboviruses across the Philippines however, which present with similar acute clinical manifestations, including Chikungunya [19, 20], Japanese encephalitis [21] and more recently Zika in 2016 [22], poses a challenge to this effort. Numerous studies have demonstrated antibody responses against dengue virus cross-react with Zika virus [23–25], making it difficult to detect the true causative agent of infection. Upon validation of an appropriate immune status algorithm, we investigated immuno-epidemiological patterns of dengue transmission across the Philippines in 2015/2016 to inform surveillance operations and targeted disease control.

Methods

Dengue surveillance in the Philippines

The Philippines, consisting of 7641 islands spanning more than 300,000 km^2, is one of the countries in the Western Pacific region most heavily burdened by dengue [2]. According to the country's Department of Health (DOH), all four serotypes of dengue (DENV1–4) co-circulate in the country and reported cases increased from 213,930 to 220,518 between 2015 and 2016, respectively [26]. In 2008, the Philippine Integrated Disease Surveillance and Response (PIDSR) system was established to synchronise and strengthen disease surveillance across the country resulting in dengue becoming notifiable across all Filipino disease-reporting units (DRUs), ranging from local barangay health facilities to major regional hospitals [27]. According to the 2009 WHO criteria [9], the PIDSR categorises patients as having no warning signs, warning signs or severe dengue symptoms. Warning signs include a sudden acute illness coupled with either abdominal pain, vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement, increased haematocrit and/or decreased platelet counts. Severe symptoms include a sudden acute illness coupled with either severe plasma leakage, severe bleeding and/or severe organ impairment.

Data collection, management and laboratory methods

Serum samples were collected from suspected dengue cases that reported to DRUs (health facilities) across the Philippines according to PIDSR criteria: a previously well person with a 2–7-day prolonged febrile illness coupled with two additional non-specific dengue symptoms. Infants under the age of 6 months were excluded from the study due to the potential persistence of maternal anti-DENV antibodies. A total of 20 sentinel and 185 non-sentinel DRUs across the Philippines participated in the study during 2015 and 2016. Sentinel DRUs supplied 5 random samples per week and included major regional hospitals. Non-sentinel DRUs included any health facility that reported a marked increase in dengue cases/deaths according to PIDSR criteria [27] and supplied samples during these outbreak periods. In total, 10,137 individuals supplied serum to the National Reference Laboratory for Dengue and Other Arboviruses at the Research Institute for Tropical Medicine (RITM), the research arm of the DOH, for further study. Coupled with sera were epidemiological data consistent with the PIDSR system including age, sex, date of birth, date of admission, date of illness onset, symptoms (no warning signs, warning signs and severe), outcome (dead and alive) and DRU address and GPS coordinates. Additionally generated variables include disease day (date of admission–date of illness onset), IgG:IgM ratio (IgG panbio units/IgM panbio units), DRU elevation (metres) and DRU population density (km^2). DRU-level covariates were generated using 100 m resolution Philippine elevation and population density raster data from 2015 (USGS; earth explorer; USA). Raster values were assigned to the midpoint of the DRUs using corresponding GPS coordinates in ArcGIS (v.10.5).

To focus this study on acute (febrile) dengue cases, those who reported more than 5 days post the onset of febrile symptoms (1318/10,137) or had missing onset/reporting date data (154/10,137) were excluded from the study. Subsequently, those with incomplete serological/molecular data (131/8665) or symptom data (176/8665) were excluded from the final dataset (Additional file 1). Those with missing serological/molecular data were excluded as our algorithm utilises both molecular and serological metrics. To assess whether excluding those

with missing data among the febrile surveillance dataset introduced selection bias, we compared percentage demographic characteristics of the final febrile dengue surveillance and those with missing serological/molecular and symptom data. To investigate whether anti-DENV responses cross-react with Zika, a random subset of serum samples from the final 2016 febrile dengue surveillance dataset (1000/3921) were selected for anti-ZIKV IgM and IgG assay.

Serum samples were stored at − 80 °C prior to molecular and serological assay. Among all viable collected samples, dengue viremia was determined using a four-plex real-time polymerase chain reaction (PCR) assay as previously described [28]. In short, dengue serotype-specific primers detect then amplify dengue RNA in serum to determine viremia. Samples were considered PCR positive or negative for dengue if they had critical threshold cycle (Ct) values below or above 36, respectively. To detect the presence of anti-dengue IgG and IgM, samples were assayed using Panbio® capture IgM and IgG ELISA kits (Cat. No.: 01PE10/01PE20, Alere, Brisbane, Australia). Briefly, kits encompass antigen capable of capturing host antibody specific to all four dengue serotypes and include plate-specific calibrators that normalise output optical density (OD) readings to generate standardised antibody panbio units. Pre-determined panbio unit serological thresholds categorised individuals as negative (IgM ≤ 9, IgG ≤ 18), equivocal (IgM 9–11, IgG 18–22) and positive (IgM ≥ 11, IgG ≥ 22) for dengue infections. Consistent with kit specifications, algorithm 1 (A1) classified primary and post-primary dengue cases as being IgM+, IgG− and IgM+, IgG+, respectively. Among samples selected for ZIKV antibody testing, samples were assayed using Euroimmune™ (Lübeck, Germany) ZIKV IgM-ELISA (El 2668–9601 M) and IgG-ELISA (El 2668–9601 G) kits according to specification instructions. The semi-quantitative ratio outputs from these tests were used to dichotomise individuals as anti-ZIKV IgM/IgG positive (OD ratio > 1.1) or negative (OD ratio < 1.1).

Serological modelling and algorithm validation

Mixture models were used to (1) establish true anti-dengue IgM and IgG seroprevalence and (2) determine, disease day-specific, IgG:IgM ratio thresholds that distinguish primary from post-primary dengue infections. All models were fitted by maximum likelihood with lognormal distributions using the command 'fmm:glm' in STATA (v.15, Texas, USA). For IgM and among the entire study population, models were fitted with 3 components to represent the seronegative, primary and post-primary populations. For IgG and among non-active DENV cases (PCR− and IgM−), the models were fitted with 2 components to characterise distributions of those with/without prior IgG exposure to DENV. We compared these models based on a single distribution models using Akaike information criterion (AIC). Lower AIC indicates better model fit. IgM and IgG seroprevalence thresholds refer to the lowest antibody titre values with a classification probability of being seropositive>seronegative.

To determine the primary and post-primary dengue immune status of active dengue cases, disease day-stratified IgG:IgM ratio distributions were fitted with 2-component mixture models to classify the distinct primary and post-primary subpopulations. For each disease day, we calculated IgG:IgM ratio thresholds corresponding to the lowest ratio value with a classification probability of being post-primary>primary. Active dengue cases with ratios above and below these disease day-specific thresholds were categorised as post-primary and primary, respectively. To determine whether IgG:IgM ratios were appropriate to distinguish immune status on specified disease days, we justified the existence of two rather than one ratio distribution using Akaike information criterion. Only 2-component models with lower AIC values compared to 1-component models were used to generate ratio thresholds for specific disease days.

To validate the commercial and novel dengue immune status algorithms, we utilised paired sera from community household members of reporting DENV RDT NS1+ patients involved in a study conducted in Nha Thang, Vietnam. Twenty-one household members reported day of fever and supplied acute and convalescent sera. Paired sera were assayed for anti-DENV NS1 (Rapid diagnostic test, Bio-Rad, France), IgM and IgG using Panbio® capture ELISA kits (as described previously). Using single acute serum samples from household members, dengue immune status was determined according to Panbio® specifications (A1) and our novel algorithm (A2). In addition, using paired sera from household members, dengue immune status was also established corresponding to WHO guidelines [22] (as described previously). The serological agreement of both A1 and A2 to the gold standard WHO technique was used to verify algorithm performance for further use characterising immuno-epidemiological trends in dengue transmission across the Philippines.

To investigate dengue transmission intensity across the Philippines, we estimated anti-DENV IgG seroconversion rates (SCRs) among those reporting with non-active dengue infections (PCR− and IgM−). SCRs, which correspond to the average annual rate individuals seroconvert from anti-DENV IgG− to IgG+, were obtained from IgG age-seroprevalence curves fitted using simple and reversible catalytic models. Assuming individuals seroconvert solely from IgG seronegative to seropositive status, Eq. 1 estimates the probability of being IgG

seropositive at specified ages (a) by fitting a constant force of infection parameter ($\boldsymbol{\lambda}$) by least squares according to the function:

$$\mathbf{P}(\mathbf{a}) = \left[\mathbf{1} - \mathbf{exp}^{-\boldsymbol{\lambda}\mathbf{a}}\right] \quad (1)$$

Given immunological protection may decay over time resulting in reporting non-active dengue cases reverting to IgG seronegative status according to our mixture model threshold, Eq. 2 fits an additional constant seroreversion parameter (ρ), by least squares, according to the function:

$$\mathbf{P}(\mathbf{a}) = \frac{\boldsymbol{\lambda}}{\boldsymbol{\lambda}+\boldsymbol{\rho}}\left[\mathbf{1} - \mathbf{exp}^{-(\boldsymbol{\lambda}+\boldsymbol{\rho})\mathbf{a}}\right] \quad (2)$$

Likelihood ratio tests were used to determine which model, simple or reversible, best characterised age-IgG seroprevalence data (p value < 0.05). All models were fitted by maximum likelihood using a constrained/unconstrained 'revcat' command in STATA (v.15).

To investigate the risk factors associated with presenting as a post-primary, rather than a primary, dengue case, we calculated unadjusted odds ratios from a univariable logistic regression model using the 'logit' command in STATA (v.15). Explanatory variables included age, sex, disease day, clinical manifestation, DRU elevation and DRU population density.

Results

Data description

Between 2015 and 2016, 8665 serum samples were collected from consenting febrile, suspected dengue cases among DRUs across the Philippines, in which 131/8665 and 176/8665 had missing molecular/serological and symptom data, respectively (Additional file 1). Similar demographic characteristics were observed between febrile dengue cases with complete data and those with incomplete molecular/serological and symptom data (overlapping 95% CIs) (Additional file 2). In the final complete dengue surveillance dataset used in this study, demographic information reveals that a slightly higher percentage were male (52.5%), whereas most were aged between 6 and 15 years (44.1%), reported with dengue-like symptoms (69.5%) and reported 3–4 days post the onset of fever (60.5%). Mortality was low among the study population with only 0.4% reported as having died from dengue (Additional file 2).

Determining dengue immune status

Upon re-assessing anti-DENV IgM seroprevalence, we identified a large proportion of the study population had elevated anti-DENV IgM titres resulting in a distribution best characterised by a 3-component, rather than a 1-component, mixture model (AIC difference −221.2) (Additional file 3). This model provided an anti-DENV IgM seropositivity threshold of 9.9 panbio units, resulting in an IgM seroprevalence of 71.8% (6050/8425) in our population (Fig. 1A). To investigate whether our anti-DENV IgM seroprevalence threshold is representative of all ages, among those aged between 0–5, 6–15, 16–30 and 31+ years, we estimated narrow-ranging anti-DENV IgM seroprevalence thresholds of 9.8, 10.1, 10.3 and 9.7 panbio units, respectively (Additional file 4). Given anti-DENV IgM responses shortly succeed viremia during a dengue infection, we concluded those either PCR+ for DENV RNA or anti-DENV IgM+ represent active dengue cases (83.1%, 6998/8425) while those anti-DENV PCR− and anti-DENV IgM− represent non-active dengue cases (misdiagnoses 16.9%, 1427/8425).

To re-assess anti-DENV IgG seropositivity, among non-active dengue cases, we assumed two subpopulations of those with/without previous IgG exposure to dengue. Rationale supported by the fact a proportion of non-active dengue cases had elevated anti-DENV IgG (Fig. 1B) resulting in a 2-component, rather than a 1-component, mixture model better characterising the IgG panbio unit distribution (AIC difference −97.7) (Additional file 5). Furthermore, a higher proportion of older non-active dengue cases had elevated IgG compared to younger non-active dengue individuals (Additional file 4). A trend likely attributed to older individuals having a higher probability of being infected with a previous dengue infection prior to reporting than younger individuals. By fitting a 2-component mixture model to the IgG panbio unit distribution of active dengue cases, this yielded a IgG seroprevalence of 2.2 panbio units; non-active dengue cases with IgG panbio units above and below this value were categorised as having historical (69.4%, 991/1427) and negative (30.6%, 436/1427) dengue exposure, respectively. Compared to kit-defined thresholds, modelled anti-DENV IgM and IgG thresholds were 1.1 and 19.8 panbio units lower, respectively.

Among active dengue cases, we determined primary and post-primary dengue immune status by investigating functional, disease day-specific, IgG:IgM ratio distributions (Fig. 2). With increasing disease day (1 to 5), we observed two increasingly distinct lower and higher ratio subpopulations consistent with predicted primary and post-primary dengue infections, respectively (Fig. 2a). These distributions were best fit by a 1-component mixture model on disease days 1 and 2, and a 2-component mixture models on disease days 3–5 (Additional file 6). For disease days 3 to 5, IgG:IgM ratio thresholds, corresponding the lowest ratio with a classification probability of being post-primary>primary, equated to 0.44, 0.44 and 0.47, respectively (Fig. 2b, c). Given the similarity between thresholds, disease day 3–5 ratio thresholds were averaged (0.45) and incorporated into algorithm 2

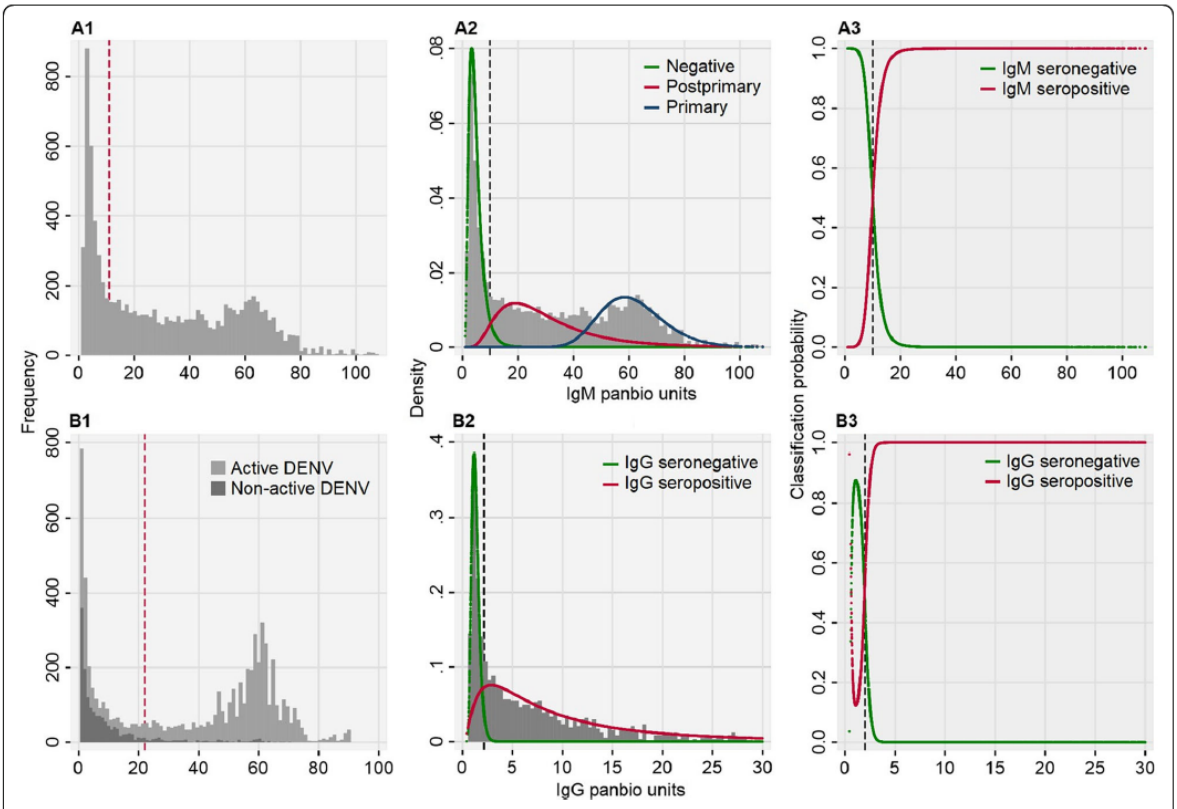

Fig. 1 Determining anti-DENV IgM and IgG seroprevalence among the study population. **A1** Histogram of study population anti-DENV IgM panbio units. Red dash: Panbio® seroprevalence threshold (11 panbio units). **A2** Mixture model of anti-DENV IgM panbio units with 3 components representing IgM seronegative, primary and post-primary subpopulations. **A3** Modelled classification probability curves of being anti-DENV IgM seronegative or seropositive. Black dash: lowest IgM panbio unit with a classification probability of being seropositive>seronegative (9.9 panbio units). **B1** Histogram of anti-DENV IgG panbio units stratified by active/non-active DENV status. Red dash: Panbio® seroprevalence threshold (22 panbio units). **B2** Mixture model of anti-DENV IgG titres among non-active DENV cases with 2 components representing IgG seronegative and seropositive subpopulations. **B3** Modelled classification probability curves of being anti-DENV IgG seronegative or seropositive. Black dash: lowest IgG panbio units with a classification probability of being seropositive>seronegative (2.2 panbio units)

to distinguish primary and post-primary dengue infections. Active dengue cases on disease days 3–5 with IgG: IgM ratios above and below 0.45 were categorised as post-primary and primary dengue, respectively. For disease days 1–2, with no statistical justification for the existence of two distinct primary and post-primary ratio distributions (1-component AIC<2-component AIC), we opted to determine dengue immune status using the previously calculated IgG seroprevalence threshold. Active dengue cases on disease day 1 or 2 with IgG panbio units above and below 2.2 were categorised as post-primary and primary, respectively. An outline of algorithm 2 (A2) is summarised in Fig. 3 while A1 and A2 study population categorisation is shown in Table 1.

After generating our novel dengue immune algorithm (A2), we compared it to commercial practice (A1). A2 assigned dengue immune status to an additional 35.1% (2955/8425) of the study population who were unclassifiable according to A1 (Table 1). Among the 21 household fever cases, A2 categorised the immune status of all members while A1 only classified 9/21 individuals. Subsequently, we investigated how well each algorithm categorised the immune status of household fever cases with paired sera according to the WHO gold standard method. A2 and A1 achieved 90.5% (19/21) and 71.4% (15/21) serological agreement, respectively (Additional files 7 and 8). These results demonstrate the superiority of A2 compared to A1 and justified its use for investigating immuno-epidemiological patterns of dengue immune status across the Philippines.

Lastly, to assess whether humoral responses against dengue were attributed to other flaviviruses, we investigated anti-ZIKV and anti-DENV cross-reactivity among those categorised as primary and post-primary according to A2 (Additional file 9). Among both primary and post-primary dengue infections, anti-ZIKV IgM responses

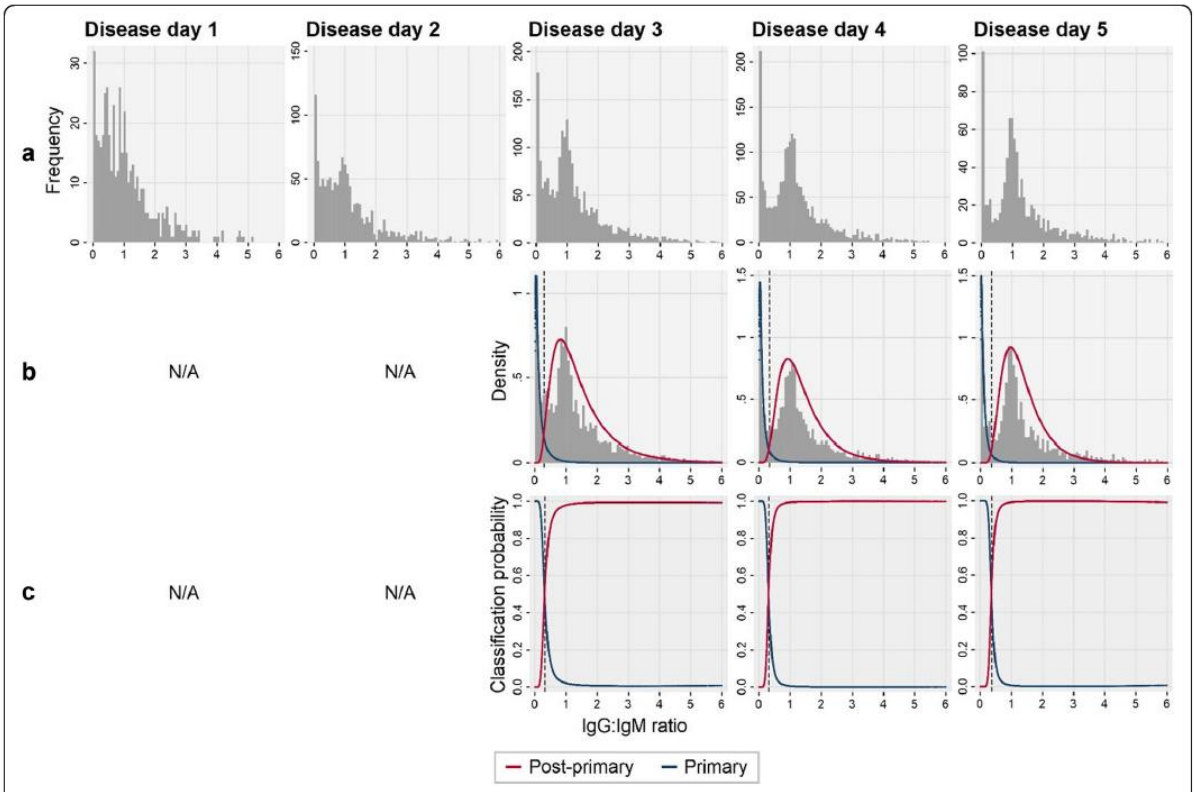

Fig. 2 Determining primary and post-primary DENV immune status among active DENV cases. **a** Histogram of individual IgG:IgM ratios among active DENV cases by disease day. **b** Mixture model of IgG:IgM ratio distributions with 2 components representing primary and post-primary subpopulations. **c** Modelled classification probability of being a primary or post-primary DENV case by disease day. Black dash: lowest ratio value with a classification probability of being post-primary>primary (Dd3, 0.44; Dd4, 0.44; Dd5, 0.47). N/A, no statistical justification for two mixture model components (1-component model AIC<2-component model AIC); Dd, disease day

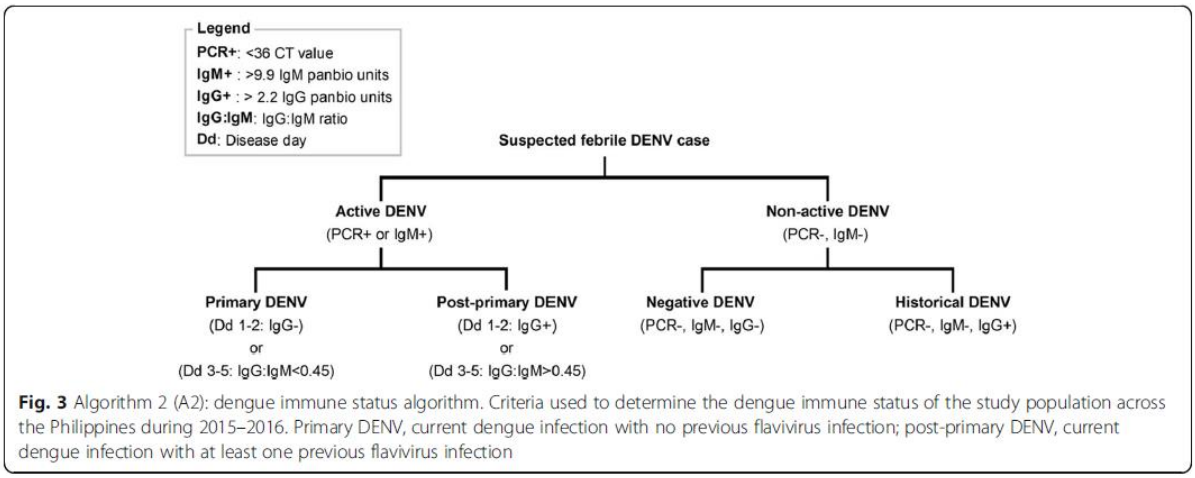

Fig. 3 Algorithm 2 (A2): dengue immune status algorithm. Criteria used to determine the dengue immune status of the study population across the Philippines during 2015–2016. Primary DENV, current dengue infection with no previous flavivirus infection; post-primary DENV, current dengue infection with at least one previous flavivirus infection

Table 1 Dengue immune status categorisation of the study population

DENV immune status	Algorithm			
	1		2	
	n	%	*n*	%
Primary	1285	15.3	1576	18.7
Post-primary	4177	49.6	5414	64.3
Historical	–	–	991	11.8
Negative	–	–	436	5.2
Unclassifiable	2963	35.2	8	0.1

Algorithm 1 (A1): Panbio® commercial algorithm. Algorithm 2 (A2): novel algorithm generated in this study

were low and only 0% (0/154) and 1% (5/508) were IgM seropositive, respectively, according to Euroimmune specifications. This suggests very few of the active DENV infections were recent ZIKV infections. In contrast, among post-primary infections, anti-ZIKV IgG responses were elevated with 23% (118/508) seropositive to anti-ZIKV IgG according to Euroimmune kit instructions. Together, these results suggest post-primary cases include current dengue infections with potential, historical, ZIKV exposure (Fig. 3).

Dengue transmission dynamics

To investigate the temporal kinetic infection patterns during acute primary and post-primary dengue infections, we calculated the mean anti-dengue viremia (Ct), IgM and IgG titres by disease day (Fig. 4). During the first 5 days of reported disease, mean IgM and IgG titres increased among both primary and post-primary infections, although IgG titres were very low among primary dengue infections. In contrast, mean dengue viremia decreased (increasing Ct) during the first 5 days of disease among primary and post-primary infections, although overall, this was significantly lower among post-primary infections.

Among active dengue cases, we investigated potential risk factors associated with post-primary compared to primary dengue status (Table 2). Individual risk factors included age (31+ compared to 0–5 years: OR 1.91 [1.54–2.38], p value < 0.001) and presenting with severe symptoms (severe compared to no warning signs: OR 1.66 [1.28–2.16], p value < 0.001) or warning signs (warning signs compared to no warning signs: OR 1.47 [1.28–1.70], p value < 0.001). DRU-level risk factors include decreasing ground elevation (150+ compared to 0–75 m: OR 0.61 [0.52–0.72], p value < 0.001) and increasing population density (200+ compared to 0–100 km^2: OR 1.32 [1.14–1.53], p value < 0.001) consistent with the known epidemiology of dengue transmission. The strong univariate association between post-primary dengue and age prompted us to explore fine-scale age trends with dengue immune status. According to percentage trends, those aged between 0.5 and 1 year with active dengue infections were more likely to be primary, rather than post-primary dengue cases. After which, the percentage of those reporting with primary dengue decreased with age while post-primary dengue cases increased, plateaued then decreased with age. Among non-active dengue cases, the percentage reporting with negative and historical dengue were mainly younger and older, respectively (Fig. 5a).

Lastly, we explored spatio-temporal trends of dengue transmission dynamics across the Philippines during 2015 and 2016. Upon investigating dengue transmission intensity across the country, we revealed a reversible

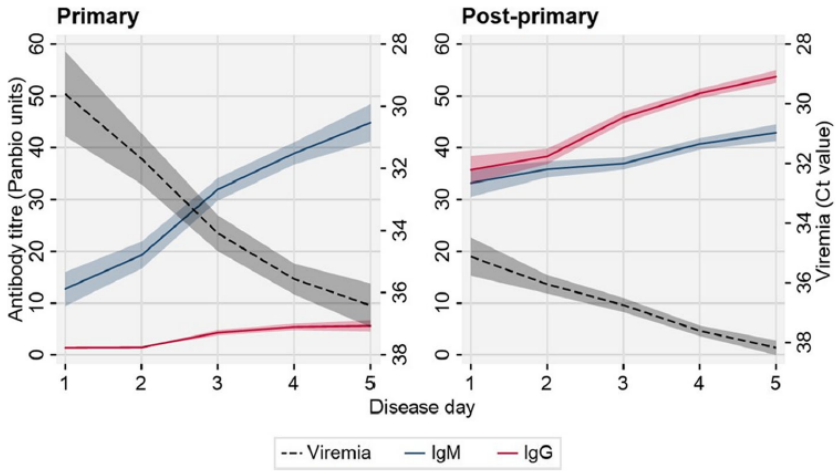

Fig. 4 Primary and post-primary temporal infection kinetics. Disease day-specific, averaged, infection kinetics (Ct value, IgM and IgG panbio units) among acute active primary and post-primary dengue infections according to algorithm 2. Shading: 95% CI

Table 2 Risk factors associated with post-primary, opposed to primary, active dengue immune status

Risk factor	Post-primary		
	OR	95% CI	*p* value
Age			
< 5	1		
6–15	1.69	1.46–1.96	< 0.001
16–30	1.80	1.53–2.11	< 0.001
> 31	1.91	1.54–2.38	< 0.001
Sex			
Female	1		
Male	0.94	0.85–1.04	0.248
Disease day			
1–2	1		
3–4	1.06	0.94–1.19	0.377
5	1.00	0.84–1.19	0.973
Clinical manifestation			
No warning signs	1		
Warning signs	1.47	1.28–1.70	< 0.001
Severe	1.66	1.28–2.16	< 0.001
Non-disclosed	0.90	0.76–1.07	0.227
DRU elevation (metres)			
0–75	1		
75–150	0.81	0.67–0.97	0.023
150+	0.61	0.52–0.72	< 0.001
DRU pop den (km^2)			
0–100	1		
100–200	0.87	0.77–0.97	0.017
200+	1.32	1.14–1.53	< 0.001

OR unadjusted odds ratio, *Pop den* population density, *DRU* disease-reporting unit

versus a simple, catalytic model best fits the age-seroprevalence data among reporting non-active dengue cases (Lrtest p value < 0.001). Using this statistically favoured model, we estimated a seroconversion rate of 0.17 [95% CI 0.14–0.20] among all non-active dengue reported cases (Fig. 6). Assuming individuals seeking care are representative of the general population, this suggests that 17% of the population were exposed to dengue annually. Additionally, bi-monthly percentage trends revealed temporal stability in the immune status of the reporting population across the Philippines between 2015 and 2016, with the majority reporting being post-primary cases (Fig. 5b). Despite this, we observed spatio-temporal heterogeneity in the immune status of the reporting population at lower administrative levels. In northern Luzon provinces during 2016, a higher percentage of primary cases reported compared to the rest of the Philippines (Fig. 5c).

Discussion

In this study, we generated and validated a novel algorithm capable of distinguishing primary and post-primary immune status among reporting, suspected dengue cases during the first 5 days of fever using a single serum sample. By incorporating molecular and serological metrics, redefining dengue antibody exposure and using IgG:IgM ratios at appropriate stages of infection, we were able to propose a dengue immune status algorithm that was superior to existing practice. Subsequently, we demonstrated how the algorithm can be applied for dengue surveillance purposes across the Philippines. We revealed that post-primary dengue cases, who are at higher risk of progressing to severe outcomes, appear to be older than primary infections and were more likely to report to health facilities in low lying, urban areas. In addition, we showed primary dengue infections, who are at risk of subsequent post-primary infections in future years, spatially clustered around the northern regions of the Philippines in 2016.

According to a solely serological commercial immune status algorithm, a large percentage of the study population were unclassifiable. This shortcoming was overcome by incorporating individual molecular metrics into our novel algorithm, which captured early stage, non-immunogenic, primary dengue infections. Our algorithm also redefined seroprevalence to IgM and IgG using mixture modelling. We questioned whether febrile primary infections could exceed the standard IgG threshold, as previously demonstrated [16], and were concerned with determining the immune status of non-active dengue cases that may have elevated anti-DENV IgG following previous dengue exposure. Upon redefining serological exposure to anti-DENV IgM and IgG, we classified those with active dengue infections as being either IgM or PCR positive given both rise and fall respectively during an active dengue infection [4, 5]. As dengue IgM persists for months following infection [4, 29], it could be argued our algorithm categorises recent dengue infections as active infections. However, elicited IgM provides temporary immunity to other serotypes and the study population included those seeking healthcare, so we considered it unlikely for individuals to seek treatment with dengue-like symptoms for a past infection. In this study, antibody seroprevalence corresponded to the lowest panbio units with > 50% probability of being seropositive according to mixture models. As a result, some individuals with panbio units close to the generated thresholds may have been misclassified. However, given the two-tiered nature of our immune status algorithm, thresholds offer the most practical solution for categorising the study population.

We used disease day-specific IgG:IgM ratios to characterise primary and post-primary dengue status as day of

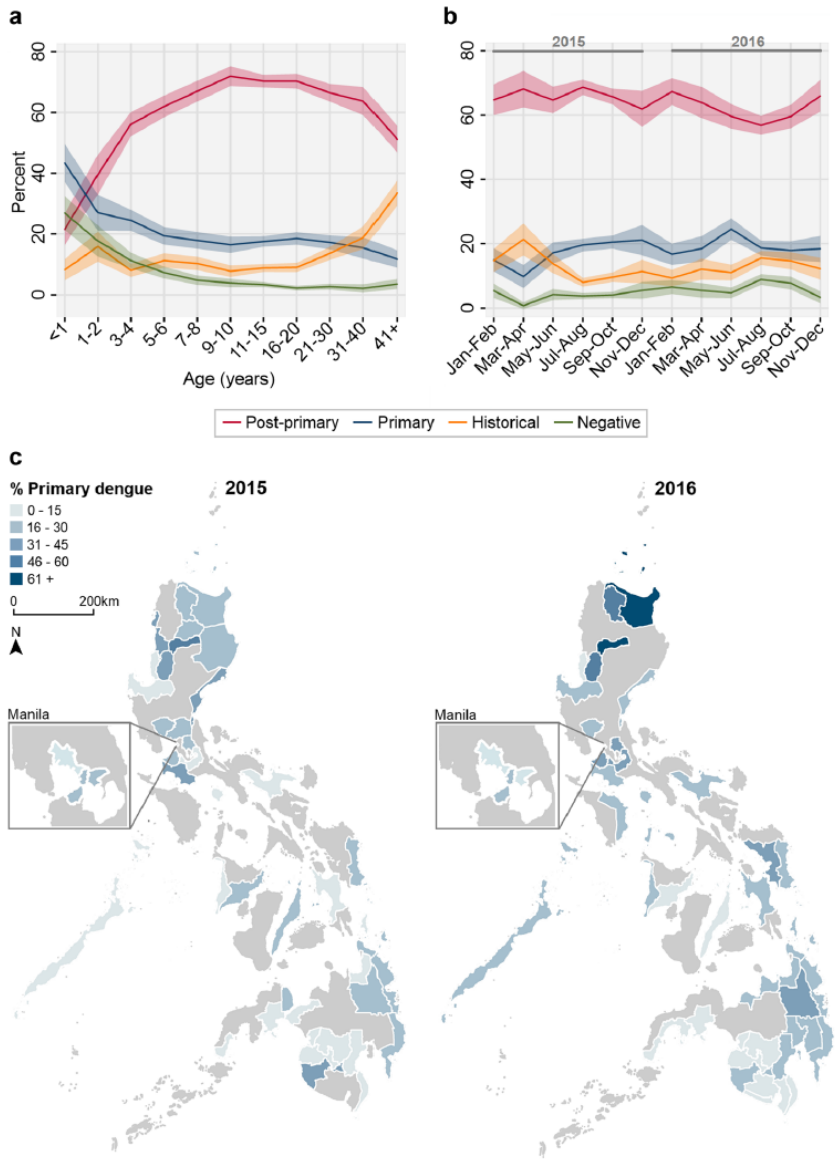

Fig. 5 Immuno-epidemiological patterns associated with DENV immune status across the Philippines. **a** Percentage age trends in dengue immune status among the study population. Shading: 95% CIs. **b** Bi-monthly percentage trends in dengue immune status among the study population between 2015 and 2016. Shading: 95% CIs. **c** Provincial spatio-temporal percentage trends in primary dengue immune status among active DENV cases reporting across the Philippines between 2015 and 2016. Provinces with less than 10 samples collected excluded. Dengue immune status determined according to algorithm 2

fever is a common variable in dengue surveillance worldwide. As anti-DENV IgG is absent or very low during febrile primary infections and pre-circulates in post-primary infections due to previous dengue exposure [17, 29, 30], we concluded the observed lower and higher IgG:IgM ratio distributions represented primary and post-primary cases, respectively. Interestingly, early during the febrile infection period, there was no statistical justification for the existence of two ratio distribution peaks, so we refrained from using antibody ratios before disease day 3. This is consistent with previous findings, which state antibody ratios are poor determinants of immune status early during infection, likely due to low antibody responses [16]. Instead for very early stage dengue infections, we opted to use our newly generated IgG exposure threshold to assess primary and post-primary

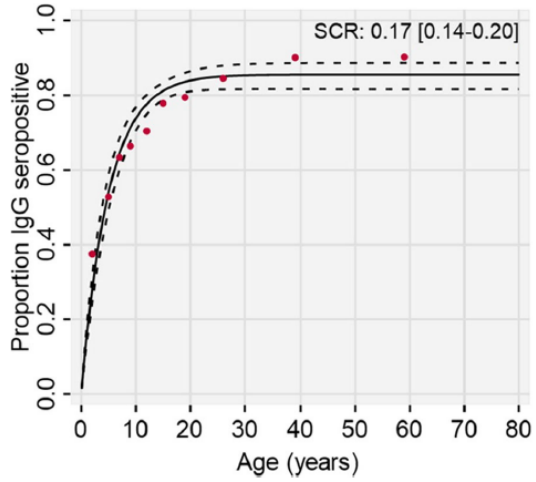

Fig. 6 Dengue transmission intensity among non-active dengue cases across the Philippines between 2015 and 2016. Red dot: observed age-IgG seroprevalence. Black solid line: predicted age-IgG seroprevalence according to reverse catalytic model. Black dash line: predicted age-IgG seroprevalence 95% CI. SCR, seroconversion rate [95% CI]

dengue given the delay in eliciting anti-DENV IgG during primary infections. Together, these findings suggest that the combination of IgG seroprevalence and IgG: IgM ratio thresholds, at appropriate stages of infection, is desirable for distinguishing dengue immune status among febrile reporting cases. In our study, we adhered to manufacturers' specifications to ensure our algorithm is compatible for dengue surveillance operations elsewhere. However, improvements in assay performance, including antibody avidity estimates, may further enhance this immune status algorithm.

Compared to a commercial algorithm, our dengue immune status algorithm had a stronger serological agreement with the WHO gold standard method [22], which demonstrated its suitability for dengue surveillance and epidemiological analysis. It should be noted, however, that observed serological discordance between our novel algorithm (A2) and the WHO gold standard may be attributed to temporal changes in dengue infection status. Individuals categorised as negative or historical according to A2 could be infected with dengue between the paired sera interim and therefore be classified as primary or post-primary, respectively, based on WHO criteria. Overall, based on the short interval between acute and convalescent dengue sera collections and the suitability of A2, we found substantial agreement between A2 and the WHO gold standard method.

In our study, we found a significant proportion of post-primary dengue infections had serological evidence of historical, yet not recent, ZIKV exposure. This supports the hypothesis that other, structurally homologous flaviviruses, including ZIKV, elicit IgG responses that serologically prime individuals for subsequent post-primary, instead of primary, dengue infections. A finding previously reported [31, 32]. However, due to unknown specificities [23–25], we cannot exclude the possibility commercial ELISA kits are detecting antibodies elicited from more than one type of flavivirus. Therefore, we assumed that post-primary dengue infections may have been preceded by any flavivirus infection. Determining whether cross-reactive antibody responses are attributed to just one or both Zika and dengue infections remains an area of ongoing investigation.

Following the immune status classification of our study population, we reported contrasting disease day-averaged infection kinetics among primary and post-primary dengue cases consistent with previous studies [16, 29, 33]. The observed, lower viremia during the acute stage of post-primary, compared to primary, dengue infections has been previously reported [34, 35]. We also revealed the majority of the reporting population were post-primary dengue cases, as previously reported in the Philippines [36], and likely a consequence of the higher risk of more severe symptoms prompting more to seek healthcare. We found lower ground elevation and higher population density as risk factors for reporting with post-primary infections among active dengue cases. This is consistent with the rationale of favourable mosquito breeding conditions in lower (warmer) altitudes and areas of high human population density promote mosquito populations [37] and increase dengue transmission intensity. However, geographical imbalances in disease awareness and healthcare access, which we were unable to adjust for in this study, may also influence this association. Together with the serological validation, these immuno-epidemiological patterns provided further evidence our algorithm accurately characterised the immune status of the study population.

Between 2015 and 2016, we estimated that 17% of our study population became serologically exposed to dengue annually, which is consistent with the previous estimated force of infection between 11 and 22% generated in Cebu, central Philippines, in 2016 [36]. However, given these cases passively reported, it could be speculated those reporting with non-dengue fever were more likely to seek treatment if they had previous dengue infection(s) due to heightened symptom awareness. Therefore, our estimates are likely a slight overestimation of true dengue transmission intensity across the Philippines. Moreover, spatio-temporal heterogeneity in dengue transmission intensity [38–41] infers this national estimate is unlikely to be representative of lower administrative areas in the Philippines. Among those

reporting with active dengue, dengue immune status remained temporally stable across the country yet spatially heterogeneous in northern Luzon. The northern cluster of increased primary dengue reporting was possibly attributed to recent dengue emergence, previously shown in Mexico [42], and/or above average healthcare access/disease awareness. Either way, these reflect populations at risk of developing post-primary infections following a novel serotype invasion. Such areas may also be worth targeting for control and/or enhanced disease surveillance.

Conclusion

In this study, we constructed a framework to accurately categorise the dengue immune status of a large reporting population of suspected dengue cases across the Philippines using routinely collected surveillance metrics. Using our algorithm, we were able to investigate detailed dengue transmission dynamics over 2 years and revealed target populations at risk of developing severe disease. It is hoped that laboratory surveillance operations, in the Philippines and elsewhere, can apply our framework to monitor primary and post-primary infection epidemiology and inform targeted dengue control.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12916-020-01833-1.

Additional file 1. Stratification flow chart of surveillance data used in this study. Exclusion steps associated with the final dataset used in this study.

Additional file 2. Study population demographics. Demographic characteristics of study population with complete data (Final dataset), those missing serological /molecular data and those missing symptom data.

Additional file 3. Anti-DENV IgM mixture model component selection. Model fit comparison of a 3-component, compared to a 1-component, mixture model characterising the anti-DENV IgM titre distribution of the study population. AIC: Akaike information criterion.

Additional file 4. Age-stratified anti-DENV IgM and IgG panbio units. (**A**) Age-stratified anti-DENV IgM distributions of the study population fitted with 3-component mixture models. Black dash: Lowest IgM panbio unit with a classification probability of being seropositive>seronegative (0-5 years: 9.8, 6-15 years: 10.1, 16-30 years: 10.3, 31+ years: 9.7). (**B**) Age-stratified anti-DENV IgG distributions of non-active DENV cases fitted with 2-component mixture models. Black dash: Lowest IgG panbio unit with a classification probability of being seropositive>seronegative (0-5 years: 2.0, 6-15 years: 2.2, 16-30 years: 2.4, 31+ years: 2.3).

Additional file 5. Anti-DENV IgG mixture model component selection. Model fit comparison of a 2-component, compared to a 1-component, mixture model characterising the anti-DENV IgG titre distribution of non-active DENV cases. AIC: Akaike information criterion.

Additional file 6. Anti-DENV IgG:IgM mixture model component selection. Model fit comparison of 2-component, compared to 1-component, mixture models characterising disease day stratified IgG:IgM ratio distributions among active DENV cases. AIC: Akaike information criterion. Bold: statistically favoured model component.

Additional file 7. Validation of A2 compared to the WHO gold standard method of determining dengue immune status. WHO immune classification: dengue immune status according to WHO guidelines. Blue: serological agreement. Red: Serological disagreement.

Additional file 8. Validation of A1 compared to the WHO gold standard method of determining dengue immune status. WHO immune classification: dengue immune status according to WHO guidelines. Blue: serological agreement. Red: Serological disagreement.

Additional file 9. Scatter plots of anti-DENV and anti-ZIKV IgM (blue) and IgG (red) among those categorised as primary and post-primary dengue according to A2. Horizontal dash: seroprevalence thresholds according to Euroimmune™ specifications (1.1 antibody ratios).

Abbreviations

ADE: Antibody-dependent enhancement; AIC: Akaike information criterion; CI: Confidence interval; Ct: Cycle threshold; DENV: Dengue virus; DOH: Department of Health; DRU: Disease-reporting unit; ELISA: Enzyme-linked immunosorbent assay; GPS: Global positioning system; HIA: Haemagglutination inhibition assay; IgG: Immunoglobulin G; IgM: Immunoglobulin M; OR: Odds ratio; PCR: Polymerase chain reaction; PIDSR: Philippine Integrated Disease Surveillance and Response; RDT: Rapid diagnostic test; RNA: Ribonucleic acid; SCR: Seroconversion rate; WHO: World Health Organization; ZIKV: Zika virus

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Authors' contributions

Project administration: AKS, SF, CI, NK, L-MY, AOT, ECLP, MRZC, CDP, JCRH and MLH. Study design: AKS, SF, MRZC, CDP, JCRH and MLH. Data curation: JRB, AKS, MAJR, MAQ, WJ-W, YHT, FLA, NLS, HKM, LTL, HDT, HATN and DDA. Data analysis: JRB, OJB, AJK, SF, JCRH and MLH. Manuscript preparation: JRB, AKS, OJB, AJK, SF, JCRH and MLH. Manuscript editing and review: all. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used in this study are available from the corresponding author, on reasonable request, following approval from appropriate institutional committees.

Ethics approval and consent to participate

Serum samples used in this study were provided from individuals in the Philippines and Vietnam. In the Philippines, suspected dengue patients provided single serum samples for the purposes of ongoing national dengue laboratory surveillance. Local ethics was awarded to the Research Institute for Tropical Medicine (RITM) (Ref: 2017-014) and London School of Hygiene and Tropical Medicine (LSHTM) Ethics Committee (Ref: 17965 and 15849) for the anonymous use of these samples for laboratory analysis. In Vietnam, paired sera were collected approximately 4 weeks apart from study participants following enrolment into a research project investigating the occurrence of dengue. Local ethical approval was obtained in Vietnam and by Nagasaki University (Ref: VN01057) and approved by LSHTM (Ref: 17853). Study participants over 18 years gave informed consent for laboratory/surveillance analysis of sera, while parental/guardian consent, coupled with minor

assent, was acquired for those under 18 years prior to data collection. All unique participant identifiers were removed before data acquisition.

Consent for publication
Non-applicable

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK. [2]Department of Virology, Research Institute for Tropical Medicine, Manila, Philippines. [3]Dengue Study Group, Research Institute for Tropical Medicine, Manila, Philippines. [4]Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK. [5]Centre for the Mathematical Modelling of Infectious Diseases, London School of Hygiene and Tropical Medicine, London, UK. [6]Philippine Epidemiology Bureau, Department of Health, Manila, Philippines. [7]Pasteur Institute of Nha Trang, Nha Trang, Vietnam. [8]National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. [9]Paediatric Infectious Diseases Department, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan. [10]Institute of Human Genetics, National Institute of Health, University of the Philippines, Manila, Philippines. [11]Philippine Genome Centre, University of the Philippines, Manila, Philippines.

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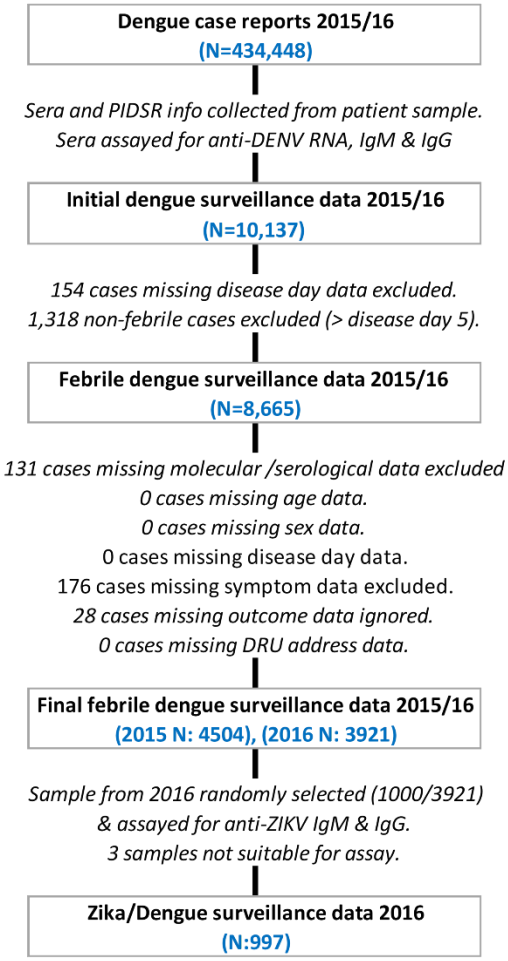
Publisher's Note

Appendix 1: Chapter 3 Supplementary material



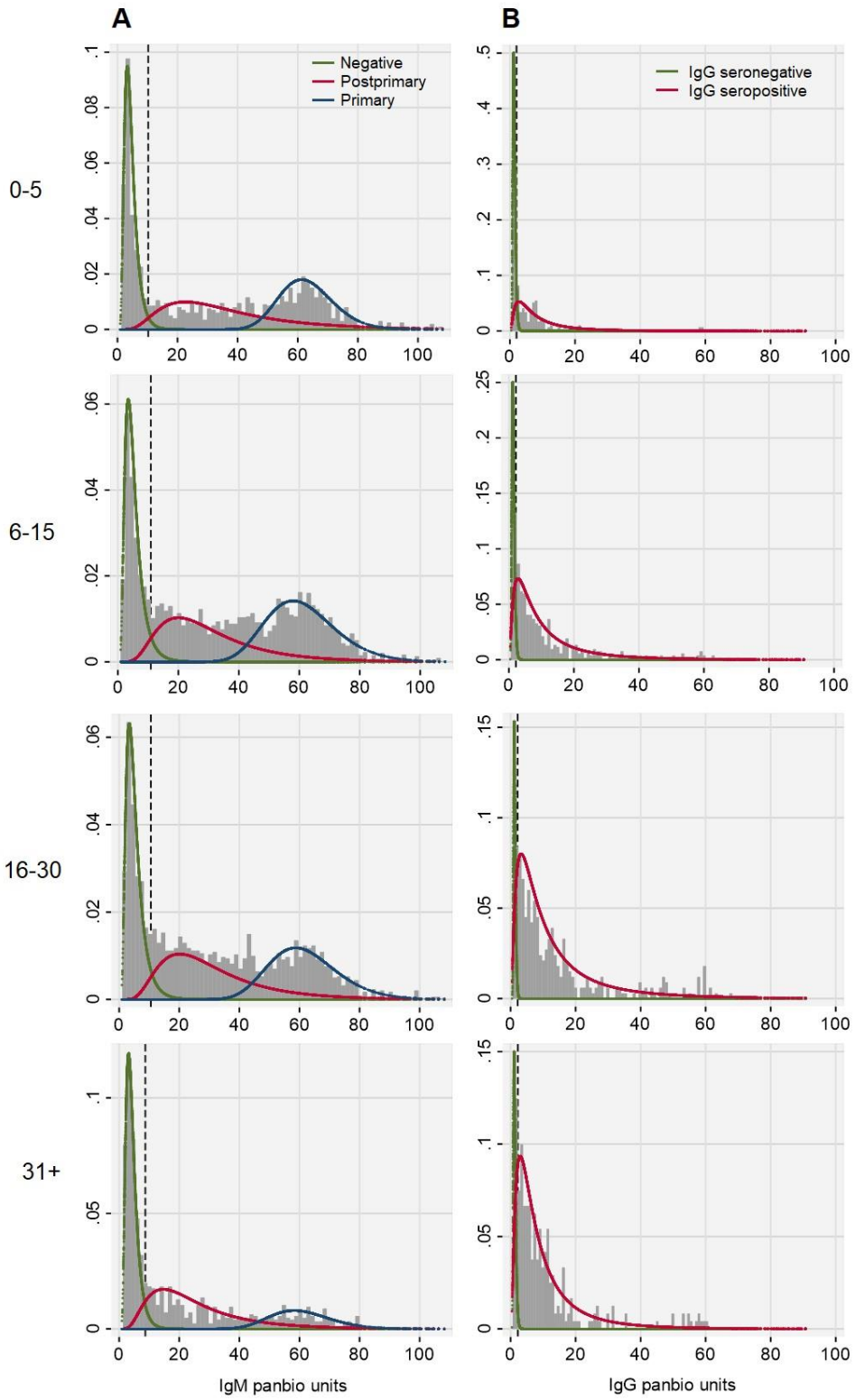
Additional file 1. Stratification flow chart of surveillance data used in this study. Exclusion steps associated with the final dataset used in this study.

Additional file 2. Study population demographics. Demographic characteristics of study population with complete data (Final dataset), those missing serological /molecular data and those missing symptom data.

Demographic characteristics	Febrile dengue surveillance data 2015/16								
	Final dataset			Missing serological/ molecular data			missing symptom data		
	N	%	[95% CI]	N	%	[95% CI]	N	%	[95% CI]
Age									
0-5	1398	16.6	[14.6-18.5]	19	14.5	[0.0-30.3]	33	18.8	[5.4-32.1]
6-15	3715	44.1	[42.5-45.7]	55	42.0	[28.9-55.0]	74	42.0	[30.8-53.3]
16-30	2390	28.4	[26.6-30.2]	44	33.6	[19.6-47.5]	53	30.1	[17.8-42.5]
31+	923	11.0	[8.9-13.0]	13	9.9	[0.0-26.2]	16	9.1	[0.0-23.2]
Sex									
Female	4006	47.6	[46.0-49.1]	56	42.7	[29.8-55.7]	81	46.0	[35.2-56.9]
male	4419	52.5	[51.0-53.9]	75	57.3	[46.1-68.4]	95	54.0	[44.0-64.0]
Disease day									
0-2	2233	26.5	[24.7-28.3]	36	27.5	[12.9-42.1]	46	26.1	[13.4-38.8]
3-4	5097	60.5	[59.2-61.8]	81	61.8	[51.3-72.4]	105	59.7	[50.3-69.0]
5	1094	13.0	[11.0-15.0]	14	10.7	[0.0-26.9]	25	14.2	[0.5-27.9]
Symptoms									
No symptoms	1861	22.1	[20.2-24.0]	35	26.7	[12.1-41.4]			
With symptoms	5854	69.5	[68.3-70.7]	79	60.3	[49.5-71.1]			
Severe symptoms	710	8.4	[6.4-10.5]	13	9.9	[0.0-26.2]			
Non-disclosed	0	0.0		4	3.1	0.019.9			
Outcome									
Alive	8365	99.3	[99.1-99.5]	126	96.2	[92.8-99.5]	170	96.6	[93.9-99.3]
Dead	32	0.4	[0.0-2.5]	2	1.5	[0.0-18.5]	1	0.6	[0.0-15.3]
Non-disclosed	28	0.3	[0.0-2.5]	3	2.3	[0.0-19.2]	5	2.8	[0.0-17.4]
Total	8425	100		131	100.0		176	100.0	

Additional file 3. Anti-DENV IgM mixture model component selection. Model fit comparison of a 3-component, compared to a 1-component, mixture model characterising the anti-DENV IgM titre distribution of the study population. AIC: Akaike information criterion.

IgM model Component	AIC	AIC difference
1	8798.4	
3	8577.1	-221.2



Additional file 4. Age-stratified anti-DENV IgM and IgG panbio units. (**A**) Age-stratified anti-DENV IgM distributions of the study population fitted with 3-component mixture models. Black dash: Lowest IgM panbio unit with a classification probability of being seropositive>seronegative (0-5 years: 9.8, 6-15 years: 10.1, 16-30 years: 10.3, 31+ years: 9.7). (**B**) Age-stratified anti-DENV IgG distributions of non-active DENV cases fitted with 2-component mixture models. Black dash: Lowest IgG panbio unit with a classification probability of being seropositive>seronegative (0-5 years: 2.0, 6-15 years: 2.2, 16-30 years: 2.4, 31+ years: 2.3).

Additional file 5. Anti-DENV IgG mixture model component selection. Model fit comparison of a 2-component, compared to a 1-component, mixture model characterising the anti-DENV IgG titre distribution of non-active DENV cases. AIC: Akaike information criterion.

IgG model Component	AIC	AIC difference
1	5035.2	
2	4934.5	-97.7

Additional file 6. Anti-DENV IgG:IgM mixture model component selection. Model fit comparison of 2-component, compared to 1-component, mixture models characterising disease day stratified IgG:IgM ratio distributions among active DENV cases. AIC: Akaike information criterion. Bold: statistically favoured model component.

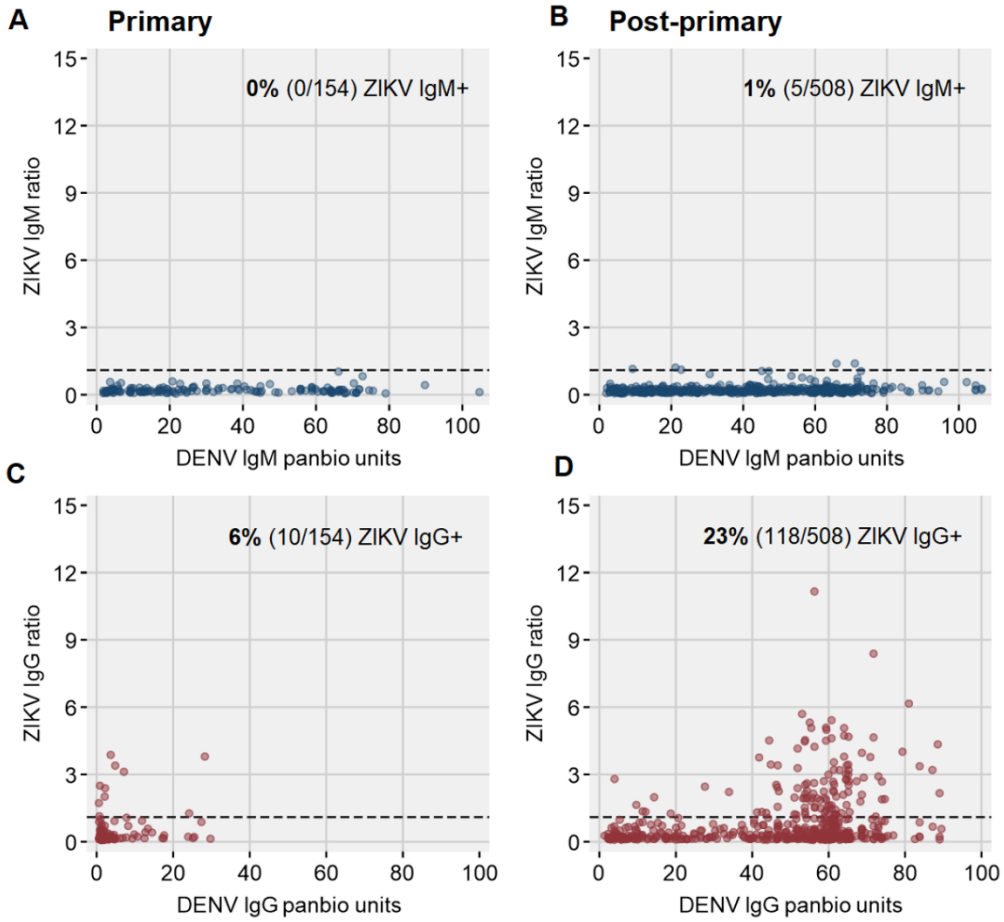
Disease Day	Model AIC 1-component	2-component	AIC difference
1	**1198.1**	1302.7	+104.6
2	**3225.4**	3290.2	+64.8
3	5331.2	**5163.5**	-167.7
4	5491.7	**5382.4**	-109.3
5	3533.6	**3195.3**	-338.3

Additional file 7. Validation of A2 compared to the WHO gold standard method of determining dengue immune status. WHO immune classification: dengue immune status according to WHO guidelines. Blue: serological agreement. Red: Serological disagreement.

Household member	Fever day	NS1 status	Acute IgM panbio units	Acute IgG panbio units	(A2) Algorithm Immune classification	IgG fold increase	Convalescent IgG panbio units	WHO immune classification
1	5	neg	1.18	6.46	Historical	3.05	19.72	Not dengue
2	2	neg	50.4	1.78	Primary	6.39	11.35	Acute primary
3	3	neg	4.88	40.46	Historical	1.04	42.06	Recent secondary
4	0	neg	13.19	2.27	Primary	4.57	10.34	Acute primary
5	1	neg	1.16	1.72	Negative	0.85	1.46	Not dengue
6	1	pos	1.74	8.43	Post-primary	11.94	100.67	Acute secondary
7	4	neg	8.43	53.35	Historical	1.16	62.05	Recent secondary
8	2	neg	11.65	0.76	Primary	14.35	10.94	Acute primary
9	2	neg	4.31	10.71	Historical	9.55	102.24	Acute secondary
10	2	neg	2.19	7.51	Historical	1.01	7.55	Not dengue
11	2	pos	0.87	0.74	Primary	32.38	23.88	Acute primary
12	2	neg	1.12	29.8	Historical	3.16	94.21	recent secondary
13	3	neg	19.19	24.98	Post-primary	4.03	100.55	Acute secondary
14	1	pos	3.5	21.3	Post-primary	4.52	96.33	Acute secondary
15	4	neg	1.41	1.06	Negative	0.56	0.59	Not dengue
16	2	pos	0.99	8.45	Post-primary	12.1	102.34	Acute secondary
17	1	neg	0.66	0.56	Negative	57.36	31.83	Acute secondary
18	2	neg	24	3.24	Primary	4.44	14.4	Acute primary
19	0	neg	1.7	12.17	Historical	1.19	14.49	Not dengue
20	2	neg	1.83	11.64	Historical	0.93	10.78	Not dengue
21	5	neg	0.75	6.46	Historical	1.17	7.54	Not dengue

Additional file 8. Validation of A1 compared to the WHO gold standard method of determining dengue immune status. WHO immune classification: dengue immune status according to WHO guidelines. Blue: serological agreement. Red: Serological disagreement.

Household member	Fever day	NS1 status	Acute IgM panbio units	Acute IgG panbio units	(A1) Panbio® Immune classification	IgG fold increase	Convalescent IgG panbio units	WHO immune classification
1	5	neg	1.18	6.46	-	3.05	19.72	Not dengue
2	2	neg	50.4	1.78	Primary	6.39	11.35	Acute primary
3	3	neg	4.88	40.46	Post-primary	1.04	42.06	Recent secondary
4	0	neg	13.19	2.27	Primary	4.57	10.34	Acute primary
5	1	neg	1.16	1.72	-	0.85	1.46	Not dengue
6	1	pos	1.74	8.43	-	11.94	100.67	Acute secondary
7	4	neg	8.43	53.35	Post-primary	1.16	62.05	Recent secondary
8	2	neg	11.65	0.76	Primary	14.35	10.94	Acute primary
9	2	neg	4.31	10.71	-	9.55	102.24	Acute secondary
10	2	neg	2.19	7.51	-	1.01	7.55	Not dengue
11	2	pos	0.87	0.74	-	32.38	23.88	Acute primary
12	2	neg	1.12	29.8	Post-primary	3.16	94.21	Recent secondary
13	3	neg	19.19	24.98	Post-primary	4.03	100.55	Acute secondary
14	1	pos	3.5	21.3	Primary	4.52	96.33	Acute secondary
15	4	neg	1.41	1.06	-	0.56	0.59	Not dengue
16	2	pos	0.99	8.45	-	12.1	102.34	Acute secondary
17	1	neg	0.66	0.56	-	57.36	31.83	Acute secondary
18	2	neg	24	3.24	Primary	4.44	14.4	Acute primary
19	0	neg	1.7	12.17	-	1.19	14.49	Not dengue
20	2	neg	1.83	11.64	-	0.93	10.78	Not dengue
21	5	neg	0.75	6.46	-	1.17	7.54	Not dengue



Additional file 9. Scatter plots of anti-DENV and anti-ZIKV IgM (blue) and IgG (red) among those categorised as primary and post-primary dengue according to A2. Horizontal dash: seroprevalence thresholds according to Euroimmune™ specifications (1.1 antibody threshold ratios)

Chapter 4. Estimating the Annual Dengue Force of Infection from the Age of Reporting Primary Infections Across Urban Centres in Endemic Countries

An online, full text version of chapter 4 is available at:
https://bmcmedicine.biomedcentral.com/articles/10.1186/s12916-021-02101-6

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646
F: +44 (0)20 7299 4656
www.lshtm.ac.uk

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Primary Supervisor	Dr Julius Clemence R. Hafalla		

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RESEARCH ARTICLE Open Access

Estimating the annual dengue force of infection from the age of reporting primary infections across urban centres in endemic countries

Joseph R. Biggs[1*†], Ava Kristy Sy[2,3†], Katharine Sherratt[4,5], Oliver J. Brady[4,5], Adam J. Kucharski[4,5], Sebastian Funk[4,5], Mary Anne Joy Reyes[2,3], Mary Ann Quinones[2,3], William Jones-Warner[1], Ferchito L. Avelino[6], Nemia L. Sucaldito[6], Amado O. Tandoc[2], Eva Cutiongco-de la Paz[7,8], Maria Rosario Z. Capeding[3,7], Carmencita D. Padilla[7,8], Julius Clemence R. Hafalla[1] and Martin L. Hibberd[1,7,8]

Abstract

Background: Stratifying dengue risk within endemic countries is crucial for allocating limited control interventions. Current methods of monitoring dengue transmission intensity rely on potentially inaccurate incidence estimates. We investigated whether incidence or alternate metrics obtained from standard, or laboratory, surveillance operations represent accurate surrogate indicators of the burden of dengue and can be used to monitor the force of infection (FOI) across urban centres.

Methods: Among those who reported and resided in 13 cities across the Philippines, we collected epidemiological data from all dengue case reports between 2014 and 2017 (*N* 80,043) and additional laboratory data from a cross-section of sampled case reports (*N* 11,906) between 2014 and 2018. At the city level, we estimated the aggregated annual FOI from age-accumulated IgG among the non-dengue reporting population using catalytic modelling. We compared city-aggregated FOI estimates to aggregated incidence and the mean age of clinically and laboratory diagnosed dengue cases using Pearson's Correlation coefficient and generated predicted FOI estimates using regression modelling.

Results: We observed spatial heterogeneity in the dengue average annual FOI across sampled cities, ranging from 0.054 [0.036–0.081] to 0.249 [0.223–0.279]. Compared to FOI estimates, the mean age of primary dengue infections had the strongest association (ρ −0.848, p value<0.001) followed by the mean age of those reporting with warning signs (ρ −0.642, p value 0.018). Using regression modelling, we estimated the predicted annual dengue FOI across urban centres from the age of those reporting with primary infections and revealed prominent spatio-temporal heterogeneity in transmission intensity.

* Correspondence: Joseph.Biggs1@lshtm.ac.uk
†Joseph R Biggs and Ava Kristy Sy are joint first authors of the study.
[1]Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK
Full list of author information is available at the end of the article

Conclusions: We show the mean age of those reporting with their first dengue infection or those reporting with warning signs of dengue represent superior indicators of the dengue FOI compared to crude incidence across urban centres. Our work provides a framework for national dengue surveillance to routinely monitor transmission and target control interventions to populations most in need.

Keywords: Dengue, Surveillance, Serology, Primary, Flavivirus, Philippines

Background

Dengue is a mosquito-borne viral disease in which individuals can suffer up to four times during their lifetime due to the existence of four distinct serotypes (DENV1-4). A primary (first) dengue infection with any serotype induces IgG antibodies that offer protection against homologous serotype infections yet allow subsequent post-primary (secondary, tertiary or quaternary) infections with heterologous serotypes [1, 2]. Severe dengue disease is associated with, though not limited to, secondary infections due to pre-existing, cross-reactive, IgG antibodies that do not protect against infection, but rather facilitate antibody-dependent enhancement of viral replication [3]. Without specific therapeutics or a widely available vaccine, costly vector control interventions remain the predominant method of dengue control [4]. To appropriately deploy these limited interventions, dengue national surveillance operations in endemic countries must accurately monitor force of infection (FOI) over space and time.

In countries where dengue is a notifiable disease, national surveillance efforts typically collate dengue case reports over specified time periods to generate incidence estimates. This readily available data can be used to inform the deployment of limited control interventions, thus maximise their impacts. However, case reporting is heavily influenced by disease awareness and variable healthcare infrastructure, which can distort generated measures [5]. Alternatively, population-based surveys or cohort studies including seroprevalence [6] and entomological surveys [7] can provide more reliable estimates of disease risk within their operational setting and are considered the gold standards for measuring long-term average transmission intensity. Yet, surveys are labour-intensive and difficult to conduct routinely over large geographical areas, so have limited use for routine surveillance proposes.

Recently, age-stratified incidence measures of case reports have been proposed as more suitable indicators of transmission intensity [5, 8, 9]. In areas of high transmission, the burden of disease is believed to disproportionately impact younger individuals compared to lower transmission areas where individuals likely report with dengue later in life due to the accumulation of immunity over time. This approach helps counter bias introduced by variable health care infrastructures and disease awareness although suffers two major caveats. First, case reporting is dependent on a variety of different diagnostics between and within endemic countries. Surveillance operations rely on non-specific diagnostics or clinical manifestations for notifying dengue [10]. Consequently, other co-endemic febrile infections, which manifest similarly to dengue and are common among children may prompt more younger individuals to seek care and be misdiagnosed as dengue infections [11, 12]. Second, among those reporting with true dengue infections, routinely used diagnostics are currently unable to distinguish those experiencing primary or post-primary infections [1]. Therefore, the age of case reports may be influenced by spatio-temporal imbalances in reported dengue immune status. Stratifying age estimates among those truly experiencing their primary infection may help to overcome these limitations.

In the Philippines, dengue has become a major contributor to mortality and morbidity over the past 50 years with all four serotypes believed to be endemic across urban centres [13]. Existing dengue surveillance operations consist of collating all reported dengue infections and surveying a subset for subsequent laboratory analysis. The serum is collected from sampled case reports for molecular and serological testing to allow spatio-temporal monitoring of dengue serotypes. Prior to this study, we utilised laboratory data from surveyed dengue patients to develop an algorithm that accurately determines individual primary or post-primary (secondary, tertiary or quaternary) immune status [14]. Despite this, it remains unknown how surveillance metrics derived from this algorithm can best be used to routinely characterise the FOI. Previous studies have demonstrated that the FOI can be reasonably estimated from age-stratified incidence rates [5, 9]. However, it remains unknown if other, easily computed, surveillance metrics represent surrogate indicators of the FOI. Here, we investigated whether laboratory/non-laboratory surveillance metrics correlate with the FOI according to age-seroprevalence and be used to routinely predict the burden of dengue across urban centres.

Methods

Data collection

Dengue is a notifiable disease among all disease reporting units (DRUs) across the Philippines and is recorded

in line with The Philippine Integrated Disease Surveillance and Response (PIDSR) manual and WHO criteria [15]. Basic epidemiological data are collected from suspected dengue patients, including age, sex, date of symptom onset, date of reporting, patient/DRU address (barangay, municipality, province, region), symptoms and outcome. According to WHO criteria, dengue symptoms are classified among patients as those with acute febrile illness coupled with either no warning signs of dengue (abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy and/or live enlargement) or severe dengue (severe plasma leakage, bleeding and/or organ impairment). All case reports were collated by the Philippine Epidemiological Bureau (Department of Health).

In addition to collated case reports, established laboratory surveillance operations performed by the Research Institute for Tropical Medicine (RITM—the research arm of the Department of Health) orchestrate annual cross-sectional surveys of dengue case reports across the country for further laboratory analysis. Participating DRUs, both sentinel and non-sentinel, collect single serum samples and basic epidemiological information (according to PIDSR criteria) from consenting, reporting dengue patients. Sentinel DRUs, including major hospitals, randomly select five case report samples per week. Non-sentinel DRUs comprising of any health facility across the Philippines that experiences a marked increase in suspected dengue case reporting according to PIDSR criteria also collected samples from case reports. For the purposes of this study, surveyed case report data were provided between 2014 and 2018 (*N* 20,666).

During the study period, a total of 13 cities across the Philippines routinely provided serum samples from dengue patients who reported and resided in the same city (*N* 11,906) (Fig. 1) (Additional file 1). In these same cities between 2014 and 2017, a total of 80,043 case reports reported and lived in these corresponding cities. The population age structures of these cities according to the Philippine 2015 census (Philippine Statistics Authority) are shown in Additional file 2.

Laboratory procedures

Serum samples were stored at −80°C at the RITM before molecular and serological assay. Serum samples were assayed using a serotype-specific, fourplex real-time reverse transcriptase PCR nucleic acid detection assay as described in [16]. Briefly, serotype-specific RNA is detected and amplified yielding a critical threshold (Ct) value that categorises samples as DENV1/2/3 or 4 positive (Ct<36) or DENV negative (Ct>36). In addition, samples were assayed for anti-DENV IgM and IgG using Panbio® capture ELISA kits (Cat No: 01PE10/01PE20, Alere, Brisbane, Australia) in accordance with the manufacturer's instructions. ELISA plates contain antigen that captures host anti-DENV NS1 antibodies specific to all four serotypes and include calibrators that normalise optical density outputs to yield IgM and IgG panbio units.

Data analysis

We categorised the dengue immune status (primary, post-primary, historical and negative) of the surveyed cases reports, using laboratory data, according to a previously developed algorithm [14]. Prior to categorisation, we generated additional variables: IgG to IgM ratio (IgG panbio units/IgM panbio units) and disease day (symptom onset date—reporting date). We excluded those under 6 months of age (0.4% 91/20,666) due to the potential influence of maternal antibodies and individuals who reported ≥6 days after symptom onset due to the algorithm's inability to categorise convalescent infections. Details of the immune status algorithm are described in Additional file 3. Briefly, suspected dengue patients were categorised as active (PCR+ or IgM+) or non-active (PCR- and IgM-) dengue infections. Active dengue infections were further categorised as primary or post-primary (dengue infection with at least one previous flavivirus infection). Non-active dengue was classified as negative (anti-DENV IgG-) or historical (anti-DENV IgG+) for dengue.

Across the Philippines, we investigated temporal patterns in reported dengue immune status and serotype by stratifying immune status (primary and post-primary) among active dengue infections and dengue serotype (DENV1-4) among those DENV PCR+ over 30-day intervals. Among those reporting with primary and post-primary infections, we explored age-stratified disease severity patterns. Univariable logistic regression models were used to calculate odds ratios of being severe opposed to non-severe (warning signs and no warning signs) with the explanatory variables being age-stratified primary or post-primary dengue immune status. Regression models were fit using the 'Logit' command in STATA (v.16).

In each of the 13 sampled cities, we estimated all age and age-stratified (under 5 years and 10 years) aggregated annual dengue incidence rates among those who reported and resided in the same city. To estimate the average city population at risk of infection, we utilised city-specific population data from the 2015 population census (Philippine Statistics Authority) and population growth rates calculated between 2010 and 2015 (Additional file 2). City-specific population growth rates were used to estimate the population in each of the non-surveyed years. Average incidence per annum per 1000 persons equated to the number of case reports over the persons years at risk (average population over the study

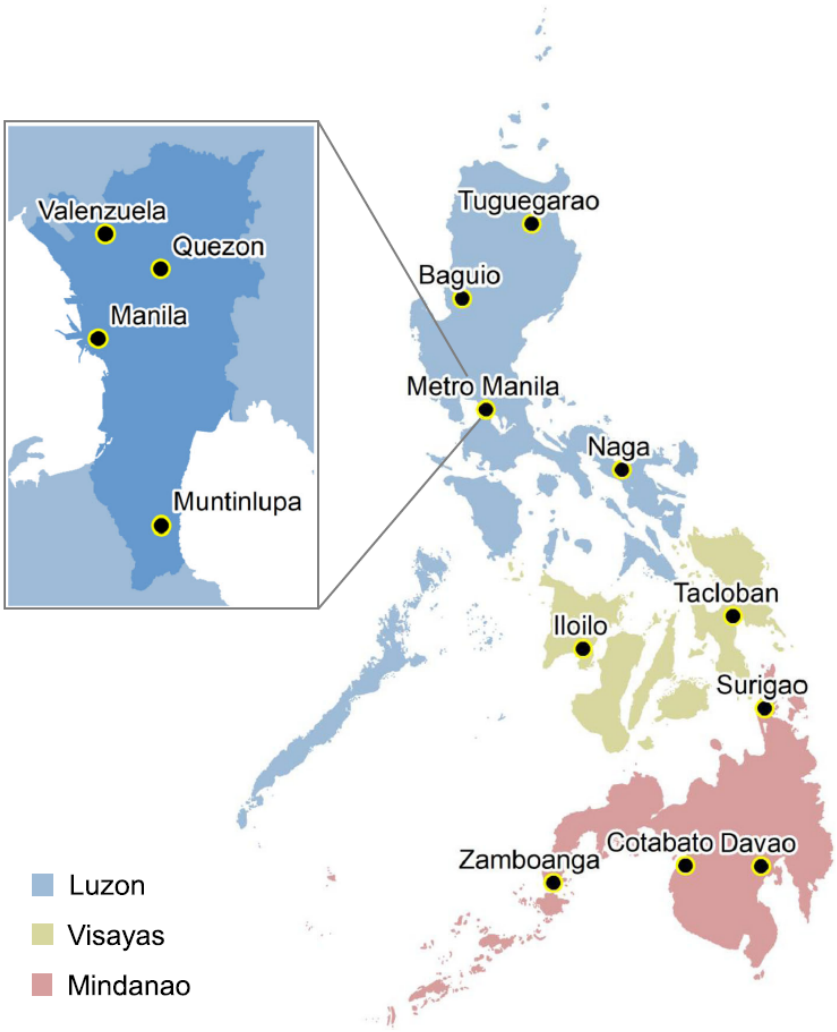

Fig. 1 Location of 13 study-participating cities across the three principle geographical divisions of the Philippines that routinely provided serum samples between 2014 and 2018

period multiplied by 4 years) multiplied by 1000 (Additional file 4).

In sampled cities, we estimated the average annual FOI estimated over the study period among non-active dengue infections (IgG+ historical and IgG-negative dengue cases). Seroprevalence corresponded to the proportion of the non-active dengue cases who were IgG seropositive to any serotype. Catalytic models were fit, by maximum likelihood, to estimate age-seroprevalence and derive seroconversion rates which correspond to the average annual rate individuals seroconvert from IgG– to IgG+ status; a rate analogous to the FOI. Under the assumption, individuals remain IgG+ after seroconversion and all circulating serotypes contributed equally to transmission, a simple catalytic model (Eq. 1) calculates the probability of being IgG seropositive at specific ages (a) by fitting a constant force of infection parameter ($\boldsymbol{\lambda}$) by least squares:

$$P(a) = \left[1 - e^{-\lambda a}\right] \quad (1)$$

Assuming IgG antibodies can wane over time to low levels undetectable according to the commercial ELISA kits, as previously shown in [17–19], a reversible catalytic model (Eq. 2) additionally estimates a seroreversion rate (the average annual rate individuals serorevert back to IgG-status) by fitting an additional seroreversion parameter (ρ), by least squares:

$$P(a) = \frac{\lambda}{\lambda + \rho}\left[1 - e^{-(\lambda+\rho)a}\right] \quad (2)$$

To determine which catalytic model, simple or reversible, was most appropriate to estimate the FOI, we used AIC (Akaike information criterion) to determine superior model fits in each city. Catalytic models were fitted using a rho-constrained/unconstrainted 'revcat' command in STATA (v.16).

We investigated whether routinely collected surveillance metrics represent surrogate indicators of the FOI at the city level, we explored their statistical association with the city FOI aggregated over the study period. Non-laboratory surveillance metrics investigated included all age/under 5/under 10 average annual dengue incidence, mean age of all reported case reports/case reports with dengue warning signs/case reports with severe dengue. Laboratory-derived surveillance metrics included the mean age of laboratory-confirmed active dengue infections/primary dengue infections/post-primary dengue infections. The strength of association between the city-aggregated FOI and averaged surveillance metrics were assessed using Pearson's correlation coefficient (ρ, p value). Among surveillance metrics that significantly correlated with the FOI at the city level (ρ, p value<0.05), exponential regression models were used to generate predicted FOI estimates. Lrtests were adopted to justify exponential over linear model fits (p value< 0.05). Models were fit using a non-linear 'nl' command in STATA (v.16). FOI estimates were subsequently converted to annual attack rates (AR) (Eq. 3) to determine the proportion of the sampled population who became exposed to dengue in each city per year:

$$\mathrm{AR} = 1 - e^{-(\lambda)} \quad (3)$$

Results

Data description

Demographic characteristics of the sampled and collated acute case reports across the Philippines are highlighted in Table 1. Similar demographic patterns were observed among both case report datasets. The majority of patients were aged 6–15 years of age, reported 3–4 days after the onset of symptoms and presented with warning signs of dengue. Among the surveyed case reports with laboratory data, we revealed 18% (3310/18,366) were primary infections, 62.9% (11,560/18,366) were post-primary infections, 12.5% (2297/18,366) were historical dengue infections and 6.5% (1199/18,366) were negative dengue infections.

Overall dengue immuno-epidemiological patterns between 2014 and 2018 revealed both primary and post-primary dengue infections were mainly children and young adults (Fig. 2A) and continuously reported to health facilities during the study period (Fig. 2B). Among PCR+ case reports, we revealed by 2017 and 2018, DENV-3 replaced DENV-1/2 as the most dominant serotype across the Philippines (Fig. 2C). Among those with disclosed symptom data (72.7% 13,357/18366), we observed similar clinical manifestations among primary and post-primary infections (Fig. 2D), whereby younger individuals tended to present with more dengue warning signs or severe dengue (Fig. 2E & F). Despite this, post-primary infections were slightly more likely to present with severe disease compared to primary infections (OR 1.22 [95%CI 1.01–1.48] p value 0.039) (Fig. 2G). Among young primary infections, we observed no age-stratified severity trends: individuals over 2 years, yet younger than 25 years, were statistically no more/less likely to present with severe disease than those under 2 years (Fig. 2H). In contrast, young children with post-primary infections were more likely to present with severe disease compared to their elders. Compared to post-

Table 1 Study population demographics. Characteristics of sampled and collated acute dengue case reports across the Philippines. Excludes patients who reported more than 5 days after symptom onset and those under the age of 6 months

Demographics	Surveyed case reports		Collated case reports	
	%n	n	%n	n
Age (years)				
<5	15.9	2920	17.1	80,046
6–15	45.6	8371	39.1	182,999
16–25	23.4	4299	24.8	116,062
26–40	9.3	1716	11.5	53,803
>41	5.8	1060	7.5	35,117
Sex				
Female	47	8624	47.3	221,446
Male	53	9742	52.7	246,581
Disease day				
<2	28.5	5233	31.8	148,933
3–4	58	10,652	54.6	255,352
5	13.5	2481	13.6	63,742
Symptoms				
No warning signs	14.2	2617	16.5	77,006
Warning signs	53.4	9811	50.3	235,619
Severe dengue	5.1	929	4.0	18,506
Non-disclosed	27.3	5009	29.2	136,896
DENV immune status				
Primary	18	3310	–	–
Post-primary	62.9	11,560	–	–
Historical	12.5	2297	–	–
Negative	6.5	1199	–	–
Total	100.0	18,366	100.0	468,027

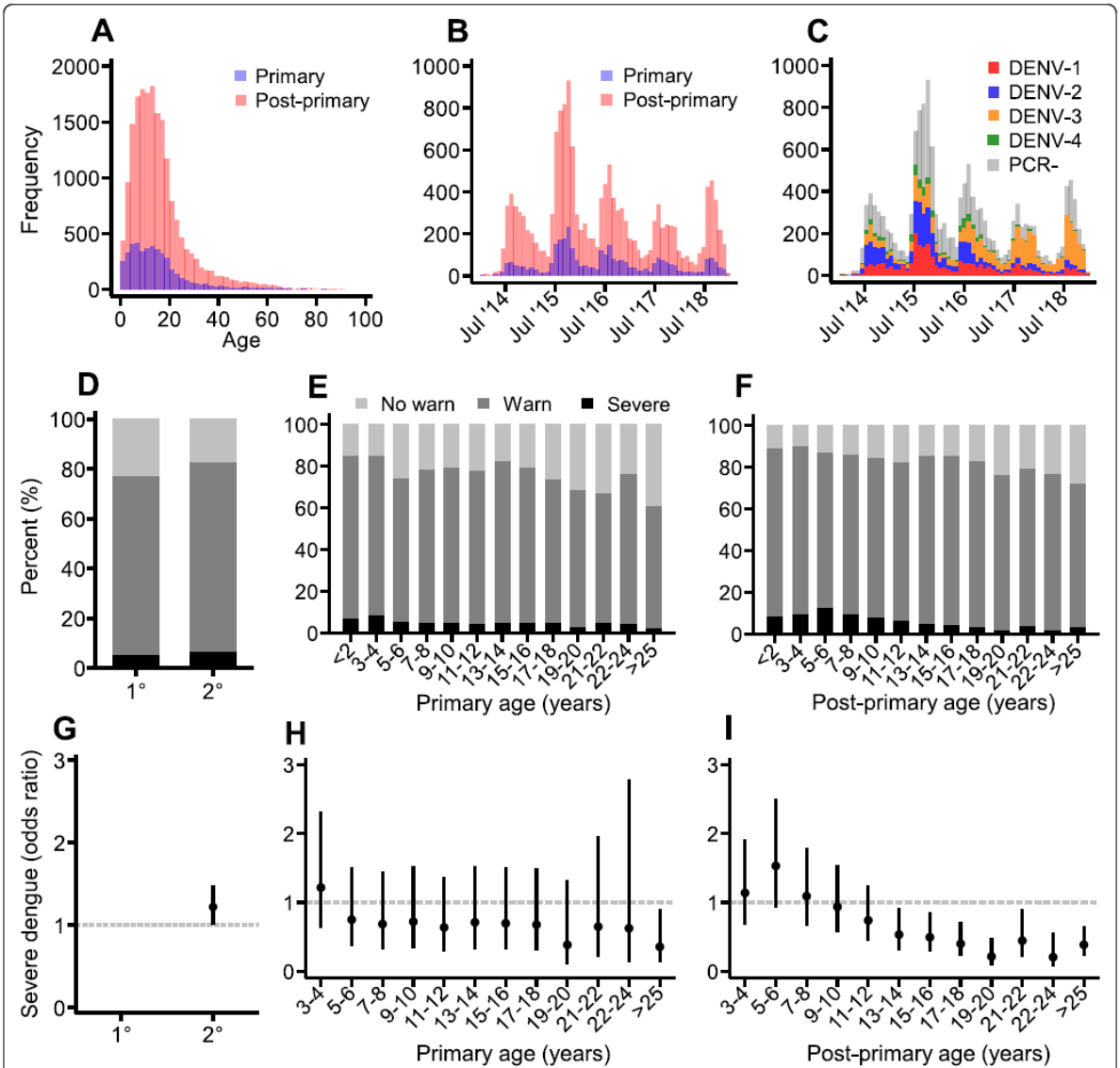

Fig. 2 Immuno-epidemiological characteristics of dengue infections reporting across the Philippines between 2014 and 2018. **A** Age distribution of the primary and post-primary dengue infections. **B** Temporal patterns in reporting primary and post-primary dengue infections. **C** Temporal patterns in reporting DENV serotypes among active dengue infections. **D** Clinical manifestations of the primary and post-primary dengue infections. **E** Age-stratified clinical manifestations of primary dengue infections. **F** Age-stratified clinical manifestations of post-primary dengue infections. **G** Odds ratio [95% CI] for post-primary dengue infections to present with severe disease compared to primary dengue infections. **H** Odds ratios [95% CI] for primary dengue infections to present with severe disease by age in years compared to those under 2 years. **I** Odds ratios [95% CI] for post-primary dengue infections to present with severe disease by age in years compared to those under 2 years. 1° primary dengue infections. 2° Post-primary dengue infections

primary infections under 2 years, those over 12 years were significantly less likely to present with severe disease (Fig. 2I).

City-level dengue transmission dynamics

Among 13 study-participating cities during the study period, we observed variation in estimated crude and age-stratified incidence rates. All-age incidence was highest in Baguio (3.75 cases per annum per 1000 [95%CI 3.65–3.86]) and lowest in Valenzuela (0.49 cases per annum per 1000 [95%CI 0.46–0.52]). After stratifying among younger age groups, city incidence rates changed. The highest incidence rate among those under ten was observed in Surigao (7.17 cases per annum per

1000 [95%CI 6.71–7.65]) while the lowest rate was again in Valenzuela (0.84 cases per annum per 1000 [95%CI 0.76–0.93]) (Additional file 4).

Across cities, we revealed prominent spatio-temporal heterogeneity in serotype dominance (Fig. 3A) and immune status reporting (primary/post-primary) (Additional file 5). During 2015/16, cities in northern Luzon were mainly burdened by DENV-1/2, yet by 2017 and 2018, DENV-3 had become the most dominant. In contrast, cities in Mindanao were burdened with DENV-3 throughout the entire study period, and in Surigao, DENV-2 replaced DENV-3 as the most dominant serotype in 2016. It should be noted that for nearly every year among all sampled cities, all four serotypes of dengue were detected among reporting PCR+ patients. Regarding the immune status of the population, patients in the cities of southern Mindanao (Zamboanga, Cotabato and Davao) had a higher probability of reporting as post-primary opposed to primary dengue infections compared to those in cities of Luzon. Within cities, we observed temporal variation in immune status reporting. For instance, in Valenzuela (Metro Manila), primary dengue reporting significantly increased during 2016, while in Tuguegarao, fell between 2015 and 2018.

Furthermore, we estimated the average annual dengue FOI among the 13 cities aggregated over the study period. In all cities, the reversible, opposed to the simple, catalytic model produced superior model fits (reversible model AIC < simple model AIC) (Additional file 6). We therefore opted to estimate the FOI (or SCR) in each city using the more complex, reversible model. Between each city, the FOI rate ranged from 0.054 [95%CI 0.036-0.081] per year in Baguio city to 0.249 [95%CI 0.223–0.279] per year in Quezon city demonstrating spatial heterogeneity in the transmission intensity (Fig. 3B).

Estimating the city-level FOI from routinely collected surveillance metrics

To assess whether data from routine dengue case surveillance activities could be used to estimate the FOI in cities, we explored statistical associations between catalytic model estimated city-level FOI against age/age-stratified incidence and the mean age of different types of dengue infections (Fig. 4). We observed a negative correlation between the FOI and all-age average annual incidence, whereby incidence increased with decreasing FOI (ρ −0.692, p value 0.009). No association was identified between the city-level FOI and average annual age-stratified (under 5 and under 10 years) incidence rates (ρ, p value>0.05). Among all those who reported suspected dengue, we identified a negative correlation between the FOI and the mean age of all case reports (ρ −0.639, p value 0.019). For those who reported with dengue warning signs, we identified a similar association where the decreasing FOI correlated with the increasing mean age (ρ −0.642, p value: 0.018). In contrast, we observed a weak association between the FOI and the mean age of severe case reports (ρ −0.497, p value 0.059). Among those with laboratory-confirmed dengue infections, we identified stronger associations between mean age and the FOI. The mean age of active dengue infections (primary and post-primary) increased with decreasing FOI (ρ:−0.749, p value 0.003). An association that was strengthened after stratifying among only primary infections (ρ −0.848, p value<0.001), yet remained similar when stratifying by post-primary infections (ρ −0.719, p value 0.006). We repeated these associations using FOI estimates generated from simple, opposed to reversible, catalytic models and still found the mean age of primary infections had the strongest association with the FOI (ρ −0.720, p value 0.005) (Additional file 7).

For each surveillance metric that demonstrated a statistically significant association with the FOI, we estimated the predicted FOI using exponential regression models where appropriate (ρ, p value<0.05). For each statistically associated metric, except incidence, exponential opposed to linear model fits were favoured (Lrtest, p value<0.05). Using these regression models, we estimated annual dengue attack rates from predicted FOI estimates from the most associated laboratory- and non-laboratory-derived metrics: the mean age of laboratory-confirmed primary dengue infections (Table 2) (Additional file 8) and the mean age of case reports with warning signs (Additional file 9). Annual trends in the mean primary age, and thus predicted attacks rates, revealed spatio-temporal heterogeneity in dengue transmission intensity among sampled cities. In Zamboanga city, the attack rate (AR) increased dramatically from 4% [95%CI 4–4%] of the population being exposed in 2014 to 30% [95%CI 19–46%] in 2018. In Manila city, the AR decreased during the study period from 27% [95%CI 20–36%] in 2015 to 10% [95%CI 8–14%] in 2018. In Quezon city, the AR remained high between 17% and 23% during the entire study period which is consistent with the estimated FOI according to the catalytic model (AR 22% [95%CI 20–24%]). For some cities (Davao, Naga), a change in serotype dominance corresponded with increase in AR; however, this was not observed in others (Tuguegarao, Manila). We also predicted the annual ARs according to the mean age of case reports with warning signs that also highlighted spatio-temporal heterogeneity in FOI, yet was less consistent with the overall AR according to the catalytic model (Additional file 10).

Discussion

We explored methods to routinely describe dengue transmission intensity among cities across the Philippines. Using data from surveillance operations, we

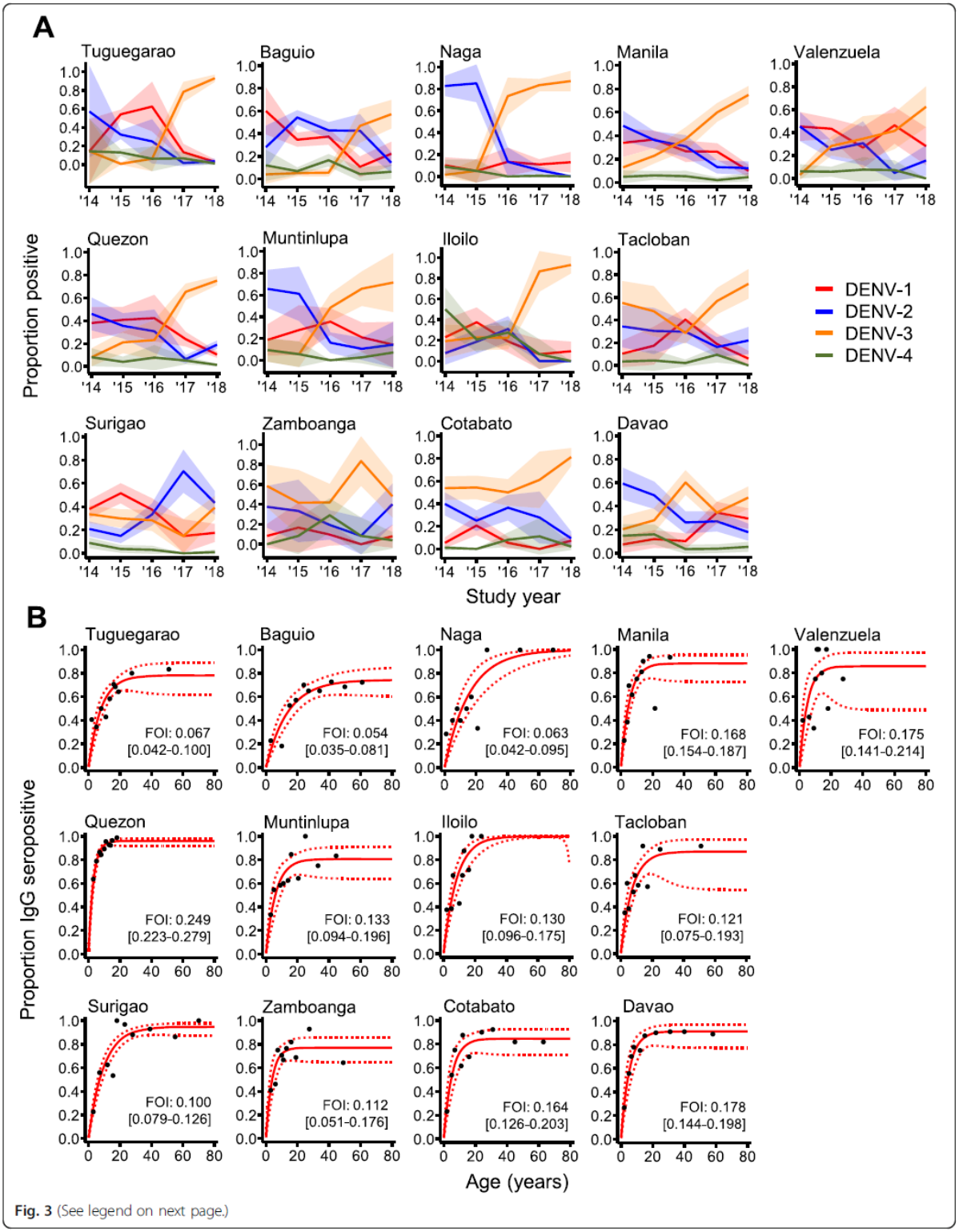

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 City-level immuno-epidemiological characteristics of the reporting dengue population between 2014 and 2018. **A** The percentage of PCR-positive patients who are DENV-1/2/3/4-positive. Shading: 95% CIs. **B** The dengue FOI in 13 cities across the Philippines aggregated between 2014 and 2018. FOI corresponds to the average annual seroconversion rate calculated among non-active dengue cases (historical and negative dengue cases). Black dots: observed age-seroprevalence. Red lines: fitted age-seroprevalence with 95%CIs (red dash)

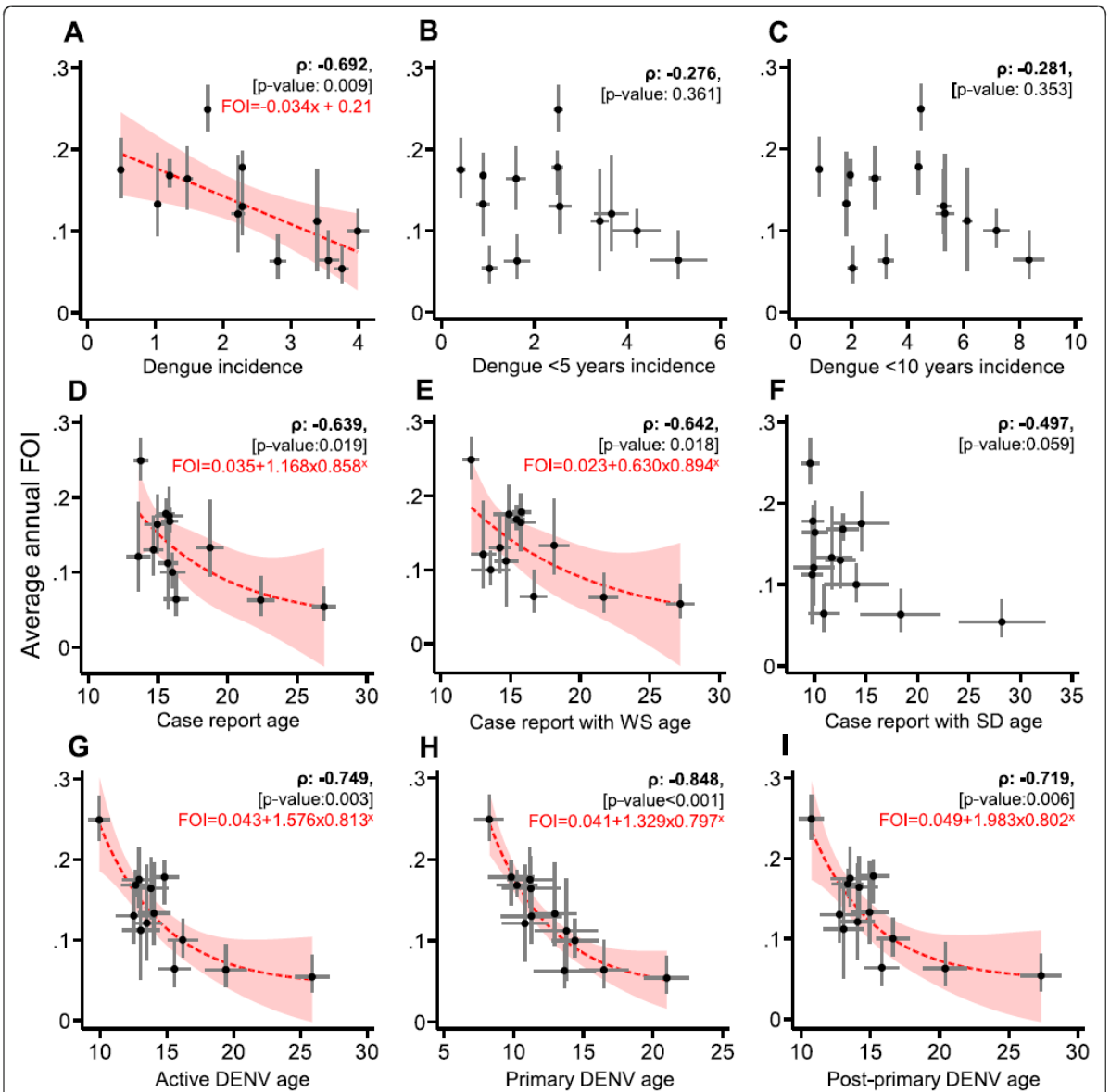

Fig. 4 The city and study period aggregated association between the average annual FOI and surveillance metrics. **A** Crude incidence. **B** Under five incidence. **C** Under 10 incidence. **D** Mean age of case reports. **E** Mean age of case reports with warning signs. **F** Mean age of case reports with severe dengue. **G** Mean age of active infections. **H** Mean age of primary dengue infections. **I** Mean age of post-primary dengue infections. *ρ* Pearson's R. **A**–**F** Data from passive surveillance. **G**–**I** Data from laboratory surveillance. Red dash: predicted FOI according to regression models for metrics with statistically significant associations with FOI (***ρ***, *p* value>0.05). Linear regression models were favoured for crude incidence while exponential regression models had superior model fits for mean case report age, case report with warning signs age, active DENV age, primary DENV age and post-primary DENV age (Lrtest, *p* value<0.05). *X* refers to the annual mean surveillance metric

Table 2 The annual predicted dengue attack rates among sampled cities between 2014 and 2018 according to the mean age of laboratory confirmed primary dengue infections. 95%CIs correspond to the upper and lower 95%CIs of the annual mean age of primary infections. Attack rates according to catalytic models were derived from FOI estimates from reversible catalytic models

City	Predicted annual attack rate (AR) [#] [a]												Attack rate (AR) [#] [b] (Catalytic model)	
	2014		2015		2016		2017		2018		2014-18 average			
	AR	[95%CI]	AR	[95%CI]	AR	[95%CI]	AR	[95%CI]	AR	[95%CI]	AR	[95%CI]	AR	[95%CI]
Tuguegarao	0.15	[0.08-0.31]	0.08	[0.07-0.10]	0.13	[0.09-0.20]	0.11	[0.08-0.17]	0.09	[0.07-0.13]	0.10	[0.09-0.12]	0.06	[0.04-0.10]
Baguio	0.04	[0.04-0.05]	0.04	[0.04-0.05]	0.04	[0.04-0.05]	0.05	[0.04-0.06]	0.07	[0.05-0.10]	0.05	[0.05-0.06]	0.05	[0.03-0.08]
Valenzuela	0.15	[0.10-0.23]	0.13	[0.10-0.19]	0.19	[0.11-0.33]	0.12	[0.09-0.17]	0.14	[0.11-0.19]	0.14	[0.11-0.18]	0.16	[0.13-0.19]
Quezon	0.17	[0.12-0.26]	0.23	[0.16-0.33]	0.21	[0.12-0.37]	0.21	[0.17-0.27]	0.22	[0.18-0.27]	0.22	[0.18-0.26]	0.22	[0.20-0.24]
Manila	0.22	[0.14-0.34]	0.27	[0.20-0.36]	0.14	[0.10-0.20]	0.14	[0.11-0.20]	0.10	[0.08-0.14]	0.16	[0.13-0.20]	0.15	[0.14-0.17]
Muntinlupa	0.11	[0.07-0.18]	0.22	[0.14-0.35]	0.18	[0.12-0.29]	0.07	[0.05-0.10]	0.10	[0.07-0.15]	0.11	[0.09-0.13]	0.12	[0.09-0.18]
Naga	0.05	[0.04-0.06]	0.13	[0.08-0.25]	0.19	[0.12-0.31]	0.11	[0.08-0.16]	0.09	[0.07-0.14]	0.10	[0.08-0.13]	0.06	[0.04-0.09]
Iloilo	0.19	[0.12-0.33]	0.17	[0.12-0.24]	0.21	[0.14-0.31]	0.12	[0.08-0.20]	0.06	[0.05-0.09]	0.13	[0.10-0.19]	0.12	[0.09-0.16]
Tacloban	0.08	[0.06-0.12]	0.14	[0.09-0.24]	0.21	[0.15-0.28]	0.19	[0.14-0.27]	0.17	[0.12-0.25]	0.14	[0.11-0.19]	0.11	[0.07-0.18]
Surigao	0.10	[0.08-0.14]	0.09	[0.07-0.12]	0.08	[0.06-0.11]	0.11	[0.08-0.19]	0.06	[0.05-0.08]	0.09	[0.07-0.11]	0.10	[0.08-0.12]
Davao	0.15	[0.10-0.21]	0.14	[0.10-0.22]	0.18	[0.13-0.27]	0.21	[0.14-0.33]	0.16	[0.11-0.22]	0.17	[0.13-0.22]	0.16	[0.13-0.18]
Cotabato	0.07	[0.05-0.10]	0.12	[0.08-0.18]	0.28	[0.20-0.39]	0.10	[0.07-0.16]	0.18	[0.12-0.28]	0.13	[0.10-0.19]	0.15	[0.12-0.18]
Zamboanga	0.04	[0.04-0.04]	0.10	[0.06-0.21]	0.15	[0.10-0.25]	0.19	[0.12-0.32]	0.30	[0.19-0.46]	0.09	[0.07-0.13]	0.11	[0.05-0.16]

[#]Attack rate (AR): $1\text{-}exp^{(-FOI)}$

[a]Estimated annual attack rate according to the FOI derived from the mean primary DENV age (FOI=0.041+1.329×0.797×), where *x* equals the annual mean primary dengue age

[b]Estimated annual attack rate according to the FOI derived from the reversible catalytic model, Eq. 2, where FOI equals λ

Attack rate

0.25-0.30 0.20-0.25 0.15-0.20 0.10-0.15 0.05-0.10 0.05-0.10

found the mean age of laboratory-confirmed primary dengue infections and the mean age of case reports with warnings signs, to a lesser extent, represented suitable surrogate indicators of the FOI. Both these easily computed metrics correlated better than crude and age-stratified incidence with the FOI estimated from seroprevalence data. Our results highlight prominent spatio-temporal heterogeneity in dengue serotype dominance and reported immune status and the FOI across urban centres in the Philippines.

It is well documented that secondary, opposed to primary, dengue infections are at a greater risk of developing severe disease [20–22], yet we found those categorised as post-primary infections were only slightly more likely to present with severe disease than primary infections. This made us suspect that some of those classified as post-primary were tertiary and quaternary dengue infections, given these types of infections are thought to be less severe than secondary infections, although it is still widely assumed post-secondary infections are often asymptomatic [2, 23]. Furthermore, we identified all four serotypes of dengue in every city almost every year making it plausible for individuals residing in urban centres to suffer multiple dengue infections over their lifetime. Lastly, we found that younger, opposed to older, post-primary dengue infections were at greater risk of presenting with severe disease. A trend possibly attributed to younger and older post-primary infections representing secondary and post-secondary infections, respectively, as shown in [2], although this may also be attributable to a different disease presentation in older people.

Our city FOI estimates, aggregated over 5 years and generated from catalytic models, were comparable to a 2016 estimate in Cebu (central Philippines) [23] and revealed a long-term spatial heterogeneity in dengue transmission intensity, similarly shown in China [24] and Colombia [25]. We found catalytic models fit with sero-reversion parameters resulted in superior model fits despite sero-reversion being very low across sampled cities. This is consistent with previous findings that documented a minority of dengue cases, who were likely exposed once, experienced IgG waning [17, 18]. To determine whether the FOI could be estimated from data routinely collected by dengue surveillance operations, we explored the association between FOI, estimated from catalytic models, against various dengue surveillance metrics. Surprisingly, aggregated at the city level and over the study period, all-age reported incidence was higher in lower FOI areas. This trend however has previously been reported in Singapore [26] and was thought to be attributed to an age shift in disease burden. In low transmission areas, individuals are more likely to experience a dengue infection at a later stage in their life when they may have heightened individual disease awareness and possibly easier access to care. Consequently, low transmission areas may experience increased case reporting. In addition, this trend could also be explained by reduced health-seeking behaviour in high FOI cities wherein individuals refrain from

reporting with symptoms as they are aware of many others with this often mild, self-limiting disease. In contrast in low FOI areas, individuals may be more prompted to seek care as they less familiar with the disease and are more concerned by unfamiliar symptoms. However, future population KAP (knowledge attitudes and practises) surveys in high- and low-transmission settings would be valuable in better describing these health-seeking patterns. Finally, this disparity could partially be explained by the FOI and incidence being measures of infection and disease, respectively. FOI estimates also include those asymptomatic with dengue who are thought to account for approximately 75% of all dengue cases and would unlikely seek treatment [27]. More surprisingly however, after stratifying incidence by age, we observed no association with the FOI which contradicts findings in [9]. We speculate that variable healthcare access and reporting across the country may account for this irregularity and remains an area of continued investigation. Together, these findings suggest that simply allocating control interventions to areas with elevated reported incidence might exclude regions with higher burdens of infection.

In cities, we found that the average age of reported dengue infections with clinical dengue outcomes increased with decreasing aggregated transmission intensity. Interestingly, the mean age of those reporting with just warning signs of dengue, opposed to severe dengue, proved a superior indicator of FOI. The poor specificity dengue clinical diagnosis is well documented as other febrile infections can present with similar disease manifestations [28–30]. Yet, compared to warning signs, severe disease is a far rarer outcome, particularly among primary and older post-primary infections. Moreover, in Thailand, reported severe dengue manifestations have been shown to be highly spatially and temporally heterogenous due to climactic factors [31]. Together, these factors likely account for the lack of association between FOI and age of case reports with severe disease.

In contrast to the age of clinically diagnosed dengue patients, the age of laboratory-confirmed dengue cases had a stronger association with the FOI, likely a consequence of excluding other co-endemic febrile infections. Yet, among laboratory confirmed infections, the age of those reporting with primary, rather than post-primary, infections proved a better indicator of FOI. We propose two main factors account for this difference. Age patterns of reporting post-primary infections might be influenced by spatio-temporal imbalances in immune status reporting, observed in this study and previously in Vietnam [32] and Taiwan [33]. In high transmission settings, where a higher proportion of the post-primary reporting population include tertiary/quaternary infections, the mean age would be higher than an equally high transmission setting where most of the post-primary infections were secondary infections. In contrast, the age of reported primary infections remains unaffected by imbalances in immune status reporting. Secondly, studies have shown that Zika, a structurally homologous virus to dengue, elicits cross-reactive IgG that primes individuals for secondary dengue infections [34, 35]. Therefore, spatio-temporal imbalances in potential Zika transmission across the Philippines may similarly impact the age of post-primary, yet not primary, dengue infection reporting. These interactions led us to propose the age at which individuals report with their first dengue infection is the better surrogate of transmission intensity which we used to estimate annual FOI across cities. It should be noted however, given that individuals are classified as primary or post-primary according to antibody titre and ratio thresholds, a minority of the study population with metrics on the cusp of these cut offs may have been misclassified [14].

Upon estimating city level, annual dengue attack rates according to the age of primary dengue infections, we demonstrated that yearly estimates correlated with overall attack rates according to the catalytic model. Patterns in predicted annual attack rates across cities revealed changing patterns in transmission intensity over time, as seen in Singapore [26]. We identified cities where transmission is stable, emerging and decreasing. Information that is critical for national dengue surveillance operations to plan for the future [36]. In Zamboanga, despite a relatively low overall FOI, annual predicted attack rates revealed that transmission intensity increased during the study period, a trend undetected in the collated incidence data. Zamboanga could therefore be earmarked for heightened surveillance activities to better characterise worrying predicted trends in the burden of infection. In Naga, we identified a jump in FOI coinciding with a switch in serotype dominance, yet this was unobserved in other cities. Approximating transmission intensity from the age of reported primary dengue infections has two major benefits. Firstly, existing surveillance operations can generate attack rates quickly and simply. Moreover, as a consequence of the exponential relationship, slight changes in the age of reported primary infections correspond to larger changes in transmission intensity. This enables FOI estimates to be generated at more granular temporal scales, which are more informative than long-term estimates derived from catalytic models. As well as describing dengue transmission intensity patterns, we speculate monitoring age patterns of reported primary cases could assist in dengue vaccine deployment. Currently, the only licenced dengue vaccine, Dengvaxia®, is recommended for individuals aged between 9 and 45 years in endemic areas, presumably as these likely include those with prior dengue exposure

[37]. Our results highlight the spatio-temporal heterogeneity in the age patterns of those reporting with their first infection; therefore, monitoring the age of these infections could potentially help identify suitable age ranges in specific locations to receive vaccination, although individual pre-vaccination testing would still be necessary and this requires legislative changes.

There are limitations associated with these findings. FOI estimates were generated from reporting, non-active, dengue infections with/without previous dengue IgG exposure. Consequently, we may have oversampled individuals who were more prompted to seek health care if they experienced a prior dengue infection. Yet, only 25% of dengue infections are thought to experience symptoms that would prompt them to seek care [27], and we concluded this health-seeking bias was minimal. Secondly, our fitted catalytic model assumes serotypes contributed equally to transmission over time. Still, given we identified every serotype in each city and that serotype dominancy changed rapidly over the 5-year sampling period, it is reasonable to assume that over the previous decades, from which the FOI is estimated, periodic serotype dominancy may have resulted in serotypes contributing equally to overall transmission. Lastly, our analysis confirms the age of primary dengue infections is associated with FOI at the city level, yet it remains unconfirmed as to whether the age of reported dengue cases accurately represent transmission intensity at lower administrative levels where patients are more likely to cross administrative borders for care. Similarly, our analysis focused on revealing annual trends in dengue transmission intensity. It remains unknown whether seasonal biases may influence the reporting age of primary dengue infections at more granular temporal scales.

Conclusion

We described methods for estimating city-level dengue transmission intensity using metrics obtained from both national laboratory and non-laboratory dengue surveillance operations. We revealed the mean age of those reporting with primary dengue infections correlated best with the FOI using laboratory data, while the mean age of those reporting with clinically diagnosed warning signs represented the best surrogate of FOI using non-laboratory surveillance data. Our work highlights the importance of laboratory dengue surveillance operations and provides a framework for other dengue-endemic countries to better characterise dengue transmission patterns and combat the global threat of this disease.

Abbreviations

AIC: Akaike information criterion; CI: Confidence interval; Ct: Critical threshold; DENV: Dengue virus; DOH: Department of Health; DRU: Disease reporting unit; ELISA: Enzyme-linked immunosorbent assay; FOI: Force of infection; IgG: Immunoglobulin G; IgM: Immunoglobulin M; OR: Odds ratio; PCR: Polymerase chain reaction; PIDSR: Philippine Integrated Disease Surveillance and Response; SCR: Sero-conversion rate; SRR: Sero-reversion rate; WHO: World Health Organization; ZIKV: Zika virus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12916-021-02101-6.

Additional file 1. Serum samples collected from dengue case reports. The number of serum samples collected from surveyed dengue case reports who reported and resided in 13 cities across the Philippines between 2014 & 2018.

Additional file 2. Population demographics of study-participating cities.

Additional file 3. Methods used to determine primary and post-primary dengue immune status.

Additional file 4. City-aggregated dengue incidence estimates.

Additional file 5. Reported dengue immune status by year and city. The reported primary/post-primary immune status of reporting active dengue infection by year and city across the Philippines between 2014 and 2018. Vertical bars: 95%CI.

Additional file 6. FOI catalytic model comparison. Catalytic model fit comparison of simple versus reversible catalytic model used to estimate FOI among sampled cities. AIC: Akaike information criterion. Lower AIC (bold) indicates superior model fit.

Additional file 7. The city and study period aggregated association between the average annual FOI, according to simple catalytic models, and surveillance metrics. A: crude incidence. B: Under five incidence. C: Under 10 incidence. D: Mean age of case reports. E: Mean age of case reports with warning signs. F: Mean age of case reports with severe dengue. G: Mean age of active infections. H: Mean age of primary dengue infections. I: Mean age of post-primary dengue infections. ρ: Pearson's R. A-F: Data from passive surveillance G-I: Data from laboratory surveillance. Red dash: predicted FOI according to regression models for metrics with statistically significant associations with FOI (ρ, p-value>0.05).

Additional file 8. Mean annual primary dengue age by city. The average annual age of reported primary dengue infections among study-participating cities between 2014 and 2018.

Additional file 9. Mean annual age of cases with dengue warning signs by city. The average annual age of reported dengue cases with warning signs among study-participating cities between 2014 and 2018.

Additional file 10. Annual city Attack rates by city according to the mean age of suspected dengue cases with warning signs.

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Authors' contributions

Study design: AKS, SF, ECLP, MRZC, CDP, JCRH and MLH. Conducted laboratory procedures: MAJR, MAQ and WJ-W. Data collation and supervision: JRB, AKS, KS, MAJR, MAQ, AOT, NLS and FLA. Data analysis: JRB, KS, AKS, OJB, AJK, SF, JCRH and MLH. Manuscript preparation: JRB, AKS, OJB, AJK, MRZC, JCRH and MLH. Manuscript editing and review: The authors read and approved the final manuscript.

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data collection and analysis, decision to publish or preparation of the manuscript.

Availability of data and materials
The datasets used in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Ethical approval for the study was obtained from the institutional ethical review boards of the Research Institute for Tropical Medicine, Philippines (2017-2014) and the London School of Hygiene and Tropical Medicine, UK (17965). Informed consent (assent for those under 18 years) was collected from study participants prior to enrolment. Participating patients gave permission for the use of sera for research purposes. All unique identifiers in the data were removed prior to data acquisition.

Consent for publication
Non-applicable

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK. [2]Department of Virology, Research Institute for Tropical Medicine, Manila, Philippines. [3]Dengue Study Group, Research Institute for Tropical Medicine, Manila, Philippines. [4]Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK. [5]Centre for the Mathematical Modelling of Infectious Diseases, London School of Hygiene and Tropical Medicine, London, UK. [6]Department of Health, Philippine Epidemiology Bureau, Manila, Philippines. [7]Institute of Human Genetics, National Institute of Health, University of the Philippines, Manila, Philippines. [8]Philippine Genome Centre, University of the Philippines, Manila, Philippines.

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Publisher's Note

Appendix 2: Chapter 4 Supplementary material

Additional file 1: Serum samples collected from dengue case reports. The number of serum samples collected from surveyed dengue case reports who reported and resided in 13 cities across the Philippines between 2014 & 2018.

Island Region	City	DRU	Annual serum samples collected (n)					
			2014	2015	2016	2017	2018	Total
Luzon	Tuguegarao	Cagayan Valley Medical Center	15	612	25	88	488	1228
Luzon	Baguio	Baguio General Hospital	49	466	168	136	194	1013
		Pines City Doctors Hospital	1	2	127	0	0	130
Luzon	Naga	Bicol Medical Center	134	75	87	321	87	704
Metro Manila	Manila	San Lazaro Hospital	91	272	200	218	215	996
Metro Manila	Valenzuela	Valenzuela Medical Center	57	69	2	1	6	135
		Valenzuela City General Hospital	28	96	38	63	42	267
Metro Manila	Quezon	Quirino Memorial Hospital	80	98	58	404	798	1438
		Quirino Community Hospital	16	35	0	10	0	61
Metro Manila	Muntinlupa	ospital ng muntinlupa	21	57	20	36	30	164
		Research Institute for Tropical Medicine	40	2	65	30	8	145
Visayas	Iloilo	Western Visayas Medical Center	65	122	176	23	59	445
Visayas	Tacloban	Eastern Visayas Regional Medical Center	75	82	337	292	115	901
Mindanao	Surigao	CARAGA Regional Hosptial	447	386	358	111	221	1523
		Surigao Medical Center	0	85	31	0	0	116
Mindanao	Zamboanga	Zamboanga City Medical Center	66	52	153	63	102	436
Mindanao	Cotabato	Cotabato Regional and Medical Center	175	201	229	68	197	870
Mindanao	Davao	Southern Philippines Medical Center	135	252	258	247	243	1135
		Davao Doctors Hospital	53	0	146	0	0	199
Total			1548	2964	2478	2111	2805	11906

Additional file 2: Population demographics of study-participating cities

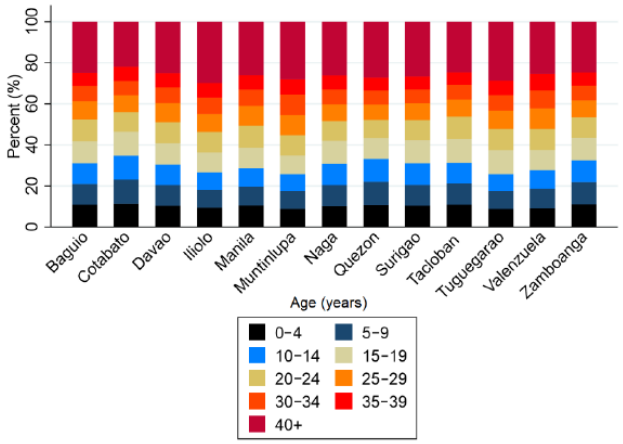
Population demographics of the 13 study participating cities in the Philippines according to the 2015 Philippine census. Source: Philippine Statistics Authority (PSA):

City	Total Population	Median age	Youth DR (<15 years)	Old age DR (>65 years)	% Annual growth rate (2010-15)
Baguio	345366	23.77	47.21	6.13	1.54
Cotabato	299438	21.88	55.62	4.54	1.86
Davao	1632991	24.46	46.31	6.14	2.3
Iliolo	447992	27.08	39.38	8.36	1.03
Manila	1780148	25.37	42.34	5.96	1.43
Muntinlupa	504509	27.68	36.47	5.44	1.78
Naga	196003	24.14	47.16	7.22	2.19
Quezon	2936116	26.39	38.1	5.75	1.17
Surigao	154137	23.91	48.39	7.76	1.77
Tacloban	242089	23.29	48.41	6.5	1.74
Tuguegarao	153502	26.2	37.1	7.19	1.93
Valenzuela	620422	26.09	40.37	5.00	1.45
Zamboanga	861799	23.26	50.98	6.07	1.26

Population age structure of the 13 study-participating cities in the Philippines according to the 2015 Philippine census:

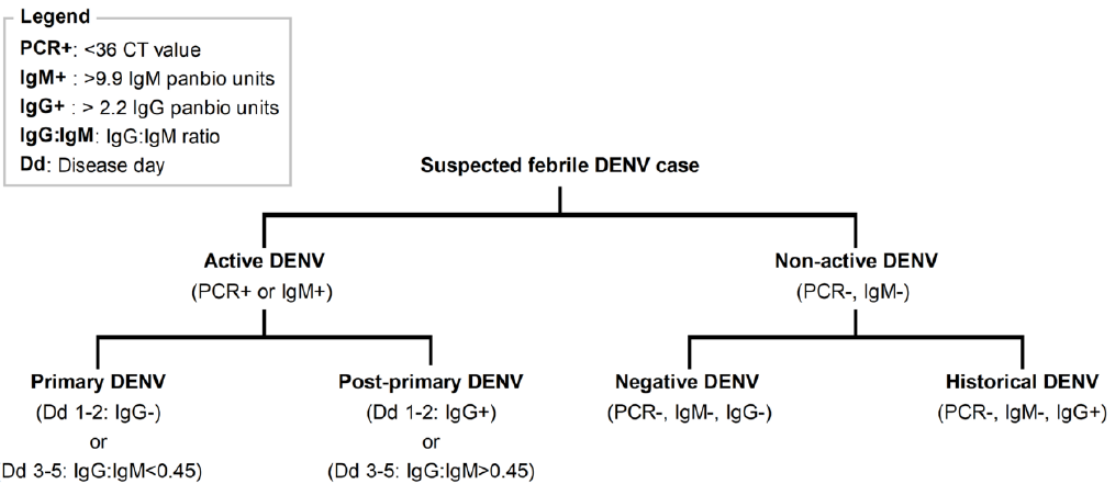
City	Age (years)									Total
	0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>40	
Baguio	37367	34588	34176	36577	36879	29876	25567	22021	88315	345366
Cotabato	34262	35300	34431	34767	29076	24432	20446	20994	65730	299438
Davao	168380	166402	161276	169826	168678	150876	126378	112341	408834	1632991
Iliolo	41946	39230	38238	43482	44468	40014	35486	31488	133640	447992
Manila	186656	164283	157333	177571	191512	170895	144641	123318	463939	1780148
Muntinlupa	44696	43068	41881	45804	50144	49807	50636	37706	140767	504509
Naga	19474	20245	20000	21889	18866	15908	14050	13085	49176	196003
Quezon	260324	259425	257923	296534	315065	282540	245160	219197	799948	2936116
Surigao	15974	15667	16123	17482	15107	12563	10534	9630	41057	154137
Tacloban	26425	24713	24517	28507	25677	20350	17469	14890	59541	242089
Tuguegarao	13462	13267	12739	18100	15949	13433	11730	10733	44089	153502
Valenzuela	55921	58939	57429	60771	63791	61439	54456	49424	158252	620422
Zamboanga	95988	93276	90500	94716	86578	70718	60836	57067	212120	861799

Percentage population age structure of the 13 study-participating cities in the Philippines according to the 2015 Philippine census:



Additional file 3: Methods used to determine primary and post-primary dengue immune status

The primary and post-primary immune status of the sampled reporting dengue population with laboratory data was categorised according to a previous developed algorithm. Suspected dengue patients either PCR positive or with IgM panbio units≥9.9 were classified as active dengue infections, while patients PCR negative and with IgM panbio units<9.9 were considered non-active dengue infections. Among active dengue infections, those on disease day 1 or 2 with IgG panbio units above and below 2.2 were categorised as post-primary and primary, respectively. Active cases on disease 3-5, with IgG:IgM ratios above and below 0.45 were classified as post-primary and primary, respectively. Non-active dengue infections were further classified as historical or negative for dengue if they had IgG panbio units above and below 2.2 panbio units, respectively. Post-primary dengue infections include infections with at least one previous flaviviral infection:



Source: Biggs JR, Sy AK, Brady OJ, *et al.* A serological framework to investigate acute primary and post-primary dengue cases reporting across the Philippines. *BMC Med* 2020; **18**: 364.

Additional file 4: City-aggregated dengue incidence estimates

City/study period-aggregated all age and age-stratified dengue incidence per annum per 1000 population. Case reports include those who resided and reported in the same city. Incidence equates to total number of cases divided by person years at risk (mean population multiplied by 4 years) multiplied by 1000:

All age dengue incidence:

City	DENV case reports (n)					Population[a] (N)					Incidence pa 1k	
	2014	2015	2016	2017	Total	2014*	2015	2016*	2017*	Mean	Rate	[95%CI]
Baguio	345	1647	2723	512	5227	340047.4	345366	350684.6	356085.2	348045.8	3.75	[3.65-3.86]
Cotabato	492	233	858	201	1784	293868.5	299438	305007.5	310680.7	302248.7	1.48	[1.41-1.54]
Davao	5578	2140	5555	1822	15095	1595432	1632991	1670550	1708972	1651986	2.28	[2.25-2.32]
Iloilo	1216	606	1831	459	4112	443377.7	447992	452606.3	457268.2	450311	2.28	[2.21-2.35]
Manila	1157	3075	2349	2122	8703	1754692	1780148	1805604	1831424	1792967	1.21	[1.19-1.24]
Muntinlupa	340	1153	286	331	2110	495528.7	504509	513489.3	522629.4	509039.1	1.04	[0.99-1.08]
Naga	288	499	341	1097	2225	191710.5	196003	200295.5	204681.9	198172.7	2.81	[2.69-2.92]
Quezon	1260	7163	3815	8716	20954	2901763	2936116	2970469	3005223	2953393	1.77	[1.75-1.80]
Surigao	788	510	918	263	2479	151408.8	154137	156865.2	159641.7	155513.2	3.99	[3.83-4.14]
Tacloban	913	379	106	772	2170	237876.7	242089	246301.3	250587	244213.5	2.22	[2.13-2.31]
Tuguegarao	218	1292	126	568	2204	150539.4	153502	156464.6	159484.4	154997.6	3.55	[3.41-3.70]
Valenzuela	225	358	276	370	1229	611425.9	620422	629418.1	638544.7	624952.7	0.49	[0.46-0.52]
Zamboanga	3837	2671	2990	2253	11751	850940.3	861799	872657.7	883653.2	867262.5	3.39	[3.33-3.45]

Under 5 years dengue incidence:

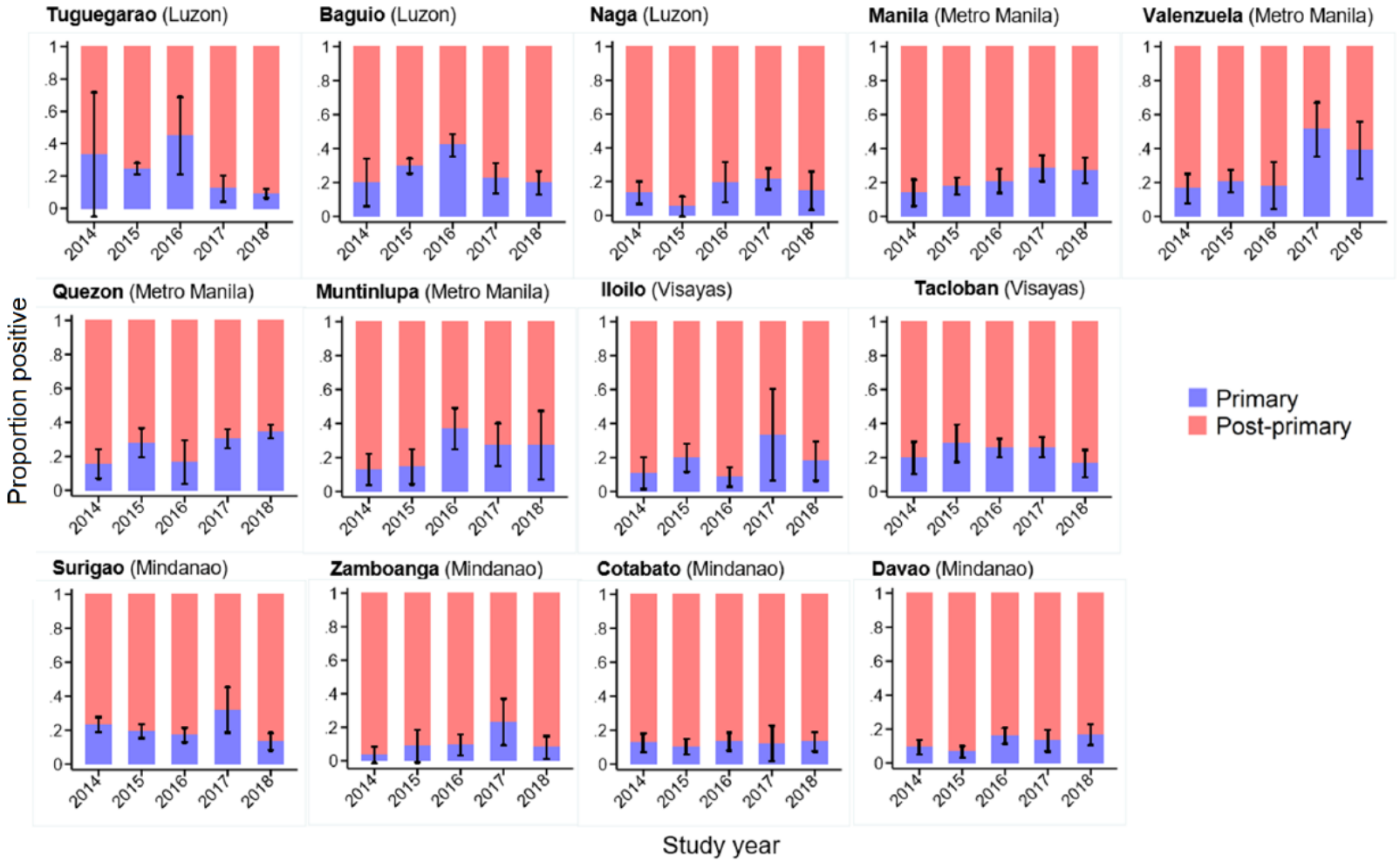
City	DENV case reports (n)					Population[a] (N)					Incidence pa 1k	
	2014	2015	2016	2017	Total	2014*	2015	2016*	2017*	Mean	Rate	[95%CI]
Baguio	8	38	91	18	155	36791.55	37367	37942.45	38526.77	37656.94	1.03	[0.87-1.19]
Cotabato	54	34	104	30	222	33624.73	34262	34899.27	35548.4	34583.6	1.60	[1.39-1.82]
Davao	586	267	635	213	1701	164507.3	168380	172252.7	176214.6	170338.6	2.50	[2.38-2.62]
Iloilo	102	90	163	74	429	41513.96	41946	42378.04	42814.54	42163.13	2.54	[2.30-2.78]
Manila	95	208	199	169	671	183986.8	186656	189325.2	192032.5	188000.1	0.89	[0.82-0.96]
Muntinlupa	23	75	32	30	160	43900.41	44696	45491.59	46301.34	45097.33	0.89	[0.75-1.02]
Naga	14	16	18	80	128	19047.52	19474	19900.48	20336.3	19689.58	1.63	[1.34-1.91]
Quezon	130	724	555	1222	2631	257278.2	260324	263369.8	266451.2	261855.8	2.51	[2.42-2.61]
Surigao	71	53	103	44	271	15691.26	15974	16256.74	16544.48	16116.62	4.20	[3.70-4.70]
Tacloban	94	79	12	205	390	25965.21	26425	26884.8	27352.59	26656.9	3.66	[3.29-4.02]
Tuguegarao	31	144	19	83	277	13202.18	13462	13721.82	13986.65	13593.16	5.09	[4.49-5.69]
Valenzuela	26	17	20	30	93	55110.15	55921	56731.85	57554.47	56329.37	0.41	[0.33-0.50]
Zamboanga	416	264	317	319	1316	94778.55	95988	97197.45	98422.14	96596.53	3.41	[3.22-3.59]

Under 10 years dengue incidence:

City	DENV case reports (n)					Population[a] (N)					Incidence pa 1k	
	2014	2015	2016	2017	Total	2014*	2015	2016*	2017*	Mean	Rate	[95%CI]
Baguio	52	151	324	62	589	70846.89	71955	73063.11	74188.28	72513.32	2.03	[1.87-2.19]
Cotabato	193	89	422	91	795	68268.15	69562	70855.85	72173.77	70214.94	2.83	[2.63-3.03]
Davao	2002	838	2355	752	5947	327082	334782	342482	350359.1	338676.3	4.39	[4.28-4.50]
Iloilo	512	263	711	239	1725	80339.89	81176	82012.11	82856.84	81596.21	5.29	[5.04-5.53]
Manila	341	869	803	747	2760	345920.6	350939	355957.4	361047.6	353466.2	1.95	[1.88-2.02]
Muntinlupa	83	333	100	125	641	86201.8	87764	89326.2	90916.21	88552.05	1.81	[1.67-1.95]
Naga	34	81	60	343	518	38849.15	39719	40588.85	41477.74	40158.69	3.22	[2.95-3.50]
Quezon	516	2719	1857	4261	9353	513667.9	519749	525830.1	531982.3	522807.3	4.47	[4.38-4.56]
Surigao	281	175	341	119	916	31080.95	31641	32201.05	32771	31923.5	7.17	[6.71-7.64]
Tacloban	327	208	42	523	1100	50248.2	51138	52027.8	52933.08	51586.77	5.33	[5.02-5.65]
Tuguegarao	76	470	55	299	900	26213.13	26729	27244.87	27770.7	26989.42	8.34	[7.79-8.88]
Valenzuela	76	117	88	109	390	113194.5	114860	116525.5	118215.1	115698.8	0.84	[0.76-0.93]
Zamboanga	1441	959	1238	1033	4671	186879.3	189264	191648.7	194063.5	190463.9	6.13	[5.96-6.31]

a: Population according to 2015 Philippine census

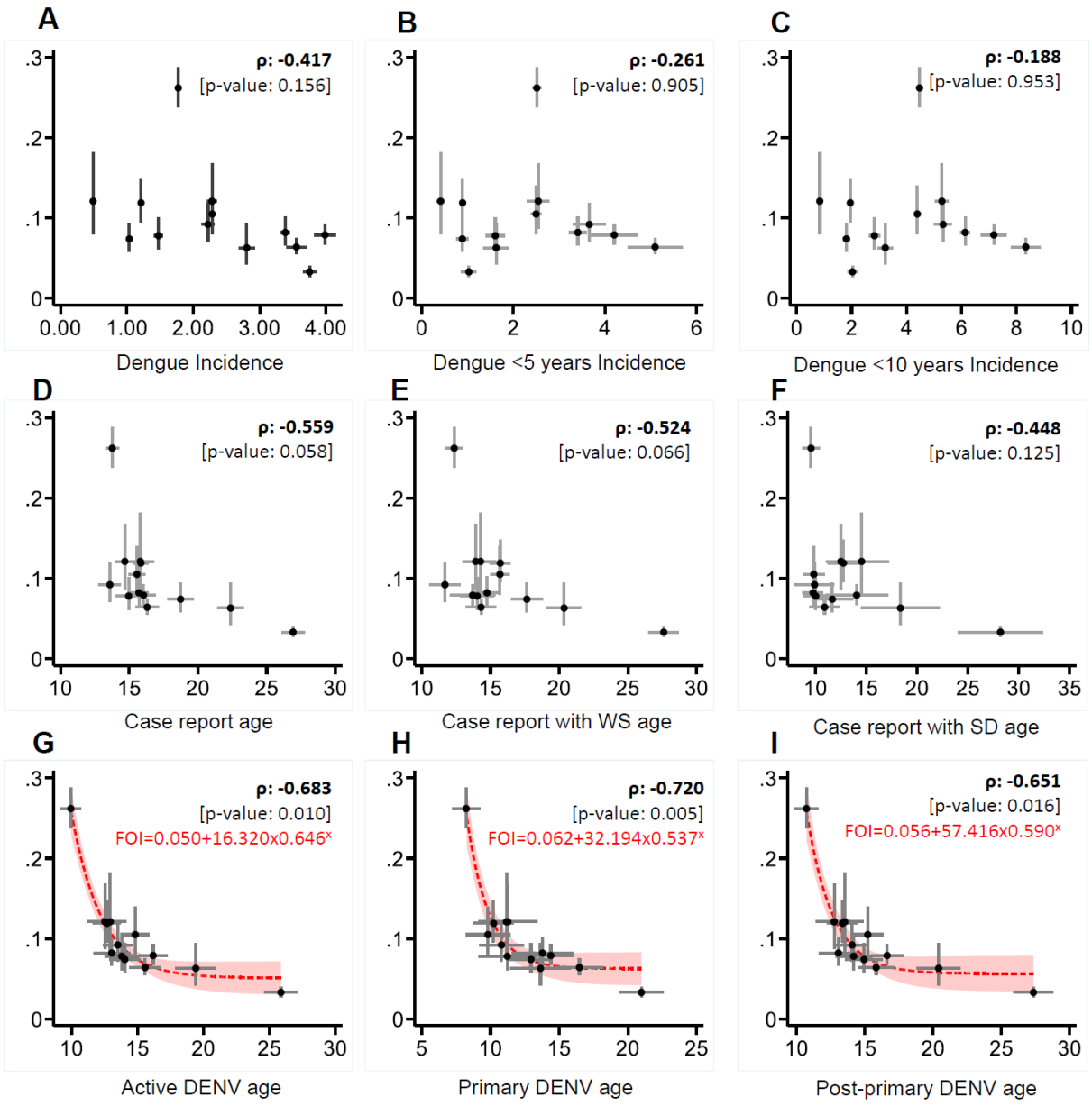
*: Estimated population according to city-specific annual growth rates between 2010 & 2015.



Additional file 5: Reported dengue immune status by year and city. The reported primary/post-primary immune status of reporting active dengue infection by year and city across the Philippines between 2014 and 2018. Vertical bars: 95%CI.

Addition file 6: FOI catalytic model comparison. Catalytic model fit comparison of simple versus reversible catalytic model used to estimate FOI among sampled cities. AIC: Akaike information criterion. Lower AIC (bold) indicates superior model fit.

City	Simple catalytic model			Reverse catalytic model				
	SCR	[95%CI]	AIC	SCR	[95%CI]	SRR	[95%CI]	AIC
Baguio	0.033	[0.027 - 0.04]	268.83	0.054	[0.035 - 0.081]	0.018	[0.007 - 0.048]	**244.30**
Cotabato	0.078	[0.061 - 0.101]	144.35	0.164	[0.126 - 0.203]	0.030	[0.009 - 0.056]	**127.51**
Davao	0.105	[0.08 - 0.14]	131.96	0.178	[0.144 - 0.198]	0.017	[0.005 - 0.035]	**111.40**
Iloilo	0.121	[0.087 - 0.168]	80.31	0.130	[0.096 - 0.175]	<0.001	[0.000 - <0.001]	**78.32**
Manila	0.119	[0.095 - 0.148]	171.75	0.168	[0.154 - 0.187]	0.022	[0.006 - 0.039]	**164.42**
Muntinlupa	0.074	[0.058 - 0.094]	159.38	0.133	[0.094 - 0.196]	0.032	[0.009 - 0.062]	**144.05**
Naga	0.063	[0.042 - 0.094]	129.39	0.063	[0.042 - 0.095]	<0.001	[0.000 - <0.001]	**112.41**
Quezon	0.262	[0.238 - 0.288]	303.29	0.249	[0.223 - 0.279]	<0.001	[0.000 - <0.001]	**291.09**
Surigao	0.079	[0.067 - 0.093]	269.10	0.100	[0.079 - 0.126]	0.006	[0.002 - 0.011]	**251.00**
Tacloban	0.092	[0.071 - 0.119]	168.12	0.121	[0.075 - 0.193]	0.018	[0.002 - 0.047]	**155.25**
Tuguegarao	0.064	[0.055 - 0.075]	142.35	0.067	[0.042 - 0.100]	0.021	[0.004 - 0.056]	**115.11**
Valenzuela	0.121	[0.08 - 0.182]	133.84	0.175	[0.141 - 0.214]	0.029	[0.002 - 0.063]	**118.66**
Zamboanga	0.082	[0.066 - 0.102]	214.52	0.112	[0.051 - 0.176]	0.061	[0.022 - 0.112]	**180.43**



Additional file 7: The city and study period aggregated association between the average annual FOI, according to simple catalytic models, and surveillance metrics. **A**: crude incidence. **B**: Under five incidence. **C**: Under 10 incidence. **D**: Mean age of case reports. **E**: Mean age of case reports with warning signs. **F**: Mean age of case reports with severe dengue. **G**: Mean age of active infections. **H**: Mean age of primary dengue infections. **I**: Mean age of post-primary dengue infections. ρ: Pearson's R. A-F: Data from passive surveillance G-I: Data from laboratory surveillance. Red dash: predicted FOI according to regression models for metrics with statistically significant associations with FOI (**ρ**, p-value<0.05).

Additional file 8: Mean annual primary dengue age by city. The average annual age of reported primary dengue infections among study-participating cities between 2014 and 2018.

City	Mean primary DENV age (years)											
	2014		2015		2016		2017		2018		2014-18	
	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]
Baguio	26.6	[22.1-31.1]	25.2	[23.1-27.2]	24.8	[22.5-27.2]	21.9	[18.6-25.1]	16.9	[13.7-20.2]	21.0	[19.4-22.6]
Cotabato	17.1	[13.3-20.8]	12.2	[9.3-15.1]	6.8	[4.7-8.8]	13.0	[10.1-15.9]	9.4	[6.7-12.0]	11.2	[9.1-13.3]
Davao	10.8	[8.4-13.1]	10.8	[8.1-13.5]	9.3	[7.1-11.6]	8.3	[5.7-10.9]	10.3	[8.1-12.5]	9.8	[8.2-11.4]
Iloilo	9.0	[5.7-12.3]	9.8	[7.6-11.9]	8.5	[6.1-10.9]	12.2	[8.7-15.7]	18.3	[14.0-22.5]	11.3	[9.1-13.4]
Manila	8.2	[5.6-10.8]	7.0	[5.3-8.7]	11.0	[8.8-13.2]	10.8	[8.8-12.8]	13.2	[11.1-15.4]	10.2	[8.8-11.7]
Muntinlupa	12.7	[9.6-15.9]	8.1	[5.5-10.8]	9.2	[6.5-12.0]	16.9	[13.3-20.4]	13.5	[10.5-16.5]	13.0	[11.4-14.5]
Naga	22.2	[17.9-26.5]	11.3	[7.5-15.0]	9.2	[6.0-12.3]	12.6	[10.0-15.2]	13.8	[11.0-16.7]	13.6	[11.4-15.8]
Quezon	9.6	[7.2-12.1]	7.9	[5.8-10.0]	8.6	[5.0-12.2]	8.4	[7.0-9.8]	8.2	[7.0-9.3]	8.2	[7.2-9.3]
Surigao	13.2	[11.1-15.3]	14.5	[12.1-16.8]	15.1	[12.6-17.6]	12.4	[9.0-15.8]	18.4	[15.5-21.3]	14.4	[12.7-16.1]
Tacloban	15.5	[12.0-19.0]	10.9	[7.7-14.1]	8.6	[6.8-10.4]	8.9	[7.0-10.9]	9.8	[7.4-12.2]	10.8	[9.2-12.4]
Tuguegarao	10.6	[6.2-14.9]	15.1	[13.1-17.1]	11.4	[8.7-14.2]	12.5	[9.6-15.4]	13.8	[11.3-16.3]	13.3	[12.1-14.5]
Valenzuela	10.8	[7.9-13.6]	11.3	[9.2-13.5]	9.2	[5.7-12.6]	11.9	[9.6-14.2]	10.9	[8.9-12.9]	11.2	[9.5-12.8]
Zamboanga	27.5	[26.1-28.9]	13.7	[8.5-18.8]	10.5	[7.5-13.6]	9.0	[6.0-12.0]	6.4	[3.7-9.1]	13.8	[11.6-16.0]

Additional file 9: Mean annual age of cases with dengue warning signs by city. The average annual age of reported dengue cases with warning signs among study-participating cities between 2014 and 2018.

City	Mean age of dengue cases with warning signs (years)											
	2014		2015		2016		2017		2018		2014-18	
	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]	Mean	[95%CI]
Tuguegarao	11.0	[7.1-14.9]	15.1	[13.8-16.4]	11.8	[9.4-14.2]	11.1	[9.2-13.0]	18.1	[16.5-19.7]	16.3	[15.1-17.5]
Baguio	21.4	[17.6-25.2]	29.8	[27.2-32.5]	29.0	[26.7-31.2]	24.2	[21.7-26.6]	22.3	[19.7-24.9]	25.4	[23.5-27.2]
Valenzuela	12.9	[11.2-14.7]	11.5	[10.1-12.9]	15.2	[12.9-17.6]	12.6	[10.9-14.3]	15.1	[12.7-17.5]	12.7	[11.4-14.0]
Quezon	12.6	[10.5-14.7]	10.7	[9.1-12.4]	15.5	[13.0-18.1]	9.4	[8.4-10.4]	9.5	[8.6-10.5]	9.9	[9.1-10.7]
Manila	10.2	[8.6-11.7]	10.8	[9.6-12.0]	12.5	[11.0-14.0]	14.2	[12.6-15.7]	15.7	[14.1-17.2]	13.1	[12.1-14.2]
Muntinlupa	13.0	[9.9-16.1]	16.4	[13.6-19.1]	12.2	[10.1-14.4]	14.5	[12.4-16.7]	14.8	[12.0-17.7]	13.9	[12.2-15.6]
Naga	22.7	[20.4-24.9]	21.0	[18.5-23.4]	19.9	[17.3-22.4]	18.4	[16.5-20.4]	13.4	[11.5-15.4]	18.2	[16.6-19.8]
Iloilo	9.9	[8.1-11.6]	10.6	[9.2-12.0]	12.8	[11.3-14.3]	8.4	[6.3-10.5]	15.8	[13.5-18.1]	12.4	[11.1-13.6]
Tacloban	25.0	[19.6-30.4]	15.1	[11.7-18.5]	11.6	[10.1-13.1]	11.5	[9.0-14.1]	12.5	[10.0-15.0]	11.9	[10.4-13.3]
Surigao	12.5	[10.5-14.5]	18.8	[15.3-22.3]	12.3	[8.4-16.1]	15.8	[12.8-18.8]	13.3	[11.2-15.5]	18.0	[14.7-21.3]
Davao	12.7	[11.0-14.3]	14.8	[13.0-16.6]	14.0	[12.5-15.4]	16.1	[14.3-18.0]	11.9	[10.5-13.2]	14.8	[13.6-16.0]
Cotabato	13.6	[11.3-16.0]	16.9	[14.9-18.8]	12.0	[10.3-13.7]	18.5	[15.6-21.4]	13.6	[11.8-15.5]	15.5	[14.0-17.0]
Zamboanga	16.6	[14.1-19.1]	11.2	[8.5-13.8]	12.4	[10.8-14.0]	10.3	[8.7-11.9]	11.0	[9.1-12.8]	11.8	[10.4-13.1]

Additional file 10: Annual city Attack rates by city according to the mean age of suspected dengue cases with warning signs

City	Predicted annual attack rate*[a]						Attack rate (AR)[b]
	2014	2015	2016	2017	2018	2014-18 average	(catalytic model)
	AR [95%CI]	AR [95%CI]	AR [95%CI]	AR [95%CI]	AR [95%CI]	AR [95%CI]	AR [95%CI]
Tuguegarao	0.19 [0.13-0.26]	0.13 [0.12-0.15]	0.17 [0.14-0.22]	0.18 [0.16-0.22]	0.10 [0.09-0.12]	0.12 [0.11-0.13]	0.06 [0.04-0.10]
Baguio	0.08 [0.06-0.10]	0.04 [0.04-0.05]	0.05 [0.04-0.05]	0.06 [0.05-0.08]	0.07 [0.06-0.09]	0.06 [0.05-0.07]	0.05 [0.03-0.08]
Valenzuela	0.16 [0.13-0.18]	0.18 [0.16-0.20]	0.13 [0.10-0.16]	0.16 [0.14-0.19]	0.13 [0.11-0.16]	0.16 [0.14-0.18]	0.16 [0.13-0.19]
Quezon	0.16 [0.13-0.20]	0.19 [0.16-0.22]	0.13 [0.10-0.16]	0.22 [0.20-0.24]	0.21 [0.20-0.23]	0.21 [0.19-0.22]	0.22 [0.20-0.24]
Manila	0.20 [0.18-0.23]	0.19 [0.17-0.21]	0.16 [0.14-0.19]	0.14 [0.12-0.16]	0.12 [0.11-0.14]	0.15 [0.14-0.17]	0.15 [0.14-0.17]
Muntinlupa	0.16 [0.12-0.21]	0.12 [0.09-0.15]	0.17 [0.14-0.20]	0.14 [0.11-0.16]	0.13 [0.10-0.17]	0.14 [0.12-0.17]	0.12 [0.09-0.18]
Naga	0.07 [0.06-0.08]	0.08 [0.07-0.10]	0.09 [0.07-0.11]	0.10 [0.08-0.12]	0.15 [0.13-0.18]	0.10 [0.09-0.11]	0.06 [0.04-0.09]
Iloilo	0.21 [0.18-0.24]	0.19 [0.17-0.22]	0.16 [0.14-0.18]	0.24 [0.20-0.28]	0.12 [0.10-0.15]	0.17 [0.15-0.19]	0.12 [0.09-0.16]
Tacloban	0.06 [0.04-0.09]	0.13 [0.10-0.18]	0.18 [0.15-0.20]	0.18 [0.14-0.22]	0.16 [0.13-0.20]	0.17 [0.15-0.20]	0.11 [0.07-0.18]
Surigao	0.16 [0.14-0.20]	0.09 [0.07-0.13]	0.17 [0.12-0.24]	0.12 [0.09-0.16]	0.15 [0.13-0.18]	0.10 [0.08-0.13]	0.10 [0.08-0.12]
Davao	0.16 [0.14-0.19]	0.13 [0.11-0.16]	0.14 [0.13-0.16]	0.12 [0.10-0.14]	0.17 [0.15-0.20]	0.13 [0.12-0.15]	0.16 [0.13-0.18]
Cotabato	0.15 [0.12-0.18]	0.11 [0.09-0.13]	0.17 [0.15-0.20]	0.10 [0.08-0.12]	0.15 [0.13-0.17]	0.13 [0.11-0.14]	0.15 [0.12-0.18]
Zamboanga	0.11 [0.09-0.14]	0.18 [0.15-0.23]	0.17 [0.14-0.19]	0.20 [0.17-0.23]	0.19 [0.16-0.22]	0.17 [0.15-0.20]	0.11 [0.05-0.16]

*Attack rate (AR): $1\text{-exp}^{(-FOI)}$

a: Estimated annual attack rate according to mean primary DENV age ($FOI=0.023+0.630x0.852^{x}$)

b: Estimated overall attack rate according to FOI estimated from catalytic model

Attack rate:

0.25-0.30

0.20-0.25

0.15-0.20

0.10-0.15

0.05-0.10

0.05-0.10

Chapter 5. Serological Evidence of Widespread Zika Transmission across the Philippines

An online, full text version of chapter 5 is available at: https://www.mdpi.com/1999-4915/13/8/1441

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646
F: +44 (0)20 7299 4656
www.lshtm.ac.uk

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Surname/Family Name	Biggs		
Thesis Title	Immuno-epidemiological analysis of dengue to enhance surveillance		
Primary Supervisor	Dr Julius Clemence R. Hafalla		

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Serological Evidence of Widespread Zika Transmission across the Philippines

Joseph R. Biggs [1,*], Ava Kristy Sy [2,3], Oliver J. Brady [4,5], Adam J. Kucharski [4,5], Sebastian Funk [4,5], Yun-Hung Tu [6], Mary Anne Joy Reyes [2,3], Mary Ann Quinones [2,3], William Jones-Warner [1], James Ashall [1], Ferchito L. Avelino [7], Nemia L. Sucaldito [7], Amado O. Tandoc [2], Eva Cutiongco-de la Paz [8,9], Maria Rosario Z. Capeding [3,8], Carmencita D. Padilla [8,9], Martin L. Hibberd [1,8,9,†] and Julius Clemence R. Hafalla [1,†]

1 Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK; william.jones-warner@lshtm.ac.uk (W.J.-W.); James.Ashall@lshtm.ac.uk (J.A.); Martin.Hibberd@lshtm.ac.uk (M.L.H.); Julius.Hafalla@lshtm.ac.uk (J.C.R.H.)
2 Department of Virology, Research Institute for Tropical Medicine, Manila 1781, Philippines; avakristysy@gmail.com (A.K.S.); maryannejoyreyes@gmail.com (M.A.J.R.); meannquinones@gmail.com (M.A.Q.); amado.tandocmd@gmail.com (A.O.T.)
3 Dengue Study Group, Research Institute for Tropical Medicine, Manila 1781, Philippines; lerosecap@yahoo.com.ph
4 Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK; Oliver.Brady@lshtm.ac.uk (O.J.B.); Adam.Kucharski@lshtm.ac.uk (A.J.K.); Sebastian.Funk@LSHTM.ac.uk (S.F.)
5 Centre for the Mathematical Modelling of Infectious Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK
6 Department of Molecular Parasitology and Tropical Diseases, Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 11031, Taiwan; jj45862001@gmail.com
7 Department of Health, Philippine Epidemiology Bureau, Manila 1003, Philippines; flavelino.chd5@yahoo.com (F.L.A.); manemia_sucaldito@yahoo.com (N.L.S.)
8 Institute of Human Genetics, University of the Philippines, Manila 1000, Philippines; eccutiongcodelapaz@up.edu.ph (E.C.-d.l.P.); cdpadilla@up.edu.ph (C.D.P.)
9 Philippine Genome Centre, University of the Philippines, Manila 1101, Philippines
* Correspondence: Joseph.Biggs1@lshtm.ac.uk
† These authors contributed to this work equally.

Abstract: Zika virus (ZIKV) exposure across flavivirus-endemic countries, including the Philippines, remains largely unknown despite sporadic case reporting and environmental suitability for transmission. Using laboratory surveillance data from 2016, 997 serum samples were randomly selected from suspected dengue (DENV) case reports across the Philippines and assayed for serological markers of short-term (IgM) and long-term (IgG) ZIKV exposure. Using mixture models, we re-evaluated ZIKV IgM/G seroprevalence thresholds and used catalytic models to quantify the force of infection (attack rate, AR) from age-accumulated ZIKV exposure. While we observed extensive ZIKV/DENV IgG cross-reactivity, not all individuals with active DENV presented with elevated ZIKV IgG, and a proportion of dengue-negative cases (DENV IgG-) were ZIKV IgG-positive (14.3%, 9/63). We identified evidence of long-term, yet not short-term, ZIKV exposure across Philippine regions (ZIKV IgG+: 31.5%, 314/997) which was geographically uncorrelated with DENV exposure. In contrast to the DENV AR (12.7% (95%CI: 9.1–17.4%)), the ZIKV AR was lower (5.7% (95%CI: 3–11%)) across the country. Our results provide evidence of widespread ZIKV exposure across the Philippines and suggest the need for studies to identify ZIKV infection risk factors over time to better prepare for potential future outbreaks.

Keywords: Zika; dengue; serology; diagnostics; force of infection; Philippines

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1. Introduction

Zika is a flavivirus predominantly transmitted by Aedes mosquitoes which typically causes asymptomatic, or occasionally mild self-limited symptomatic, infections in humans. Consequently, previous global Zika outbreaks during the 20th century were underreported, and the disease was of limited public health concern [1]. In 2016, Zika gained global prominence due to an outbreak in Brazil coinciding with an unprecedented rise in severe birth abnormalities [2]. Subsequent studies linked Zika virus infections with Guillain-Barré syndrome [3] and microcephaly in infants [4]. Today, heightened surveillance operations report evidence of autochthonous Zika transmission in approximately 87 countries [1]. However, population exposure rates and transmission patterns at subnational levels remain poorly characterized, at least partially because of the difficulties in distinguishing Zika from other flavivirus infections [5–7].

Similar to other flaviviruses, including dengue, Zika virus (ZIKV) infection in humans is characterized by an initial viremic, followed by an immunogenic phase. A few days post-infection, viremia increases rapidly in hosts, during which time viral RNA is detectable in the blood for a few days [8,9]. Shortly after this peak in viremia, hosts elicit IgM antibodies that likely persist for months post-infection [10]. Approximately a week after the peak in viremia, hosts mount a long-term IgG antibody response that offers protection from successive Zika infections and is thought to be detectable for decades [11]. In contrast, for flaviviral infections caused by dengue virus (DENV), the existence of four serologically distinct serotypes (DENV1-4) means that immunity only offers protection from subsequent homologous, not heterologous, serotypes enabling post-primary (secondary, tertiary, or quaternary) dengue infections [12]. During a secondary infection, previously elicited IgG no longer neutralizes, but instead cross-reacts and surges with the new serotype to trigger the antibody-dependent enhancement (ADE) of viral replication. Increased virus replication during a secondary infection is thought to result in more severe disease because host-elicited cytokine storms can trigger vascular leakage [12–14]. Interestingly, the extensive structural and antigenic homology between ZIKV and DENV has generated speculation as to whether cross-reactive IgG responses from a Zika infection can result in the enhancement of dengue [15]. Indeed, a recent cohort study conducted in Nicaragua revealed that infection by Zika enhances the future risk of severe disease in subsequent DENV-2 infections, comparable to a previous heterologous dengue serotype, suggesting possible ADE mechanisms [16]. A pattern also found in vivo when rhesus macaques, previously infected with ZIKV, experienced higher viremia and proinflammatory cytokines during a subsequent DENV-2 infection compared to those previously uninfected with ZIKV [17]. In contrast, a Brazilian study reported a decline in dengue infections following a Zika outbreak, eluding to cross-protection, not enhancement [18]. However, given that cross-protective ZIKV IgG antibodies wane over time [19], the remaining cross-reactive antibodies may facilitate adverse ADE mechanisms later in life.

With disease presentation largely asymptomatic and with a short window of viral detection, serological diagnosis is crucial for capturing Zika cases. However, cross-reactive antibody responses between ZIKV and DENV present a challenge for differential diagnosis. Numerous commercial serological diagnostic tests have been developed recently, including the Euroimmun (Lübeck, Germany) indirect IgM and IgG ELISAs (enzyme-linked immunosorbent assays), which state that the kits are highly specific for Zika [20]. Recent studies have utilized Euroimmun and have reported Zika specificity >90% [21,22], although the test subjects often included small groups of infected travelers who resided outside flavivirus-endemic countries. More recently, however, the accuracy of these commercial tests has been brought into question by studies in flavivirus-endemic regions, including Salvador (Brazil) [23], Rio de Janerio (Brazil) [24], and Carabobo (Venezuela) [25]. Studies have revealed that IgM ELISAs have adequate specificity, yet poor sensitivity for capturing active ZIKV infections. Conversely, studies have shown that ZIKV IgG ELISAs have favorable sensitivities, yet variable specificities in distinguishing Zika from dengue infections. Moreover, one group demonstrated that ZIKV IgG kits reasonably differentiated Zika

infections from primary dengue infections, yet not secondary dengue infections [24], which may be a potential consequence of ZIKV IgG simply cross-reacting with pre-circulating IgG during a secondary infection elicited from either a prior dengue or Zika infection. This corresponds to findings described by the authors of [26], who evaluated a novel immunomagnetic assay for ZIKV and found that ZIKV IgG was elevated among secondary DENV infections, but not primary DENV infections. Further understanding into how ZIKV IgG responses change during the acute stage of a dengue infection, and of those reporting without active dengue infections, may help distinguish whether patients have experienced prior dengue or Zika infections.

In the Philippines, Zika transmission remains poorly understood. Prior to 2016, isolated reports of confirmed Zika infections among non-travelling individuals in two cities—Quezon City in 2010 [27] and Cebu City in 2012 [28]—eluded to autochthonous transmission rather than imported Zika. Then, in 2016, a total of 47 non-travelling, PCR-confirmed Zika cases were detected after enhanced surveillance operations incorporated fever, rash, arthralgia and conjunctivitis into their case definition [29]. Considering that Zika cases are often asymptomatic and symptomatic infections resemble other co-endemic febrile illnesses, relying on passive case reports likely underestimates the true burden of disease. In Thailand, a recent study revealed evidence of persistent Zika transmission throughout the whole country [6]. We therefore explored whether those reporting with suspected dengue across the Philippines, who are regularly sampled in accordance with existing laboratory surveillance, had evidence of recent or historical Zika exposure.

2. Methods

2.1. Flavivirus Surveillance in the Philippines

Zika and dengue are both notified at the point of care in health facilities, called disease reporting units (DRUs), across the Philippines in accordance with WHO and PIDSR (Philippine Integrated Disease Surveillance and Response) guidelines [30]. Suspected Zika case reports include those presenting with fever, conjunctivitis, skin rash and either of the following: headache, malaise, myalgia, malaise, joint pain, retro-orbital pain, travel to a Zika-reporting area, or a history of Guillain-Barré syndrome. Suspected cases also include infants/fetuses with neurological conditions with unknown etiologies, including reduced occipitofrontal circumference and/or intracranial calcifications. Serum, urine, and placental tissues collected from suspected cases undergo laboratory confirmation at the Research Institute for Tropical Medicine (RITM—Department of Health) and are assayed for anti-ZIKV PCR, IgM and IgG.

For dengue, suspected cases include those reporting with a sudden prolonged febrile illness accompanied by at least two additional symptoms: headache, body malaise, myalgia, arthralgia, nausea, vomiting, diarrhea, flushed skin and rash. All suspected dengue case reports are collated by the country's Epidemiological Bureau. Additional laboratory surveillance, coordinated by the RITM, survey a representative of sample of suspected dengue cases and collect single serum samples at the point of care for further laboratory analysis. Five samples per week are randomly collected from patients who visit sentinel DRUs, which include major regional hospitals. Samples are also collected from those who visit non-sentinel DRUs following a surge of dengue case reporting in accordance with PIDSR criteria. Basic epidemiological data are collected from dengue patients: age, sex, date of symptom onset, date of reporting, health facility/home location (Region, Province, Barangay) and symptoms. Symptoms are categorized as no warning signs, warning signs (vomiting, fluid accumulation, mucosal bleeding, abdominal pain and liver enlargement) and severe dengue (severe plasma leakage, organ impairment and bleeding). Individuals excluded include those under 6 months or those who reported 5 days post-symptom onset.

2.2. Data Collection

For the purposes of this study, we selected a random subset of all dengue serum samples collected in 2016. In total, 1000 viable serum samples out of 3921 (25.5%) were

selected and subjected for further Zika and dengue laboratory analysis. Additional covariates generated included disease day (date of symptom reporting—date of symptom onset), adverse clinical symptoms (severe dengue or warning signs) and urban barangay (>1500 persons per km^2). Barangay population density refers to the 2015 barangay population over the barangay area (km^2) (2015 Philippine census, Philippine Statistics Authority).

2.3. Laboratory Analysis

Patient serum samples were stored at −80 °C at the RITM prior to laboratory analysis. Using the semiquantitative Euroimmun™ indirect ELISA (Lübeck, Germany), we assayed samples for ZIKV IgM and IgG according to manufacturer's instructions (Cat No: EI 2668–9601 M and EI 2668–9601 G). Output ratio values were subsequently termed 'ZIKV IgM/G ELISA values'. ZIKV IgM and IgG ELISA values above 1.1 represented ZIKV IgM and IgG-seropositive samples, respectively. We also assayed samples for anti-DENV viremia, IgM and IgG. Using methods described by the authors of [31], a fourplex, real-time polymerase chain reaction (PCR) assay was used to detect serotype specific RNA to DENV1-4. An output critical threshold value below 36 was used to determine PCR-positive samples. For DENV IgM and IgG, Panbio™ capture indirect ELISA (Alere, Australia) kits were utilized in accordance with manufacturer guidelines (Cat. No: 01PE10 and 01PE20) to detect antibodies specific to any DENV serotype. Output index values were subsequently termed 'DENV IgM/G ELISA values'. DENV IgM and IgG seroprevalence thresholds were previously generated by the authors of [32], corresponding to 0.99 and 0.22 ELISA units (Panbio index values), respectively.

2.4. Data Analysis

We determined individual DENV immune status according to the dengue laboratory and epidemiological data using methods previously described by the authors of [32]. Suspected dengue case reports were initially classified as active (PCR+ or IgM+) or non-active (PCR- and IgM-), as at least one of these markers should be present during an ongoing dengue infection. Active cases were further categorized as primary (IgG- on disease day 1–2 or IgG:IgM ratio < 0.45 on disease day 3–5) or post-primary (IgG+ on disease day 1–2 or IgG:IgM ratio > 0.45 on disease day 3–5). Post-primary cases included those with either secondary, tertiary or quaternary dengue infections with previous exposure to flaviviruses. Non-active dengue infections were further classified as historical (IgG+) or negative (IgG-) for dengue.

Mixture modelling methods were used to determine ZIKV IgM and IgG seroprevalence as described by the authors [32–34] using the 'fmm' command in STATA (v.16). Mixture models were fit to the ZIKV IgM/IgG ELISA value data by maximum likelihood with lognormal titer distributions and two components to characterize the seronegative and seropositive subpopulations. The existence of two components opposed to one subpopulation was justified according to Akaike information criterion (AIC), whereby lower AIC indicates superior model fit. Seroprevalence thresholds correspond to the lowest IgM/G ELISA values with >95% probability of being in the seropositive distribution.

Catalytic models were used to determine the DENV and ZIKV force of infection (and attack rates) across the Philippines among those without active ZIKV or DENV infections as antibody levels are heavily influenced by day of infection [11,32]. Models fitted with maximum likelihood were used to characterize anti-ZIKV/DENV IgG age-seroprevalence and generate seroconversion rates—estimates equivalent to the force of infection. For ZIKV, the seroconversion rate refers to the average annual rate at which the ZIKV-susceptible population (ZIKV IgG-) converts to ZIKV IgG+ status. For DENV, the seroconversion rate refers to the average annual rate at which the unexposed DENV IgG- population converts to DENV IgG+ status to any DENV serotype. Under the rationale that IgG wanes to low/undetectable levels with time, as shown by the authors of [35], we fitted both simple and reversible catalytic models in STATA using the 'revcat' command, which estimates the force of infection parameter using least squares. Consistent with individuals serocon-

verting to IgG+ following infection and remaining seropositive, Equation (1) estimates the probability of being seropositive by age (a) assuming constant force of infection (λ):

$$P(a) = 1 - e^{-\lambda a} \quad (1)$$

In contrast, assuming individuals gradually lose IgG antibodies over time following infection, the reversible model (Equation (2)) fits an additional seroreversion parameter (ρ) to estimate the seroreversion rate: the average annual rate at which individuals serorevert back to IgG- status. AIC (Akaike information criterion) was used to determine whether simple or reversible catalytic models had the best model fit. To estimate the annual risk of ZIKV and DENV infection, FOI rate estimates were converted to attack rates (AR) according to Equation (3) [36].

$$P(a) = \frac{\lambda}{\lambda + \rho}\left[1 - e^{-(\lambda+\rho)a}\right] \quad (2)$$

$$AR = 1 - e^{-(\lambda)} \quad (3)$$

Last, using univariate logistic regression modelling, we investigated whether those with post-primary DENV infections with/without ZIKV IgG exposure were as likely to present to clinics with adverse clinical and severe outcomes than primary DENV infections. We calculated the unadjusted odds ratios of presenting to DRUs with adverse clinical symptoms (warning signs of dengue or severe dengue) and severe dengue among those classified as post-primary dengue with/without Zika IgG exposure compared to primary dengue infections (ZIKV IgG-).

3. Results

3.1. Data Description

We successfully assayed 997/1000 suspected dengue case reports who visited 102 DRUs situated in all 17 regions of the Philippines during 2016 (Figure 1) (Supplementary Materials Table S1). The demographic characteristics of the sampled population are shown in Supplementary Materials Table S2. Overall, most cases were aged between 6 and 15 years (43.3%, 432/997), reported between disease day four and five (51.4%, 512/997) and presented with warning signs of dengue (54.4% 542/997). Of those who reported with suspected dengue, we estimated that 80.1% (794/991) presented with an active DENV infection (PCR+ or IgM+). Among active dengue infections, we classified 23.8% (189/794) as primary and 76.2% (605/794) as post-primary infections.

3.2. ZIKV and DENV Cross-Reactive Antibody Responses

The majority of the sampled population reported with elevated dengue antibody responses, with 70.2% (696/991) DENV IgM+ and 80.7% (800/991) DENV IgG+ (Figure 2A). This infers a high degree of short- and long-term exposure to dengue among the sampled population.

For ZIKV IgM and IgG, seroprevalence thresholds were determined using mixture models. For ZIKV IgG, a two-component, as opposed to a one-component model, best fit the data (AIC difference: −284.2) (Supplementary Materials Figure S1). This generated a new ZIKV IgG seroprevalence threshold of 0.57 ELISA values, which resulted in 31.5% (314/997) of the study population having ZIKV IgG exposure (Figure 2A). For ZIKV IgM, a mixture model fitted with just one, instead of two, components best fit the data (AIC difference: +159.9), as most of the study population reported with very low ZIKV IgM levels. We were therefore unable to determine IgM seroprevalence. Thus, we concluded that, despite a proportion of the study reporting with long-term exposure, no evidence of recent Zika exposure was found in the study population.

Cross-reacting IgG responses between Zika and dengue are shown in Figure 2B. Among those with elevated (seropositive) DENV IgG, most reported with low levels of ZIKV IgG (63.1% (505/800) ZIKV IgG-). Contrastingly, among those with elevated (seropositive) ZIKV IgG, nearly all reported with elevated DENV IgG responses (93.6%

(295/314) DENV IgG positive). This suggests the Panbio IgG ELISA kits detected IgG from either ZIKV or DENV, while Euroimmun IgG ELISA kits were more specific to ZIKV antibodies. Notably, after stratifying by DENV immune status, we found a significantly higher proportion of post-primary (38.0% (95%CI: 4.2–42.0%)) and historical (38.1% (95%CI: 30.2–46.5)) cases were classified as ZIKV IgG-positive compared to primary (12.7% (95%CI: 8.6–18.2%)) and negative (14.3% (95%CI: 7.7–25.0%)) dengue cases. Moreover, despite post-primary cases experiencing higher DENV IgG responses (median: 5.6 (IQR: 3.7–6.2)) than historical cases (median: 0.8 (IQR: 0.5–1.4)), the same proportion of these cases reported ZIKV IgG-seropositive (38%) (Figure 2C). Therefore, if ZIKV IgG was elevated solely due to elevated DENV IgG, then a higher proportion of post-primary than historical cases would be ZIKV IgG positive. However, this was not observed. Last, despite negative cases (clinically misdiagnosed dengue cases) being DENV IgG-negative, 14.3% (9/63) reported with distinctly elevated ZIKV IgG levels (ZIKV IgG-seropositive), which, notably, cannot be attributed to ZIKV/DENV cross-reactivity (Figure 2D).

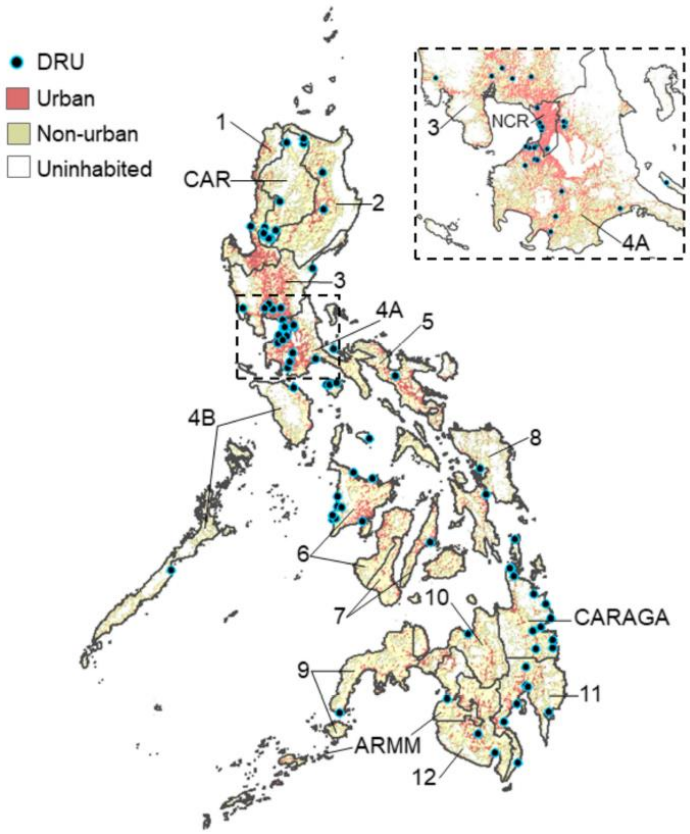

Figure 1. Map of the Philippines showing the location of the 102 DRUs where dengue patients were sampled from across all 17 regions during 2016. Urban zones: >1500 persons per km^2. Non-urban zones: <1500 persons per km^2.

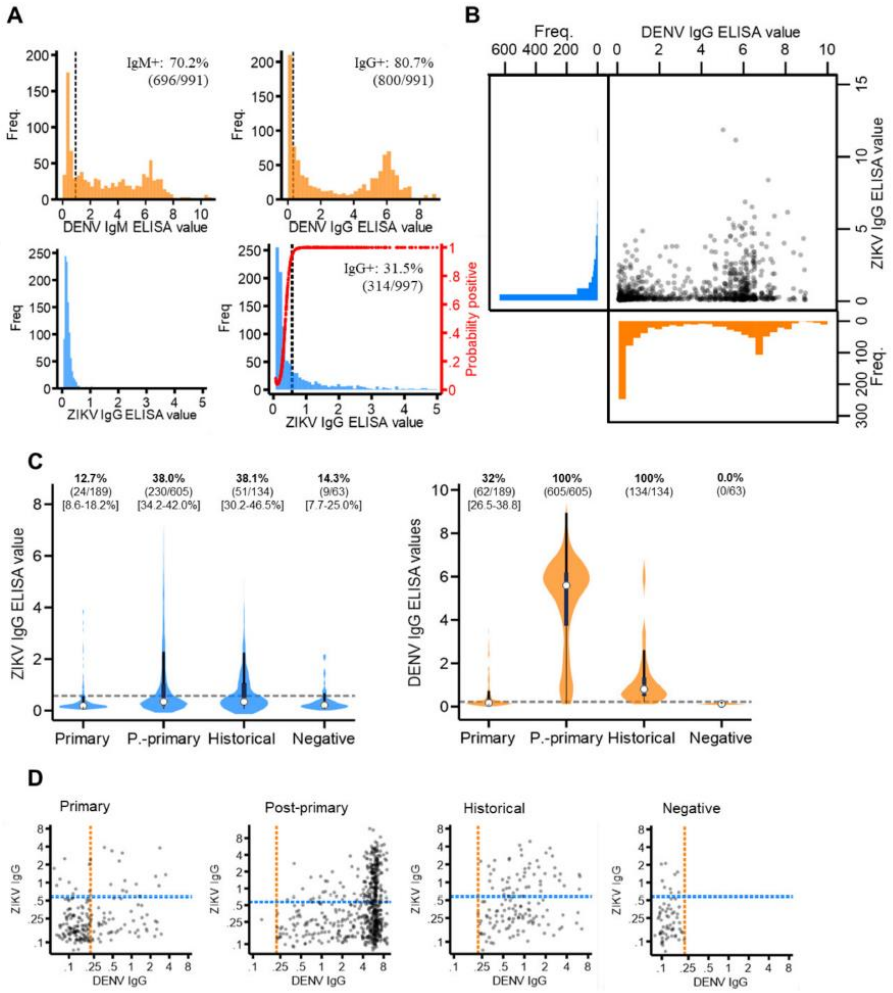

Figure 2. ZIKV and DENV antibody responses among the study population. (**A**) Distribution of ZIKV and DENV IgM and IgG antibody responses (ELISA values) among the study population. Red line: probability of being ZIKV IgG seropositive according to the mixture model. (**B**) Scatterplot of ZIKV versus DENV IgG ELISA values among the study population. (**C**) Violin plots of ZIKV and DENV IgG ELISA values among those classified as DENV primary, post-primary, historical and negative. White circles: median, thick black bar: IQR. Grey dash: IgG seroprevalence thresholds (ZIKV IgG: 0.57 ELISA values, DENV IgG: 0.22 ELISA values). (**D**) Scatterplots of ZIKV versus DENV IgG ELISA values among DENV primary, post-primary, historical and negative cases plotted on a log scale. Orange dash: DENV IgG seroprevalence threshold (0.22 ELISA values). Blue dash: ZIKV IgG seroprevalence threshold (0.57 ELISA values).

3.3. ZIKV Immunoepidemiology in the Philippines

We next investigated how the progression of an active DENV infection influenced ZIKV IgG responses in the study population. Among those reporting with primary, historical or negative DENV infections, we observed no difference in the proportion ZIKV IgG positive by day of disease (Supplementary Materials Figure S2). This suggests that ZIKV IgG levels remained stable during the acute stage of primary DENV infections and in those reporting with historical or negative DENV infections. In contrast, ZIKV IgG positivity significantly increased by disease day among those reporting with post-primary DENV infections, particularly those under 10 years of age (Figure 3A). In total, 14.3% (95%CI:

0.00–35.3%) of post-primary infections under 10 years of age were ZIKV IgG-seropositive between disease days 0–1, which increased to 53.8% (95%CI: 44.7–62.9%) between disease days 4–5. Interestingly, this increasing trend was not observed among older post-primary infections (Figure 3A), but was observed among post-primary infections stratified by serotype, although these differences did not reach statistical significance (Supplementary Materials Figure S1). Together, this infers that ZIKV IgG surges during the acute phase of a post-primary DENV infection, particularly among those who are younger. We then explored how age impacted ZIKV IgG seroprevalence among the study population and found contrasting age-ZIKV IgG seropositivity trends between post-primary and historical DENV cases (Figure 3B). ZIKV IgG seroprevalence decreased with increasing age among post-primary DENV infections, whereby 54.8% (95%CI: 43.1–66.5%) of those under 5 years of age were ZIKV IgG-seropositive, which gradually decreased to 26.8% (95%CI 14.8–38.8%) among those aged between 21–25 years. ZIKV IgG seroprevalence appeared to increase with age among historical cases, although this was not statistically significant (Figure 3B). Taken together, these results show that younger, as opposed to older, age post-primary DENV infections had high levels of ZIKV IgG which surged rapidly during the acute stage of disease.

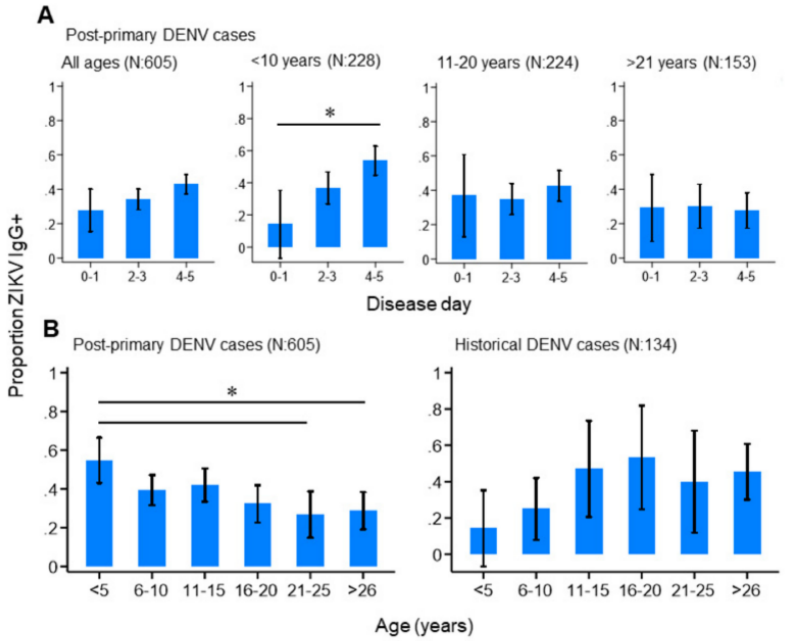

Figure 3. ZIKV IgG seroprevalence patterns among the study population. (**A**) ZIKV IgG seroprevalence by disease day among all, and age-stratified, post-primary dengue cases. (**B**) ZIKV IgG seroprevalence among post-primary and historical dengue cases. Vertical lines: 95%CI (confidence interval), (* non-overlapping 95%CI).

We then explored the spatial patterns in ZIKV and DENV IgG exposure across the Philippines in 2016 among those without active dengue infections, as ZIKV IgG is impacted by changing levels of DENV IgG during an active DENV infection (Figure 4A). We found that ZIKV historical exposure was widespread across the Philippines, with ZIKV IgG+ individuals identified in 15/17 Philippine regions. Moreover, we observed no statistical correlation between regional ZIKV and DENV IgG exposure, inferring that elevated ZIKV IgG is not attributed to higher DENV IgG (ρ: 0.26, p-value: 0.184) (Supplementary Materials Figure S3). We also found further evidence of widespread ZIKV exposure, identifying

seven regions across the Philippines where DENV-negative (DENV IgG-) cases reported as ZIKV IgG-seropositive (Supplementary Materials Table S3). However, numbers were small (a total of 63 negative dengue cases in 11/17 regions across the Philippines).

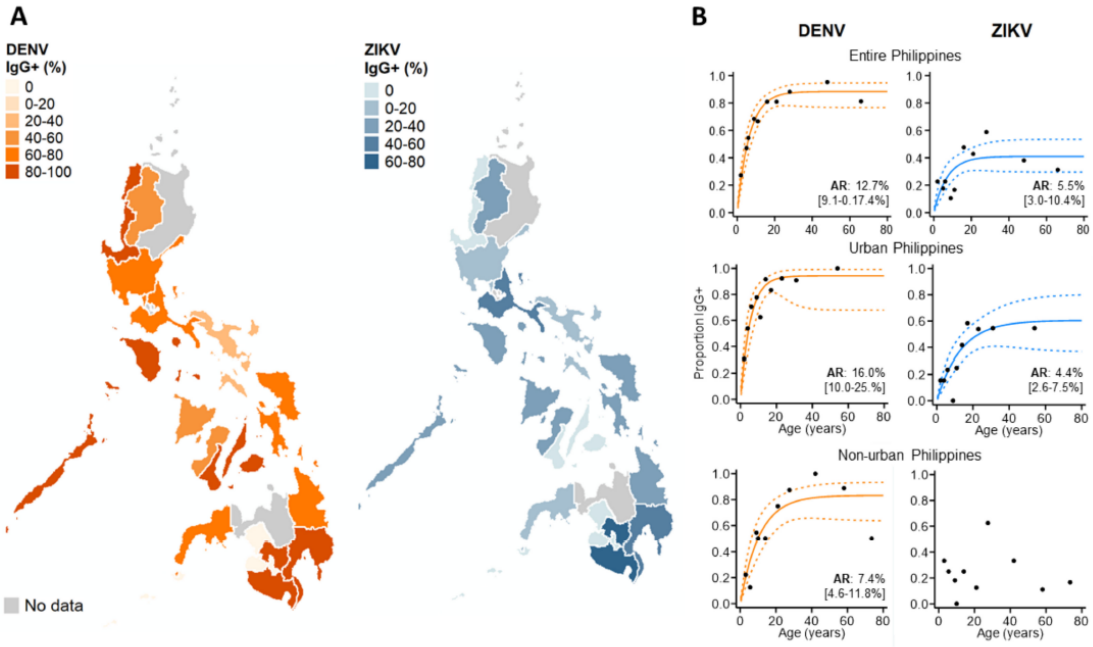

Figure 4. Immunoepidemiology of ZIKV and DENV across the Philippines during 2016. (**A**) Regional ZIKV/DENV IgG seroprevalence among those reporting without active DENV infections. (**B**) ZIKV/DENV age seroprevalence across the Philippines and stratified by urban/non-urban areas. ARs (attack rates) were calculated from the seroconversion rate (SCR) estimated among those without current DENV infections (historical and negative DENV cases) using reverse catalytic models. Black dots: observed age IgG seroprevalence. Curve: predicted age IgG seroprevalence. Dash: 95%CI.

To investigate the DENV and ZIKV annual attack rate, we used catalytic models to characterize age seroprevalence among those reporting without active DENV infections (DENV PCR- & IgM-). According to AIC, reversible, as opposed to simple catalytic models, had superior model fits. For dengue, increasing DENV IgG seroprevalence with age generated an AR estimate which suggests that 12.7% (95% CI: 9.1–17.4%) of the study population became exposed to DENV annually. DENV AR estimates were slightly higher in urban settings (AR: 16.0% (95%CI: 10.0–25.0%)) and lower in non-urban settings (7.4% (95%CI: 4.6–11.8%)). For Zika, the overall AR was lower than for dengue, with an estimated 5.5% (95%CI: 3.0–10.4%) of the study population becoming exposed annually. After stratifying by population density, the Zika FOI remained similar in urban centers across the Philippines. In contrast, we observed no increasing age ZIKV IgG seroprevalence in non-urban areas and were unable to estimate the FOI (Figure 4B).

Last, we explored whether post-primary dengue cases with/without prior ZIKV IgG exposure had a similar risk of adverse clinical/severe symptoms compared to primary DENV infections (Table 1). Adverse clinical symptoms included reporting with either warning signs of dengue or severe dengue disease. Among the 81.6% (814/997) of the study population with symptom data, 73.3% of primary DENV infections, 80.7% of post-primary DENV infections without ZIKV IgG exposure and 87.4% of post-primary DENV infections with ZIKV IgG exposure presented with adverse clinical symptoms. Moreover, compared to primary DENV infections, post-primary infections with prior Zika exposure were statistically more likely to experience adverse clinical outcomes (OR: 2.52 (95%CI:

1.42–4.49), *p*-value: 0.002). However, no such association was identified upon stratifying the outcome by just severe disease. Post-primary DENV infections with prior exposure to Zika did not have a significantly higher risk of presenting with severe disease compared to primary infections (OR: 1.31 (95% CI: 0.66–2.60), *p*-value: 0.438). This pattern is likely attributed to severe dengue being a rare disease outcome. It should also be noted that, among the study population with active disease, those with elevated DENV IgG, on average, had higher ZIKV IgG. Moreover, those with active disease and elevated DENV IgG were more likely to present with adverse clinical symptoms (Supplementary Materials Figure S4).

Table 1. Clinical manifestations associated with primary DENV infections (ZIKV IgG-), post-primary DENV infections (ZIKV IgG-) and post-primary DENV infections (ZIKV IgG+). Adverse clinical symptoms: dengue warning signs or severe symptoms. OR: odds ratios.

Reported DENV/ZIKV	N	Adverse Clinical Symptoms				Severe Symptoms			
Immune Status		%	OR	[95% CI]	*p*-Value	%	OR	[95% CI]	*p*-Value
Primary DENV (ZIKV IgG-)	131	73.3	1 (ref)			9.2	1 (ref)		
Post-primary DENV (ZIKV IgG-)	306	80.7	1.53	[0.94–2.47]	0.084	6.9	0.69	[0.33–1.34]	0.254
Post-primary DENV (ZIKV IgG+)	190	87.4	2.52	[1.42–4.49]	0.002	12.6	1.31	[0.66–2.60]	0.438

4. Discussion

Our results show that, during 2016, suspected dengue cases from the Philippines had evidence of long-term, yet not short-term, serological exposure to Zika. This suggests that widespread ZIKV epidemiological investigations are warranted to determine the future risk of disease outbreaks across the country. We confirmed substantial IgG cross-reactivity between ZIKV and DENV, particularly among those reporting with post-primary DENV infections, where ZIKV IgG responses increased with disease progression. Among those reporting with non-active DENV infections (clinically misdiagnosed dengue cases), however, ZIKV IgG levels remained constant by reported day of disease, and some of those without any evidence of DENV IgG still had elevated ZIKV IgG. Last, we showed that the Zika FOI was lower than the dengue FOI across the Philippines, and that Zika exposure accumulated with age across urban settings, suggesting persistent transmission.

During the early stages of a secondary dengue infection, hosts experience a storm of specific and non-specific DENV IgG originally elicited from a previous, heterologous, serotype infection [12–14]. In our study, we revealed significant ZIKV/DENV assay cross-reactivity among those presenting with post-primary dengue infections. Our findings are consistent with those previously reported in Brazil, which found that Euroimmun kits are capable of distinguishing Zika infections from primary, yet not secondary, dengue infections [24]. We suggest two factors that account for this finding. First, Zika kits may simply detect pre-circulating, non-specific DENV IgG during a post-primary dengue infection that originated from a prior heterologous DENV serotype due to cross-reactivity. Alternatively, kits may detect IgG in post-primary dengue infections that was elicited from a prior Zika, not dengue, infection which cross-reacted with the subsequent dengue infection to mimic secondary-like disease. The latter is consistent our findings that ZIKV IgG seroprevalence increased with disease progression during post-primary infections, suggesting ZIKV IgG-induced ADE mechanisms. Moreover, we found that only a subset of those with post-primary DENV infections had elevated ZIKV IgG. Furthermore, we found that post-primary dengue cases with ZIKV IgG exposure, like post-primary DENV infections without ZIKV exposure, were at higher risk of presenting with adverse clinical symptoms than primary infections, a trend similarly reported by the authors in [16], and possibly a consequence of prior ZIKV exposure, priming individuals for a worse secondary-like dengue infection. However, considering that those with elevated DENV IgG were

more likely to have higher ZIKV IgG and that elevated DENV IgG is associated with severe disease outcomes, this association may be confounded by cross-reactivity. Interestingly, we showed that younger reporting post-primary cases were more likely to be ZIKV IgG-positive compared to older age post-primary DENV infections. We speculate that younger post-primary DENV infections include more secondary DENV infections while older age post-primary DENV infections include more post-secondary infections (tertiary/quaternary dengue), as older individuals are more likely to have experienced more than two DENV infections in their lifetime [37]. Moreover, secondary DENV infections experience larger surges in IgG levels compared to post-secondary infections due to ADE mechanisms [13]. Therefore, younger post-primary DENV infections, which likely included more secondary, as opposed to post-secondary, infections, were more likely to be ZIKV IgG-positive due to ZIKV IgG ELISAs detecting non-specific surging IgG responses compared to older post-primary infections with subdued IgG responses. Consequently, we were unable to conclusively determine whether ZIKV IgG exposure among post-primary infections was due to true prior exposure or simply cross-reactivity. Novel IgG assays that truly distinguish cross-reactive antibodies from true exposure are needed to overcome these limitations [26].

Following a dengue infection, heightened IgG levels wane, leaving hosts with DENV-specific IgG that persists for decades at lower levels [12]. Consequently, we speculated that historical and negative dengue cases reporting with elevated ZIKV IgG truly experienced a prior a ZIKV infection. We attribute this to two factors. First, only a subset of historical and negative cases presented with elevated ZIKV IgG. If the Euroimmun kits were also detecting DENV-specific IgG, then those with elevated DENV IgG would have elevated ZIKV IgG. Second, some of those reporting with negative dengue infections, without evidence of any DENV IgG, were ZIKV IgG-positive, which cannot be attributed to cross-reactivity. Among those reporting with non-active DENV infections, we revealed evidence of widespread ZIKV exposure across the Philippines similar to previous findings in Thailand [6]. This suggests that focal Zika surveillance practices in the Philippines would likely miss Zika infections and justifies further investigations into Zika transmission dynamics across the country.

Across the Philippines, the ZIKV FOI was lower than for DENV FOI, as expected, given the huge difference in reported cases. However, considering that DENV Panbio IgG ELISAs also detect ZIKV IgG, similarly shown by the authors of [23], and our study population included reported patients, our DENV FOI estimate is likely slightly overestimated. However, after stratifying by population density, we found that DENV transmission intensity was higher in urban compared to non-urban settings, as demonstrated previously in Bangladesh [38]. Moreover, accumulating DENV exposure with age in urban and non-urban areas eludes to well-established, historical dengue transmission in both these settings [7]. For ZIKV, however, we found that FOI in urban areas was very similar to the overall country. However, in non-urban areas, there was no evidence of increasing ZIKV exposure with age. This is consistent with the rationale that historical, or potentially ongoing ZIKV transmission, is more common in urban settings. We suggest two factors that may account for this finding. First, ZIKV transmission in the Philippines is more recent than DENV and is still only dominant in urban areas where transmission originated [39]. Second, widespread DENV exposure across the Philippines offers the population protection from ZIKV, as suggested by the authors of [7], and hampers the spread of ZIKV into more rural areas. The observation that ZIKV seroprevalence is higher in younger individuals in non-urban settings also suggests more recent ZIKV outbreaks. However, this requires further epidemiological validation.

No evidence of recent ZIKV exposure among the study population was observed in this study, which we believe is attributed to several factors. Earlier studies have shown ZIKV outbreaks to be periodic in nature [7,16,40]. Therefore, we may have collected samples during a non-outbreak period. However, this contradicts with DOH reports of laboratory-confirmed cases of Zika across the country during 2016. Second, Zika is thought

to be a largely asymptomatic infection, so most of those infected would be unlikely to seek care. Third, as only 997 dengue case reports were sampled across the country and Euroimmun IgM ELISAs have previously shown to suffer low sensitivity [22], we may have missed recent ZIKV infections. Therefore, future surveillance and epidemiological programs should consider the type of samples that should be collected from individuals and what laboratory procedures could be used to maximize the chances of identifying those with recent ZIKV infections. A recent study showed that ZIKV RNA and IgM compartmentally persist in hosts and that novel diagnostic methods might extend the window of detection [9]. Despite not capturing recent Zika infections, our study still revealed evidence of long-term exposure to Zika. Therefore, we believe that further epidemiological studies into ZIKV transmission across the Philippines are warranted. Population-based seroprevalence studies would provide better understanding into the spatiotemporal nature of ZIKV transmission across the country and identify regions with or without the disease. Moreover, despite not capturing recently reported ZIKV infections in this study, routinely assaying suspected DENV cases for ZIKV, particularly those without active DENV infections, may still assist in identifying future outbreaks.

5. Conclusions

In this study, we provided the first evidence of widespread ZIKV exposure across the Philippines and suggest ZIKV transmission has potentially been ongoing in urban areas for many years. Despite detecting cross-reactivity between DENV and ZIKV IgG responses, our analysis provides evidence of ZIKV transmission by considering dengue serological findings. Our results highlight the need for continued investigations into ZIKV transmission across the Philippines and justify combining ZIKV surveillance with other flaviviruses. Together, this could better describe ZIKV exposure over time and help curb possible future outbreaks of severe outcomes associated with ZIKV.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13081441/s1, Table S1: Regional administrative boundaries of the Philippines, Table S2: Demographic characteristics of the study population, Figure S1: Methods used to determine ZIKV IgM and IgG seroprevalence, Figure S2: ZIKV IgG seroprevalence, Figure S3: Scatter plot of regional DENV versus ZIKV IgG seroprevalence among those with non-active DENV infections, Table S3: The percentage of reporting negative DENV cases (DENV PCR-, IgM- and IgG-) across the Philippine regions who were ZIKV IgG positive, Figure S4: Cross-reactive ZIKV/DENV IgG responses.

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Informed Consent Statement: Not applicable.

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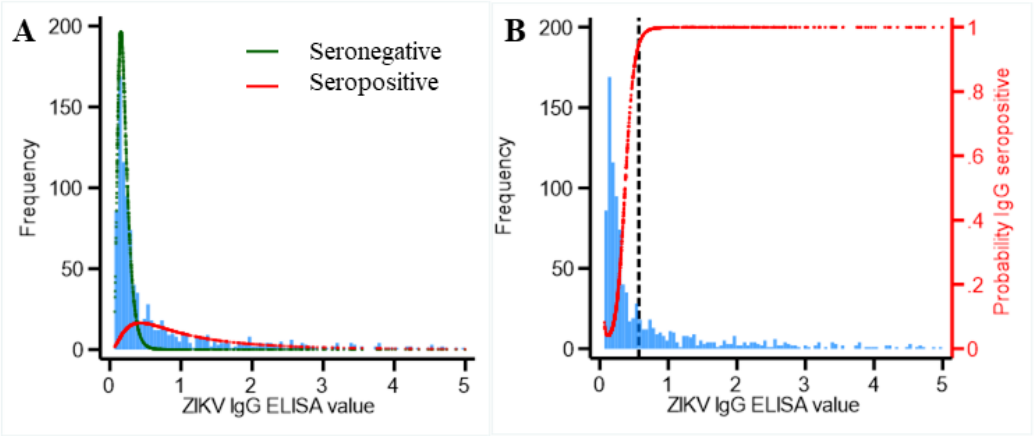
Appendix 3: Chapter 5 supplementary materials

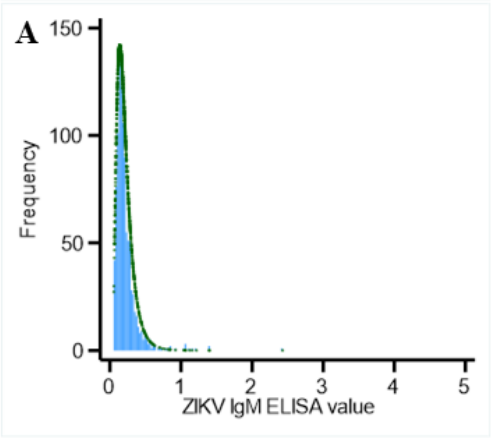
Supplementary table S1: Regional administrative boundaries of the Philippines.

Island group	Region	
	Code	Name
Luzon	1	Ilocos Region
	CAR	Cordillera Administrative Region
	2	Cagayan Valley
	3	Central Luzon
	4A	Calabarzon
	4B	Mimaropa
	5	Bicol Region
	NCR	National Captial Region
Visayas	6	Western Visayas
	7	Central Visayas
	8	Eastern Visayas
Mindanao	9	Zamboanga Peninsula
	10	Northern Mindanao
	11	Davao Region
	12	Soccsksargen
	CARAGA	Caraga Region
	ARMM	Autonomous Region in Muslim Mindanao

Supplementary table S2: Demographic characteristics of the study population.

Study demographics		
	%	n
Age		
<5	16.5	165
6-15	43.3	432
16-30	27.8	277
>30	12.3	123
Sex		
Female	48.6	485
Male	51.4	512
Disease day		
0-1	8.8	88
2-3	39.8	397
4-5	51.4	512
DENV symptoms		
No warning signs	17.5	174
Warning signs	54.4	542
Severe dengue	9.8	98
Non-disclosed	18.4	183
Island group		
Luzon	48.5	484
Visayas	17.5	174
Mindanao	34.0	339
DENV immune status		
Primary	19.1	189
Post-primary	61.0	605
Historical	13.5	134
Negative	6.4	63
Total	100	997



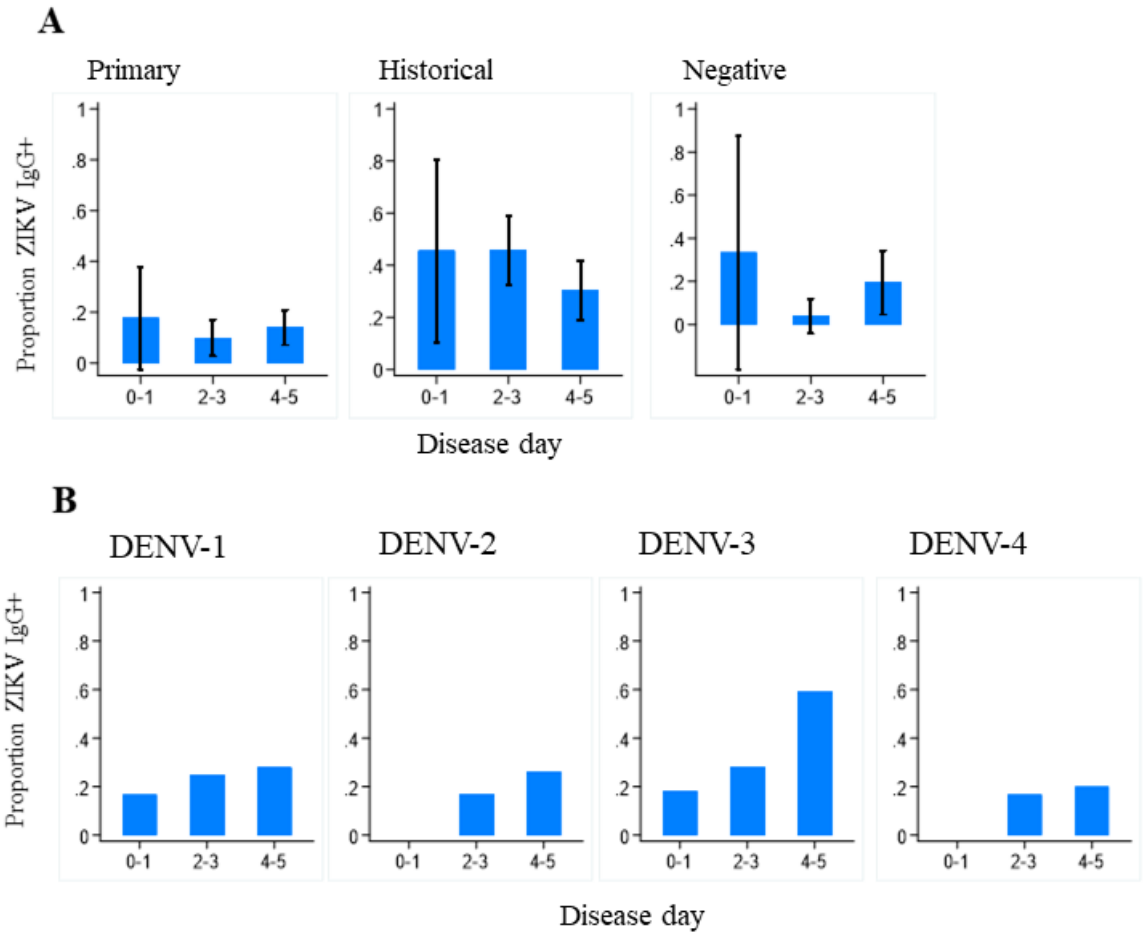


C

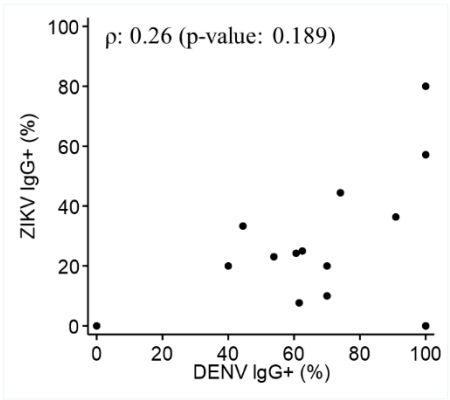
IgG model Component	AIC	AIC difference
1	920.5	
2	636.3	-284.2

IgM model Component	AIC	AIC difference
1	876.2	
2	1036.1	+159.9

Supplementary figure S1: Methods used to determine ZIKV IgM and IgG seroprevalence. **A**: Histogram plots of the study populations ZIKV IgM and IgG distributions fitted with two mixture model subpopulations: green: seronegative, red: seropositive. **B**: Histogram of ZIKV IgG overlaid by the probability of being seropositive according the mixture model. Vertical dash (revised cut off): >95% probability of IgG seropositive to ZIKV. **C**: Model fit comparison of ZIKV IgM and IgG distributions according to AIC.



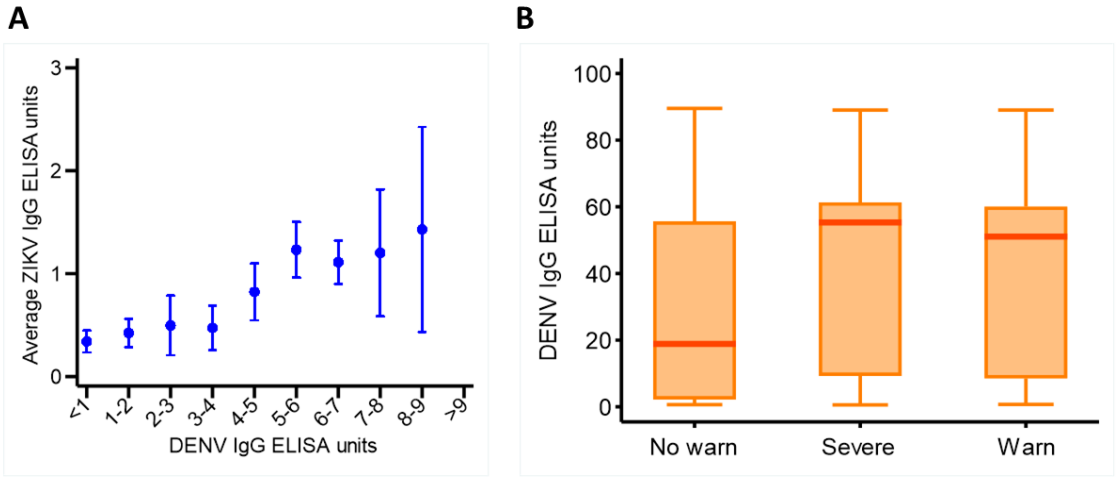
Supplementary figure S2: A: ZIKV IgG seroprevalence by day of disease among those reporting with primary, historical and negative dengue infections. B: ZIKV IgG seroprevalence by day of disease among those reporting with DENV1-4 infections.



Supplementary figure S3: Scatter plot of regional DENV versus ZIKV IgG seroprevalence among those with non-active DENV infections. Rho: Pearson's R coefficient.

Supplementary table S3: The percentage of reporting negative DENV cases (DENV PCR-, IgM- and IgG-) across the Philippine regions who were ZIKV IgG positive.

Region	N	ZIKV IgG seropositive	
		%	n
3	5	0.0	0
4A	14	7.1	1
4B	1	100.0	1
5	3	33.3	1
6	5	0.0	0
8	3	66.7	2
9	3	33.3	1
ARMM	1	0.0	0
CAR	12	8.3	1
CARAGA	13	15.4	2
NCR	3	0.0	0
Total	63	14.3	9



Supplementary figure S4: Cross-reactive ZIKV/DENV IgG responses. A: Average ZIKV IgG ELISA value over stratified DENV IgG ELISA values. Vertical bars: 95%CIs. B: DENV IgG responses among active dengue infections presenting with warning signs (warn), severe dengue (severe) and no warning signs (no warn).

Chapter 6. Combining Rapid Diagnostic Tests to Estimate Primary and Post-primary Dengue Immune Status at the Point-of-Care

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646
F: +44 (0)20 7299 4656
www.lshtm.ac.uk

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SECTION A – Student Details

Student ID Number	1402440	**Title**	Mr
First Name(s)	Joseph Robert		
Surname/Family Name	Biggs		
Thesis Title	Immuno-epidemiological analysis of dengue to enhance surveillance		
Primary Supervisor	Dr Julius Clemence R. Hafalla		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?			
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SECTION C – Prepared for publication, but not yet published

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Please list the paper's authors in the intended authorship order:	Joseph R. Biggs, James Ashall, Ava Kristy Sy, Marsha S Santoso, Oliver Brady, Sebastian Funk, Mary Anne Joy Reyes, Mary Ann Quinones, William Jones-Warner, Amadou O Tandoc, Nemia L. Sucaldito, Huynh Kim Mai, Le Thuy Lien, Hung Do Thai, Hien Anh Thi Nguyen, Dang

	Duc Anh, Chihiro Iwasaki, Noriko Kitamura, Marnix Van Loock, Guillermo Herrera-Taracena, Joris Menten, Freya Rasschaert, Liesbeth Van Wesenbeeck, Sri Masyeni, Sotianingsih Haryanto, Benediktus Yohan, Eva Cutiongco-de la Paz, Lay-Myint Yoshida, Stephane Hue, Maria Rosario Z. Capeding, Carmencita D. Padilla, Tedjo R Sasmono, Julius Clemence R. Hafalla & Martin L. Hibberd
Stage of publication	**Accepted for publication**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I was first author. I was responsible for the data analyses and writing the draft manuscript.

SECTION E

Student Signature	[redacted]
Date	30/09/2021

Supervisor Signature	[redacted]
Date	30/09/21

Combining Rapid Diagnostic Tests to Estimate Primary and Post-primary Dengue Immune Status at the Point of Care

Authors and affiliations

Joseph R. Biggs[1#¶*], Ava Kristy Sy[2,3¶], James Ashall[1], Marsha S Santoso[4], Oliver J. Brady[5,6], Mary Anne Joy Reyes[2,3], Mary Ann Quinones[2,3], William Jones-Warner[1], Amadou O Tandoc[2], Nemia L. Sucaldito[7], Huynh Kim Mai[8], Le Thuy Lien[8], Hung Do Thai[8], Hien Anh Thi Nguyen[9], Dang Duc Anh[9], Chihiro Iwasaki[10], Noriko Kitamura[10], Marnix Van Loock[11], Guillermo Herrera-Taracena[12], Joris Menten[13], Freya Rasschaert[11], Liesbeth Van Wesenbeeck[11], Sri Masyeni[14], Sotianingsih Haryanto[15], Benediktus Yohan[4], Eva Cutiongco-de la Paz[16,17], Lay-Myint Yoshida[10], Stephane Hue[5,6], Maria Rosario Z. Capeding[3,16], Carmencita D. Padilla[16,17], R. Tedjo Sasmono[4], Julius Clemence R. Hafalla[1] & Martin L. Hibberd[1,16,17]

1. Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK.
2. Department of Virology, Research Institute for Tropical Medicine, Manila, Philippines.
3. Dengue Study Group, Research Institute for Tropical Medicine, Manila, Philippines.
4. Dengue Research Unit, Eijkman Institute for Molecular Biology, National Agency for Research and Innovation of the Republic of Indonesia, Jakarta, Indonesia.
5. Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK.
6. Centre for the Mathematical Modelling of Infectious Diseases, London School of Hygiene and Tropical Medicine, London, UK
7. Philippine Epidemiology Bureau, Department of Health, Manila, Philippines
8. Pasteur Institute of Nha Trang, Nha Trang, Vietnam
9. National Institute of Hygiene and Epidemiology, Hanoi, Vietnam
10. Paediatric Infectious Diseases Department, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan
11. Janssen Global Public Health, Janssen Pharmaceutica NV, Beerse, Belgium.
12. Janssen Global Public Health, Janssen Research & Development, Pennsylvania, United States of America.
13. Quantitative Sciences, Janssen Pharmaceutica NV, Beerse, Belgium.
14. Department of Internal Medicine, Faculty of Medicine and Health Sciences, Universitas Warmadewa, Denpasar, Bali, Indonesia.
15. Raden Mattaher Hospital, Jambi, 36361, Indonesia.

16. Institute of Human Genetics, University of the Philippines, Manila, Philippines
17. Philippine Genome Centre, University of the Philippines, Manila, Philippines

Corresponding author: Joseph.Biggs1@lshtm.ac.uk

¶ These authors contributed equally to this work

Abstract

Background

Characterising dengue virus (DENV) infection history at the point of care is challenging as it relies on intensive laboratory techniques. We investigated how combining different rapid diagnostic tests (RDTs) can be used to accurately determine the primary and post-primary DENV immune status of reporting patients during diagnosis.

Methods and findings

Serum from cross-sectional surveys of acute suspected dengue patients in Indonesia (N:200) and Vietnam (N: 1,217) were assayed using dengue laboratory assays and RDTs. Using logistic regression modelling, we determined the probability of being DENV NS1, IgM and IgG RDT positive according to corresponding laboratory viremia, IgM and IgG ELISA metrics. Laboratory test thresholds for RDT positivity/negativity were calculated using Youden's J index and were utilized to estimate the RDT outcomes in patients from the Philippines, where only data for viremia, IgM and IgG were available (N:28,326). Lastly, the probabilities of being primary or post-primary according to every outcome using all RDTs, by day of fever, were calculated. Combining NS1, IgM and IgG RDTs captured 94.6% (52/55) and 95.4% (104/109) of laboratory-confirmed primary and post-primary DENV cases, respectively, during the first 5 days of fever. Laboratory test predicted, and actual, RDT outcomes had high agreement (79.5% (159/200)). Among patients from the Philippines, different combinations of estimated RDT outcomes were indicative of post-primary and primary immune status. Overall, IgG RDT positive results were confirmatory of post-primary infections. In contrast, IgG RDT negative results were suggestive of both primary and post-primary infections on days 1-2 of fever, yet were confirmatory of primary infections on days 3-5 of fever.

Conclusion

We demonstrate how the primary and post-primary DENV immune status of reporting patients can be estimated at the point of care by combining NS1, IgM and IgG RDTs and considering the days since symptoms onset. This framework has the potential to strengthen surveillance operations and dengue prognosis, particularly in low resource settings.

Author summary

Combined NS1, IgM and IgG dengue rapid diagnostic tests (RDTs) have previously been shown to accurately diagnose those experiencing dengue virus (DENV) infections at the point of care and are now available as single commercial kits. Using such kits to additionally determine those experiencing primary (first) or post-primary (second, third or fourth) DENV infections however remains challenging as accurate immune status classification currently relies on laboratory analysis. We used logistic regression modelling methods to estimate RDT positive and negative outcomes according to corresponding PCR and ELISA laboratory-based methods, which showed high sensitivity and specificity. Dengue RDT outcomes were then predicted among a large sample of suspected dengue case reports, to calculate the probability of being primary or post-primary for dengue according to every possible set of dengue RDT outcomes, by day of fever. Different RDT outcomes, at certain stages of infection, were indicative of primary and post-primary immune status. Using our framework to determine dengue immune status at the point of care in low resource settings, regional surveillance systems could estimate and monitor dengue transmission intensity. Additionally, this framework could potentially support dengue prognosis and identify primary cases who would benefit from current vaccination regimes to prevent subsequent secondary infections associated with severe disease.

Introduction

Dengue is an emerging arboviral infectious disease, transmitted through the bite of an *Aedes* mosquito, that burdens much of the urbanised tropical and subtropical world. According to World Health Organisation (WHO) figures, global case reporting has risen 8-fold in the past 20 years with a record 5.2 million reports in 2019 [1]. However, these data only account for the minority of symptomatic dengue cases who actively sought care and were successfully documented. Therefore, figures exclude most of the 105-390 million annual estimated dengue infections across the globe [2,3]. Dengue virus (DENV) is a member of the *Flaviviridae* family consisting of four distinct serotypes (DENV1-4) [4]. A primary infection with any serotype is typically associated with a self-limiting disease which elicits a long-lived IgG response that protects against subsequent homologous serotype infections [5]. Consequently, individuals can suffer successive, post-primary (i.e. secondary, tertiary and quaternary), DENV infections during their lifetime with heterologous serotypes. A secondary DENV infection is associated with more severe clinical outcomes, including severe organ impairment and bleeding [6,7], due to a phenomenon known as antibody-dependent enhancement [8,9]. Previously elicited, sub-neutralising, IgG resurges upon infection and intensifies viral replication to trigger immuno-modulated severe disease. Without specific dengue chemotherapies, severe disease management is currently limited to intravenous hydration therapy (IHT) that requires careful monitoring and adequate health care infrastructure [10].

Current dengue diagnostics are primarily concerned with capturing active infections, thus no such method for determining primary or post-primary DENV infections, at the point of care, exists [6]. Nonetheless, WHO serological laboratory techniques can be utilized to distinguish DENV immune status retrospectively using patient paired sera collected at the acute and convalescent stage of disease [11]. By assaying for changes in both DENV IgM and IgG antibodies, a rise in IgM titres coupled with high and low convalescent IgM:IgG ratios indicates active primary and secondary infections, respectively. A major caveat to this approach however is the necessity of paired sera which makes it impractical for large scale epidemiological studies and detects the result too late to inform many case management decisions. Fortunately, more recent studies have shown dengue immune status can be determined using single serum samples collected during the acute phase of disease according to disease day-specific IgG:IgM ratios [12–15]. Yet again however, these algorithms have limited value in point-of-care testing, particularly in low resource settings, as they rely on individual laboratory metrics which take time to generate, are labour intensive and require extensive equipment.

Recently, the WHO has advocated for the use of rapid diagnostic tests (RDTs) to improve dengue case detection and management in low resource, regional health care facilities [16]. Numerous quick, easy-to-use and inexpensive commercial RDTs are now available which can detect different markers of

infection [17,18]. The dengue NS1 RDT detects the dengue non-structural protein 1 (NS1), which is secreted into the blood during, and slightly after, the viraemic phase of disease. In contrast, The IgM and IgG RDTs detect IgM and IgG antibodies during the later immunogenic phase of infection, respectively. Many studies have shown how well these kits capture true active dengue infections, particularly when used in combination, although their performance varies according to specific commercial brands [17,19–21]. Moreover, studies have documented how the DENV immune status of patients influences the performance of RDTs. When used alone, NS1 RDTs have better sensitivities for capturing primary, as opposed to secondary, infections [18,22]. This is likely a consequence of the contrasting viremia kinetics, whereby viremia is higher and persists longer in the febrile stage during primary compared to post-primary infections [12,23,24]. Conversely, studies have demonstrated IgG RDTs are better at capturing post-primary infections as IgG is believed to be absent during the acute phase of primary disease [22,25]. Although, it has been shown during a primary infection, patients can begin to elicit IgG towards the end of the acute phase while very early stage post-primary infections still experience increasing titres of IgG which may not be high enough to yield IgG RDT positive results [12,26]. Indeed one study revealed the IgG RDT sensitivity for capturing secondary DENV infections was lower among those reporting before disease day 4 [27]. Consequently, assuming all primary and post-primary dengue infections would yield IgG RDT negative and positive results, respectively, could result in misclassification. Despite studies demonstrating that combining the three NS1, IgM and IgG RDTs improves diagnostic performance, it remains unclear what exact combination of RDT outcomes, at specific stages of infection, indicate primary or post-primary dengue infections.

Documenting DENV immune status at the point of care could assist surveillance operations. The age at which patients present with their first (primary) DENV infection has been shown to correlate with the force of infection in endemic cities [28] and establishing immune status promptly might assist in the deployment of vaccinations targeted at those with primary DENV exposure [29]. Moreover, as patients can deteriorate quickly during a DENV infection, determining primary and post-primary immune status prior to the development of severe symptoms could potentially assist in clinical case management in health care settings [30]. For instance, post-primary DENV patients could receive closer monitoring and be prioritised for limited IHT compared to primary DENV patients. Yet whether this would be appropriate for effective case management remains unknown and would require further investigation.

Prior to this study, we generated and validated an algorithm capable of distinguishing individual primary and post-primary DENV immune status that relies on basic epidemiological and laboratory-obtained metrics from single serum samples [12]. The framework utilises individual molecular and serological DENV metrics from the CDC fourplex DENV1-4 PCR assay and commercial IgM and IgG capture ELISAs (Panbio®, Abbott, Cat no. 01PE10 & 01PE20), respectively. Panbio® serological assays were chosen based on a WHO report which compared their performance to other commercial assays which revealed similar sensitivities [31]. The novel algorithm achieved 90% agreement with the WHO

gold standard method for categorising immune status based on paired sera and proved superior to the Panbio® method for classifying immune status [11,12]. Given dengue serological assays have been shown to detect cross-reactive antibodies elicited from other flavivirus infections, including Zika virus (ZIKV) which is often co-endemic with dengue [32–34], previous analysis explored the impact of ZIKV exposure on the generated DENV immune status algorithm [12]. Results revealed a proportion of those with post-primary, opposed to primary, DENV infections had evidence of ZIKV IgG but not IgM exposure. This suggested some patients had historical ZIKV exposure that primed individuals for a subsequent post-primary-like, instead of a primary-like, DENV infection upon their first infection with DENV. Moreover, further analysis suggested post-primary DENV infections with prior ZIKV exposure were at risk of adverse clinical symptoms [34] which has been previously reported in Nicaragua [35]. These findings suggested that individuals categorised as post-primary DENV infections include those with either prior exposure to DENV and/or other flaviviruses [12]. A major challenge associated with the generated immune status algorithm is the reliance on laboratory-derived metrics (PCR and ELISA), consequently this framework has limited value in low-resource, regional health care settings [16]. In this study, we investigated whether RDTs can be utilised to accurately determine primary and post-primary immune status of reporting patients at the point of care. Specifically, we examined: 1) the utility of combining the outcomes of NS1, IgM and IgG RDTs in accurately capturing both primary and post-primary dengue infections; 2) the translatability between dengue laboratory tests and RDTs; and 3) The probability of being primary or post-primary dengue cases by every possible NS1, IgM and IgG RDT outcome at specific days of disease.

Methods

Ethics statement

This study was approved by the ethical review boards of the London School of Hygiene and Tropical Medicine (Ref: 17853), the Research Institute for Tropical Medicine (Ref: 2017-014), Nagasaki University (Ref: VN01057) and Eijkman Institute for Molecular Biology (Ref: 136/2019). Verbal consent was obtained from patients over 18 years, while verbal assent was acquired from those under 18 years coupled with parent/guardian consent, for the use of serum samples. All unique participant identifiers were removed before data acquisition.

Data collection

Data were obtained from suspected dengue patients who visited health care facilities during the acute stage of disease in the Philippines (N: 28,326), Vietnam (N: 1,217) and Indonesia (N: 200). Suspected dengue patients included those with a self-reported sudden acute fever coupled with at least two

additional warning signs: headache, malaise, myalgia, arthralgia, retro-orbital pain, anorexia, nausea, vomiting, diarrhoea, flushed skin and/or rash in accordance with WHO criteria [6]. Specific data collected from patients in each dataset are highlighted in (S1 Table).

In the Philippines, data were collected from a survey of dengue patients who visited disease reporting units (DRUs) situated across the country between 2014 and 2018. In major, regional DRUs, five weekly random serum samples were collected from suspected dengue patients during the acute phase of disease. In smaller regional health care centres across the Philippines, samples were collected from patients during an upsurge in case reporting defined according to Philippine Integrated Disease Surveillance and Response (PIDSR) criteria [36]. Additional epidemiological data were collected from patients including age, sex, disease day (date of reporting – date of symptom onset), symptoms (no warning signs, warning signs, severe dengue). Serum samples were sent to the Research Institute of Tropical Medicine (Department of Health, Manila, Philippines) for further laboratory testing.

In Vietnam, data used in this study were obtained from those who reported with suspected dengue to a Polyclinic or the Tropical Disease Hospital out-patient clinic in Nha Trang city between October 2016 and May 2019. We enrolled patients who gave home addresses from four communes in Nha Trang City: Vinh Hai, Vinh Phuoc, Vinh Tho, and Vinh Hoa. Serum samples underwent subsequent laboratory testing at the Pasteur Institute in Nha Trang. Epidemiological data collected from patients included: Age, sex, symptoms, and disease day.

In Indonesia, serum samples were collected from suspected dengue patients that reported across regions of Indonesia between July 2014 and July 2019 originally obtained for a previous study [37]. Additional epidemiological data provided for each sample included age and disease day. Samples were stored and assayed at the Eijkman Institute, Jakarta, Indonesia.

Dengue testing

All serum samples collected from patients included in this study (N: 29,743) received laboratory dengue testing in their respective institutes. Samples were assayed for the presence of DENV1-4 viremia using the CDC fourplex, real-time polymerase chain reaction (RT-PCR) test according to methods described in [38]. Briefly, dengue serotype-specific primers amplify viral RNA and yield critical threshold (Ct) values which inversely corresponds to the level of viral RNA (viremia). Samples with Ct values $\leq$36 were considered PCR positive for DENV. The presence of DENV IgM and IgG antibodies was performed using Panbio® capture ELISAs according to manufacturer's instructions (Cat no: 01PE20; 01PE21, Abbott). Assays detect IgM/G antibodies specific to all serotypes and provide plate-calibrated titre outputs termed 'panbio units'.

Additional laboratory and RDT testing were conducted among samples obtained from Vietnam and Indonesia. In Vietnam, patients were tested, at the point of care in the Polyclinic, for the presence and absence of NS1 using DENV NS1 RDTs (Cat no: 70700, Bio-Rad, Inc) (N: 1,217). Among samples from Indonesia, patient serum samples were tested for DENV NS1 using both NS1 capture ELISAs (Cat no: 01PE40, Abbott) and NS1 RDTs (Cat no: 09DEN10D, SD Biosensor) according to commercial guidelines and in the laboratory. NS1 capture ELISAs generated plate-calibrated titres termed 'NS1 panbio units'. Finally, samples collected from Indonesia were further tested for the presence or absence of DENV IgM/G using IgM and IgG RDTs in line with manufacturers specifications in the laboratory (Cat no: 09DEN20D, SD Biosensor). A summary of the data collected from reporting patients in each country are shown in (S1 Table).

Statistical analysis

Using laboratory and basic epidemiological data, we categorized the reference DENV immune status (primary, post-primary, historical and negative) of the entire study population using the exact methods described in [12]. Patients who reported as either PCR+ or IgM+ (Ct≤36 or IgM panbio units>9.9) were classified as active DENV infections as both these markers are detectable during infection. PCR- and IgM- (IgM panbio units<9.9) cases were categorised as non-active DENV infections and represent patients misdiagnosed as suspected active dengue. Non-active DENV infections were further classified as historical or negative if they were DENV IgG positive (IgG panbio units>2.2) or negative (IgG panbio units<2.2), respectively. Historical and negative cases included misdiagnosed patients who reported without a current dengue infection yet with and without previous exposure to DENV, respectively. IgG:IgM ratios (IgG panbio units/IgM panbio units) were used to distinguish active DENV infections as primary or post-primary cases. Among active dengue patients at the early stage of disease (disease day 1 or 2), those DENV IgG+ and IgG- were classed as post-primary and primary respectively. Among active cases on disease day 3 to 5, individuals with IgG:IgM ratios above and below 0.45 were categorised as post-primary and primary respectively. As a consequence of previous findings [12,34], post-primary cases included current DENV infections with at least one previous flavivirus infection including DENV and or ZIKV. An overview of the reference DENV immune status classification is shown in S1 File.

Using binomial logistic regression modelling, we estimated the probability of being RDT positive according to corresponding laboratory-derived metrics with 95% confidence intervals (ELISA & PCR). Using data from Indonesia, we estimated the probability of being IgM and IgG RDT positive according to IgM and IgG panbio units, respectively. From the Vietnam dataset, we predicted the probability of being NS1 RDT positive according to DENV viremia (Ct value). To account for the lag in NS1 production during the viraemic stage of infection, we stratified NS1 logistic regression models by disease day. To assess the validity of logistic regression modelling, Hosmer–Lemeshow tests were used

to determine appropriate model fits (p-value>0.05). For each model, the optimal laboratory-derived metric cut off for RDT positivity was determined using Youden's J index (sensitivity + specificity – 1) [39]. The threshold refers to the optimal estimated probability of being RDT positive according to sensitivity/specificity based on actual RDT outcomes. This approach was adopted to minimise the misclassification of RDT outcomes according to corresponding laboratory metrics. Moreover, the percentage agreement between of the combined (NS1, IgM and IgG) estimated and actual RDT outcomes were calculated. To estimate immune status according to RDTs, we estimated the NS1, IgM and IgG RDT status of all patients from the Philippines with defined primary, post-primary, historical and negative DENV immune status according to laboratory testing (S1 File). Lastly, we calculated the probability of being primary, post-primary, historical and negative according to every combination of RDT result possible using all three rapid tests, stratified by disease day.

Results

In our study population, we observed similar demographic characteristics among suspected dengue patients who reported in Indonesia, Vietnam, and the Philippines (S2 Table). Most were aged between 6-15 years (≥33.4%), reported 3-4 days after the onset of disease symptoms (≥51.0%) and presented with post-primary DENV infections (≥48.7%). There were contrasting patterns in the DENV serotypes patients presented with. In Vietnam only 0.5% (4/803) of patients assayed by PCR were DENV-3, while among those assayed for PCR in the Philippine dataset, 17.1% were DENV-3 (4535/26,494).

Dengue infection kinetics

We explored disease-day stratified DENV infection kinetics among primary and post-primary dengue patients according to the laboratory and RDT data collected among patients from Indonesia (Fig 1) (N:200). Viremia, as measured by Ct value, and NS1 levels, measured by ELISA, plateaued at higher levels during the acute phase of primary infections (Fig 1A&B) yet were lower and dropped more rapidly during the acute phase of post-primary infections (Fig 1E-F). This was mirrored by the higher proportion of primary cases who were NS1 RDT positive (81.8% 45/55) compared to post-primary cases (45.9% 50/109) during the acute phase of disease (Fig 1I&M). Likewise, we found both IgM and IgG RDT outcomes matched IgM and IgG ELISA laboratory values, respectively. For IgG, ELISA titres among primary cases remained low during the acute phase with only 20.0% (11/55) IgG RDT positive (Fig 1D&K). In contrast, median IgG ELISA values increased to high levels among post-primary cases (Fig 1H) which was reflected by an increase in IgG RDT positivity from 13.3% (2/15) to 78.4% (29/37) on disease days 1-2 and 4-5, respectively (Fig 1O). Given not all early-stage post-primary cases yielded IgG RDT positive outcomes, these results indicate assuming post-primary and primary DENV cases would present as IgG RDT positive and negative, respectively, would result in immune

status misclassification. Lastly, we found combining RDTs maximised the chances of identifying primary and post-primary DENV infections at all stages of acute disease. For primary and post-primary cases, 94.6% (52/55) and 95.4% (104/109) were positive to either NS1, IgM or IgG RDTs, respectively (Fig 1L&P).

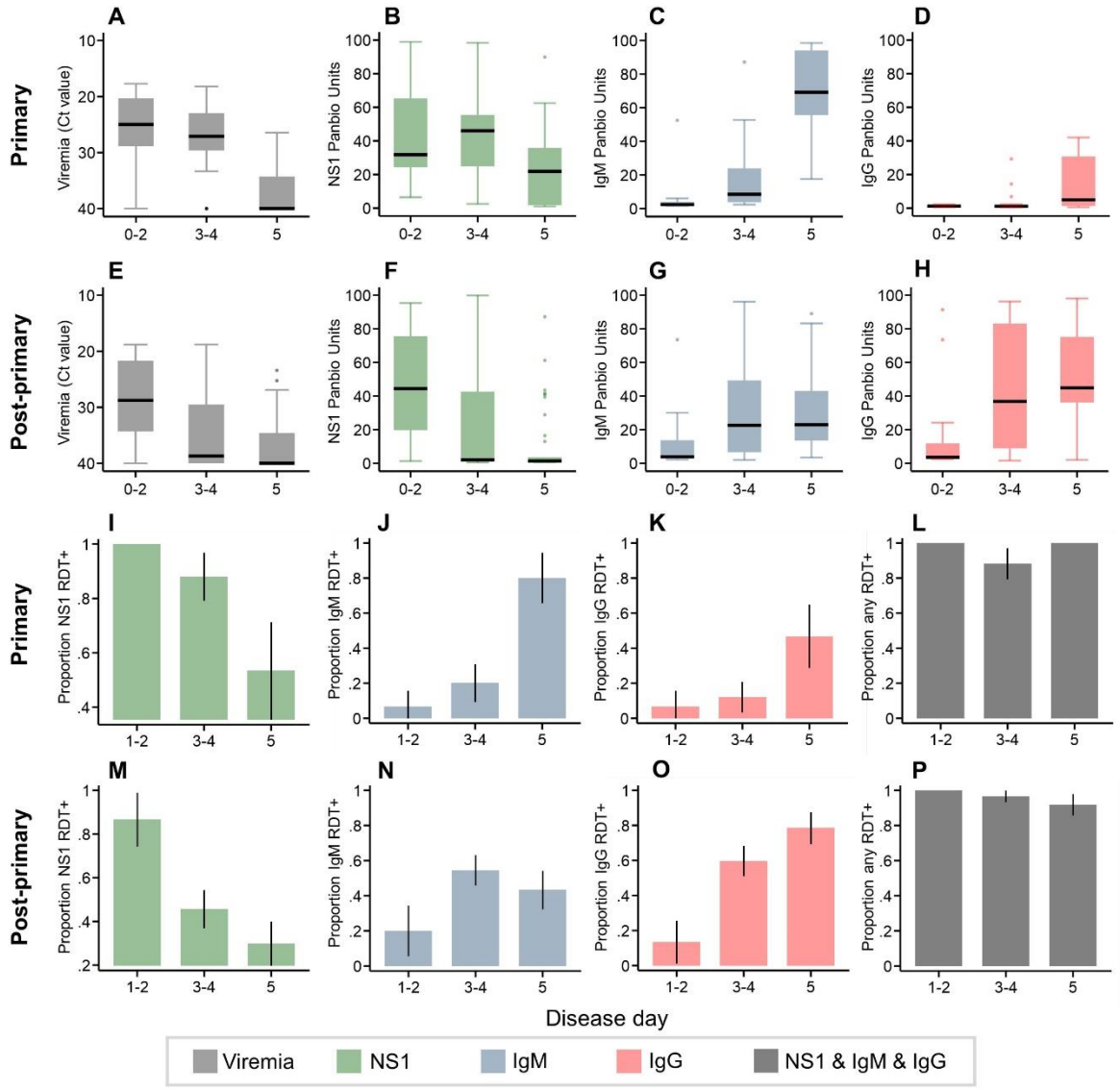

Figure 1. Infection kinetics among primary and post-primary DENV patients from Indonesia by disease day according to laboratory and rapid tests. A-H: Boxplots of viremia (Ct), NS1, IgM and IgG by disease day among primary and post-primary cases according to PCR and ELISA tests. **I-P**: Proportion RDT positive to NS1, IgM, IgG and all combined by disease day among primary and post-primary cases. Black error bars: 90% confidence intervals based on t-distributions. (Primary N: 55) (Post-primary N:109).

Among patients from the Philippines with serotype and PCR (Ct values) data, we explored whether the contrasting viremia kinetics among primary and post-primary cases during the acute stage of disease were driven by the infecting serotype (S1 Fig). Irrespective of serotype, viremia decreased from higher

levels in primary cases compared to post-primary cases. We also investigated whether contrasting NS1 patterns observed between primary and post-primary cases was influenced by certain serotypes secreting more/less NS1 proteins (S3 Table). We found NS1 RDTs effectively captured most DENV1-4 PCR+ infections, regardless of immune status (Sensitivity>80% for each serotype).

Estimating RDT outcomes according to laboratory metrics

To investigate the translatability between laboratory and rapid dengue diagnostics, we explored whether DENV RDT outcomes could be predicted from DENV laboratory test results using logistic regression models. Models were assessed using Hosmer–Lemeshow tests which revealed no significant poor model fits (p-value>0.05) (S2 Fig). Among dengue patients from Indonesia (N:200), logistic regression models were used to estimate the probability of being IgM/G RDT positive according to IgM/G ELISA panbio units, respectively (Fig 2A & 2B). For both IgM and IgG, the estimated probability of being RDT positive increased with increasing ELISA panbio units. According to the highest Youden's J-index values, thresholds of 15.2 and 11.9 panbio units provided the optimal sensitivity and specificity for determining IgM and IgG RDT positivity, respectively. Individuals with ELISA values below and above these thresholds were considered RDT positive for each antibody. This resulted in 43.0% (86/200) with estimated IgM RDT positive outcomes which corresponded to 37.5% (75/200) with actual IgM RDT positivity (Sensitivity: 74.7% [95%CI: 63.3-84.0%], Specificity: 76.0% [95%CI: 67.5-83.2%]) (Table 1). For IgG, our optimal threshold resulted in 47.0% (94/200) with estimated IgG RDT positivity which corresponded to 44.5% (89/200) actual IgG RDT positivity (Sensitivity: 82.0% [95%CI: 72.5-89.4%], Specificity: 81.1% [72.5-87.9%]) (Table 1).

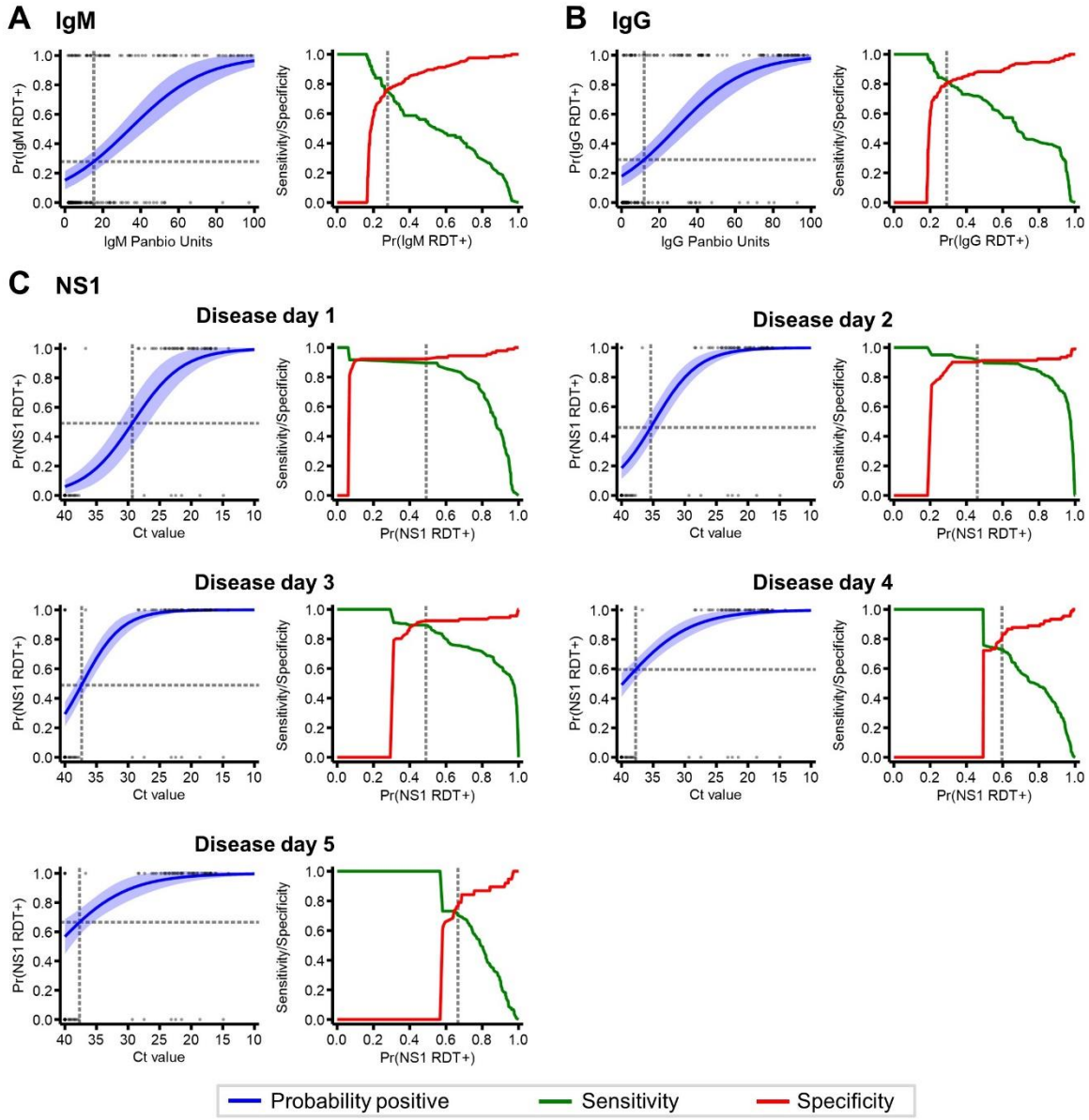

Figure 2: Estimated probability of being DENV RDT positive according to corresponding laboratory-derived metrics using logistic regression modelling. **A:** IgM RDT positivity according to IgM panbio units and the corresponding sensitivity/specificity among patients from Indonesia (N:200) **B**: IgG RDT positivity according to IgG panbio units and the corresponding sensitivity/specificity among patients from Indonesia (N:200) **C**: NS1 RDT positivity according to viremia (Ct value) and the corresponding sensitivity/specificity stratified by disease day among patients from Vietnam (N: 1,217). Grey dash: estimated laboratory-derived metric threshold for RDT positivity according to the optimal Youden's J index value.

Table 1: Estimated laboratory-test values that yield RDT positive results compared to actual RDT outcomes. Estimated RDT positivity threshold refer to the optimal Youden's J index value. Estimated/actual NS1 RDT positivity determined among patients from Vietnam (N: 1,217). Estimated/actual IgM/IgG RDT positivity determined among patients from Indonesia (N:200).

DENV metric	Disease day	Estimated RDT positivity threshold according to lab metrics	Estimated RDT positive[#]			Actual RDT positive			Sensitivity		Specificity	
			%	N	n	%	N	n	%	[95% CI]	%	[95% CI]
NS1	1	<29.3 Ct	36.2	138	50	34.8	138	48	89.6	[77.3-96.5]	92.2	[84.6-96.8]
	2	<35.4 Ct	65.9	293	193	68.9	293	202	91.1	[86.3-94.6]	90.1	[82.1-95.4]
	3	<37.4 Ct	62.7	279	175	67.4	279	188	89.4	[84.0-93.4]	92.3	[84.8-96.9]
	4	<37.8 Ct	56.1	289	162	68.9	289	199	72.9	[66.1-78.9]	81.1	[71.5-88.6]
	5	<37.7 Ct	57.8	142	82	73.2	142	104	70.2	[60.4-78.8]	76.3	[59.8-88.6]
IgM	-	>15.2 panbio units	43.0	200	86	37.5	200	75	74.7	[63.3-84.0]	76	[67.5-83.2]
IgG	-	>11.9 panbio units	47.0	200	94	44.5	200	89	82.0	[72.5-89.4]	81.1	[72.5-87.9]

Among patients from Vietnam (N: 1,217), we estimated NS1 RDT outcomes according to DENV viremia (PCR Ct value) (Fig 2C). As PCR assays detect DENV RNA directly from the virus and NS1 RDTs detect virus-secreted proteins that peak during and after viremia, we opted to stratify logistic regression models by disease day to account for the delayed NS1 production. For each day of disease, the probability of being NS1 RDT positive increased with decreasing Ct values (increasing viremia). According to the logistic function however, as day of disease progressed, individuals were more likely to be NS1 RDT positive at lower levels of viremia. For instance, among those with a DENV PCR Ct value of 34, we estimated 22.9% [95%CI: 11.8-33.7%] were NS1 RDT+ on disease day 1 while we estimated 79.6% [95%CI: 71.4-87.6%] were NS1 RDT positive on disease day 5 (Fig 2C). This infers NS1 levels are impacted by both the amount of virus and the stage of infection. Consequently, this yielded disease-day specific NS1 RDT thresholds according to PCR Ct values which increased with disease day (Table 1). Upon predicting NS1 RDT outcomes according to disease day-stratified thresholds, we estimated 36.2% (50/138) were NS1 RDT positive on disease day 1 which corresponded to 34.8% with actual NS1 RDT positive results on disease day 1 (Sensitivity: 89.6% [95%CI: 77.3-96.5%; Specificity: 92.2% [95%CI: 84.6-96.8%]). By disease day 5, this agreement decreased slightly as 57.8% (82/142) and 73.2% (104/142) had estimated and actual NS1 RDT results, respectively (Sensitivity: 70.2% [95%CI: 60.4-78.8%; Specificity: 76.3% [95%CI: 59.8-88.6%]) (Table 1).

After generating DENV PCR and ELISA test thresholds that we estimated gave rise to NS1 and IgM/G RDT positive results, respectively, we explored how well our laboratory thresholds could estimate all 3 RDTs combined. Among the Indonesian sample population (N:200) who were tested using all 3 RDTs, we investigated the combined estimated RDT outcome agreement with the actual combined DENV RDT results (Table 2). Overall, our combined RDT outcome estimates achieved 79.5% (159/200) agreement overall. After stratifying by immune status, estimated and actual RDT agreement for primary and post-primary cases equated to 87.3% (48/55) and 78.0% (85/109), respectively. Together these results demonstrated that we were able to accurately determine the outcomes of DENV RDTs according to patient DENV laboratory metrics.

Table 2: Agreement between the estimated and actual combined DENV RDT results of patients in Indonesia.

DENV infection status	Combined RDT agreement		
	%	n	N
Age			
0-5	82.4	28	34
6-15	78.2	68	87
16-30	82.6	38	46
≥31	75.8	25	33
Disease day			
1-2	84.2	32	38
3-4	79.4	81	102
5	76.7	46	60
Serotype			
DENV-1	92.0	23	25
DENV-2	80.0	20	25
DENV-3	80.0	20	25
DENV-4	76.0	19	25
PCR-	77.0	77	100
DENV immune status			
Primary	87.3	48	55
Post-primary	78.0	85	109
Historical	70.0	14	20
Negative	75.0	12	16
Total	79.5	159	200

Combining RDTs to estimate primary and post-primary DENV status

According to the optimal dengue laboratory metric thresholds, we estimated the NS1, IgM and IgG RDT positive and negative status of study population in the Philippines which lacked RDT data (N: 28,326). For every possible RDT outcome using all three tests by disease day, we calculated the probability of being primary, post-primary and historical for dengue (Table 3). It should be noted, all those with at least one predicted positive RDT result were either primary, post-primary or historical for dengue. The most common combination of RDT outcomes in the study population was NS1-, IgM+ and IgG+ (5,745) while the least common was NS1+, IgM- and IgG+ (542). For many combinations of RDT outcomes on specified disease days, RDT results corresponded to very clear immune status outcomes. The presence of an IgG+ RDT result nearly always represented a post-primary DENV infection. For instance, on disease day 3, 100% (1,613/1,613) of patients with an estimated NS1- IgM+ IgG+ RDT outcome combination were post-primary dengue infections. At the early stages of infection (disease day 1-2), IgG negative RDT results yielded uninformative immune status outcomes. Yet towards the later stages of acute disease (disease day 3-5), IgG negative RDT results were often confirmatory of primary infections. For instance, patients with estimated NS1- IgM+ IgG- RDT

outcomes on disease days 4 and 5 had a >99% probability of being a primary case. These results reveal certain combinations of RDT results, at different stages of infection, can be confidently used to determine immune status while some combinations yield more uncertain conclusions.

Table 3: The probability of being primary, post-primary or historical for DENV according to every outcome combination of NS1, IgM and IgG RDTs stratified by disease day. RDT results estimated among patients from across the Philippines (N: 28,326).

Estimated RDT result	Disease day 1				2				3				4				5			
	Total	Probability			Total	Probability			Total	Probability			Total	Probability			Total	Probability		
		1°	2°	Hist		1°	2°	Hist		1°	2°	Hist		1°	2°	Hist		1°	2°	Hist
(1 positive RDT)																				
NS1+ IgM- IgG-	**248**	0.51	0.49	0.0	**460**	0.47	0.53	0.0	**612**	0.36	0.64	0.0	**299**	0.44	0.56	0.0	**78**	0.46	0.54	0.0
NS1- IgM+ IgG-	**180**	0.45	0.55	0.0	**224**	0.51	0.49	0.0	**364**	0.98	0.02	0.0	**376**	0.99	0.01	0.0	**270**	0.99	0.01	0.0
NS1- IgM- IgG+	**160**	0.0	0.48	0.52	**246**	0.0	0.44	0.56	**388**	0.0	0.46	0.54	**308**	0.0	0.49	0.51	**184**	0.0	0.47	0.53
(2 positive RDTs)																				
NS1+ IgM+ IgG-	**18**	0.33	0.67	0.0	**118**	0.48	0.52	0.0	**237**	0.97	0.03	0.0	**265**	0.97	0.03	0.0	**98**	0.96	0.04	0.00
NS1+ IgM- IgG+	**19**	0.0	1.0	0.0	**119**	0.0	1.0	0.0	**199**	0.0	1.0	0.0	**151**	0.0	1.0	0.0	**54**	0.0	1.0	0.0
NS1- IgM+ IgG+	**551**	0.0	1.0	0.0	**908**	0.0	1.0	0.0	**1613**	0.0	1.0	0.0	**1747**	0.0	1.0	0.0	**926**	0.0	1.0	0.0
(3 positive RDTs)																				
NS1+ IgM+ IgG+	**6**	0.0	1.0	0.0	**204**	0.0	1.0	0.0	**658**	0.0	1.0	0.0	**818**	0.0	1.0	0.0	**331**	0.0	1.0	0.0

1°: primary DENV

2°: post-primary DENV

Hist: Historical DENV

Discussion

In this study, we demonstrated that dengue rapid tests corresponded well to associated laboratory metrics and that combining different types of RDTs accurately captured laboratory-determined primary and post-primary DENV infections. At certain stages of an acute DENV infection, different combination of NS1, IgM and IgG RDT results gave rise to clear predictions of immune status, yet at other stages of disease, ambiguous immune status classifications were estimated. We found that IgG RDT positivity was almost always confirmatory of a post-primary DENV infection. In contrast, an IgG RDT negative result on fever days 1 and 2 were suggestive of both primary and post-primary infections while at fever 3 to 5 were confirmatory of a primary infection. This infers simply classifying reporting primary and post-primary DENV cases according to IgG RDT negative and positive results, respectively, would lead to immune status misclassification.

As shown previously, combining NS1, IgM and IgG DENV RDTs maximises the chances of capturing both primary and post-primary DENV infections and that using NS1 RDTs individually, risks misdiagnosing infections [17,22]. We revealed the poor performance of NS1 RDTs in diagnosing post-primary cases is attributed to the lower overall viremia post-primary experience cases during the acute phase of disease (relative to primary cases) - a trend that has been shown before [23,24]. It has been suggested that enhanced, T-cell modulated, viral clearance may account for patients with post-primary DENV to present with lower viremia than primary cases [40,41]. Alternatively, post-primary cases could just be typically reporting earlier than primary infections [42]. Concerning IgG RDTs, we found many early acute stage (fever day 1-2) post-primary infections were IgG RDT negative due to their low IgG titres. This may be a consequence of pre-elicited IgG titres rising from low levels during the early stage of a post-primary infection which are not high enough to generate a positive IgG RDT result due to elevated test thresholds [25].

In our study, we revealed individual laboratory metrics (PCR, IgM, IgG) were good predictors of corresponding NS1 IgM and IgG RDT outcomes. Despite this, we did observe some discordance between RDT results and laboratory metrics. For instance, several individuals with low antibody ELISA values still produced IgM/G RDT positive results. This might be due to the contrasting commercial brands used for the ELISAs and RDTs that rely on different epitopes present on DENV antigen that have contrasting immunogenicities. In contrast, some with elevated ELISA antibody response were negative for corresponding antibody RDTs. This trend could be attributed to ELISAs, yet not RDTs, cross-reacting with other flaviviruses including ZIKV which has shown to be potentially widespread across dengue-endemic countries [32–34]. It is now well established commercial DENV ELISAs cross-react with ZIKV [43,44] yet whether DENV antibody rapid tests cross-react with ZIKV remains poorly characterised and deserves further attention [45,46]. For NS1, we found as the disease progressed, the

probability of being RDT positive increased for any level of viremia. This is likely a consequence of the time lag between DENV viremia and NS1 secretion whereby NS1 proteins persist longer in the bloodstream than detectable nucleic acid [23,47]. This was likely a key factor for why we were less able to accurately predict NS1 RDT positivity later during the acute phase of disease. By disease day 5, our models predicted Ct value of less than 37 (very low viremia) had >50% probability of yielding a NS1 RDT positive result. Overall however, our estimated combined RDT outcomes achieved a high level of agreement with actual RDT outcomes in the Indonesian study population demonstrating we could reasonably estimate the RDT status of those without RDT data.

Our combined rapid test framework for determining primary and post-primary dengue immune status has the potential to assist dengue control efforts. It could strengthen regional surveillance systems in settings where laboratory testing is unfeasible [16]. For instance, health care workers could utilise the framework to calculate the age of those reporting primary infections to estimate and monitor the dengue force of infection as described in [28]. Furthermore, this framework could be used to inform vaccination deployment. Currently, the only fully licensed vaccine against dengue, Dengvaxia®, is recommended to those with prior dengue exposure in endemic areas aged between 9 and 45 years [48]. This is to ensure dengue-naïve recipients are not primed for a subsequent severe secondary infection by vaccination [49,50]. However, current screening methods are unable to distinguish those with one or multiple previous infection(s) [51]. Consequently, numerous individuals could be targeted, at cost, for vaccination yet would not benefit from the protection as they may have experienced multiple DENV infections beforehand. Our framework could be used to identify reporting patients with primary infections who represent suitable targets for vaccination. Moreover, monitoring the age of reporting primary infections in certain settings could be informative for population-based pre-vaccination screening. If in high endemicity areas patients report with their first dengue infection at an earlier age than 9 years, this could warrant other younger children in these areas for pre-vaccination screening.

Our rapid test framework also has the potential to benefit dengue case management. Given a secondary DENV infection is a risk factor for severe disease [5,52], determining immune status using these simple point-of-care tests could assist health care workers in prioritising patients for further monitoring and additional supportive treatment [10]. However, it should be noted that most suspected dengue cases who report to health facilities are post-primary DENV infections as these infections are associated with more symptomatic outcomes than primary infections [53,54]. Consequently, prioritising all post-primary patients for additional severe disease monitoring in health care facilities would likely be unviable. Nonetheless, there are other potential prognostic markers of severe disease, including serum chymase [55] NS1 [56] and RNA/proteins [57]. Furthermore, it has been previously shown that post-primary dengue infections under the age of 10 years are at greater risk of severe disease than those over ten years [28]. Therefore, whether this immune status rapid test framework could be integrated with

other prognostic markers into an early severe disease warning system, such as those described in [58,59], warrants further investigation.

There are some noteworthy limitations associated with this study. Firstly, our results are limited to the commercial diagnostics used in this analysis. Other commercial kits may have varying sensitivities and specificities that may yield slightly contrasting results. Despite this, our work provides a methodological framework for other kits to be evaluated. Secondly, the accuracy of this immune status RDT framework was based on a laboratory immune status framework that had 90.5% serological agreement with the gold standard WHO method for categorising primary and secondary DENV [12]. Therefore, our accuracy estimates are likely slightly overestimated. Lastly, our combined RDT outcome classification of immune status is based upon estimated, not actual, RDT results. This was necessary as just estimating the immune status based on the minority with actual RDT results would yield less confident results.

Conclusion

We describe methods for estimating the primary and post-primary immune status of dengue patients at the point of care, using a combination of simple-to-use rapid diagnostic tests. Using all three NS1, IgM and IgG RDTs, we demonstrate how at certain stages of infection health care workers and surveillance operations could confidently determine types of DENV infections. It is hoped our framework might lead to improved dengue case management and disease surveillance by identifying those who may benefit from close monitoring and could be utilised to estimate dengue transmission intensity.

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Competing interests

I have read the journal's policy and the authors of this manuscript have the following competing interests: MVL, GHT, JM, FR and LVM are employees of Johnson & Johnson.

Data availability

The data used in this study are available from the corresponding author, on reasonable request, following approval from appropriate institutional ethical committees (Joseph.biggs@hotmail.co.uk).

Author Contributions

Conceptualisation: JRB, AKS, ECdlP, MRZC, CDP, RTS, JCRH, MLH

Data Curation: JRB, AKS, JA, MSS, NLS, HATN, DDA, SM, SHa,

Formal analysis: JRB

Funding acquisition: AKS, MVL, GHT, JM, FR, LVW, LMY, SHu, ECdlP, MRZC, CDP, RTS, JCRH, MLH

Investigation: AKS, JA, MAJR, MAQ, WJW, HKM, LTL, HDT,

Methodology: JRB, JA, OJB, JM, FR, LVW, JCRH, MLH

Supervision: AKS, AOT, CI, NK, MVL, LMY, ECdlP, MRZC, CDP, SHu, RTS, JCRH, MLH

Visualisation: JRB

Writing – Original Draft Preparation: JRB, AKS, OJB, RTS, JCRH, MLH

Writing – Review and Editing: All

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Appendix 4: Chapter 6 Supplementary material

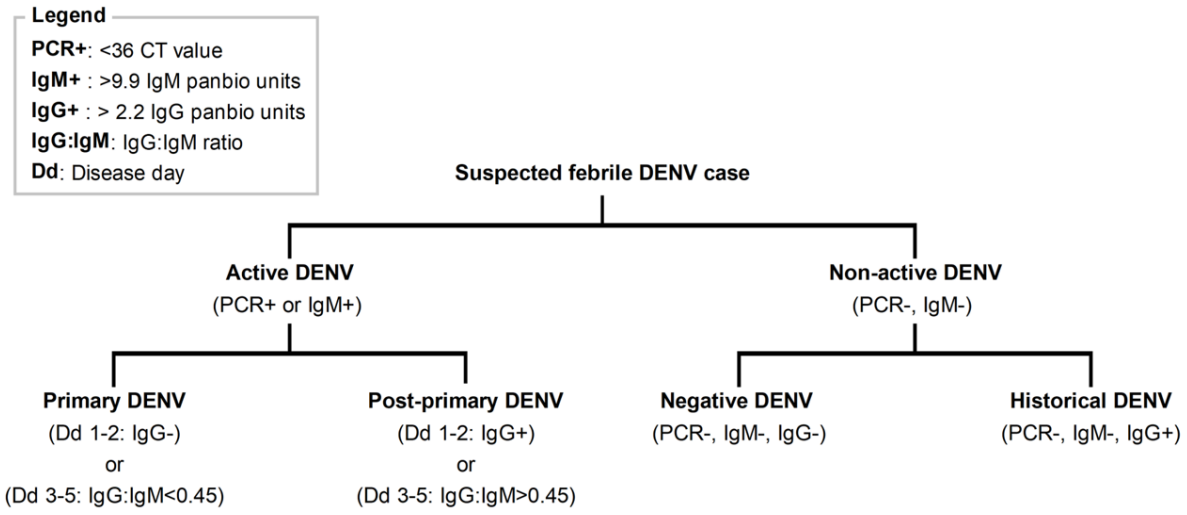
S1 Table. A summary of data collected from suspected dengue patients included in the study population from Indonesia, Vietnam and the Philippines.

Variables	Indonesia (N:200)	Vietnam (N:1,217)	Philippines (N: 28,326)
Age	+	+	+
Sex		+	+
Symptoms		+	+
Outcome			+
Disease day	+	+	+
DENV PCR status	+	+	+
DENV serotype status	+	+	+
DENV NS1 ELISA	+		
DENV NS1 RDT	+	+	
DENV IgM ELISA	+	+	+
DENV IgM RDT	+		
DENV IgG ELISA	+	+	+
DENV IgG RDT	+		

+: Data collected

S1 File. An overview of the methods used to characterise the DENV primary and post-primary immune status according to laboratory test methods.

The primary and post-primary immune status of the sample population categorised according to a previous developed algorithm [1]. Suspected dengue patients either PCR positive or with IgM panbio units≥9.9 were classified as active dengue infections, while patients PCR negative and with IgM panbio units<9.9 were considered non-active dengue infections. Among active dengue infections, those on disease day 1 or 2 with IgG panbio units above and below 2.2 panbio units were categorised as post-primary and primary, respectively. Active cases on disease 3-5, with IgG:IgM ratios above and below 0.45 were classified as post-primary and primary, respectively. Non-active dengue infections were further classified as historical or negative for dengue if they had IgG panbio units above and below 2.2 panbio units, respectively. Post-primary dengue infections include infections with at least one previous flaviviral infection:

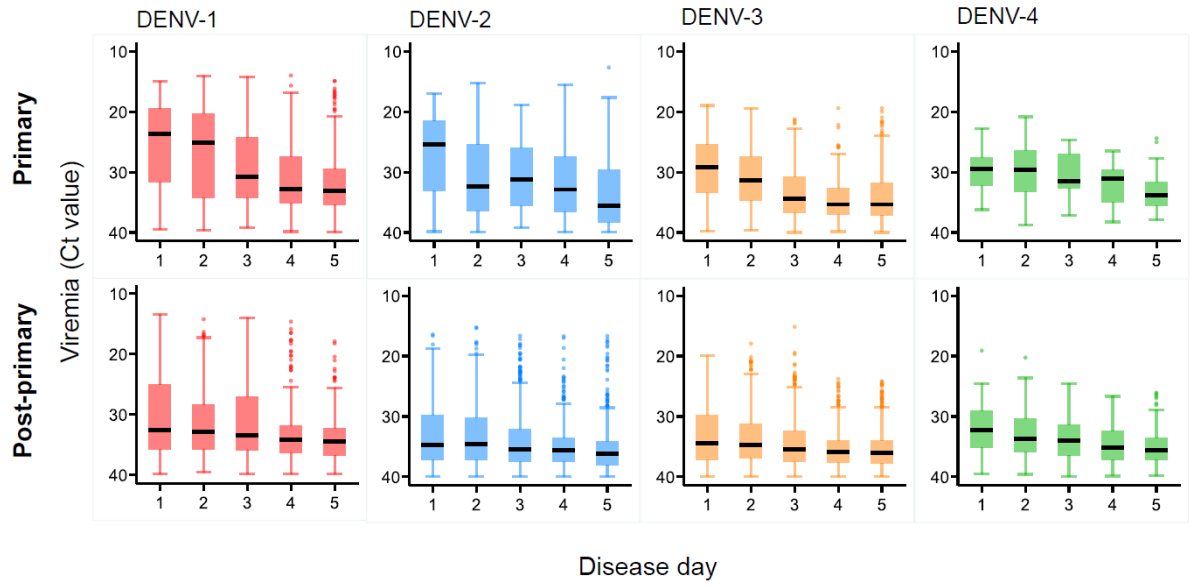


Reference

1. Biggs JR, Sy AK, Brady OJ, *et al*. A serological framework to investigate acute primary and post-primary dengue cases reporting across the Philippines. *BMC Med*. 2020; 18: 364.

S2 Table. Demographic characteristics of the reporting study population at enrolment. Includes suspected dengue case reports who reported in Indonesia (N:200), Vietnam (N:1,217) and the Philippines (N: 28,326).

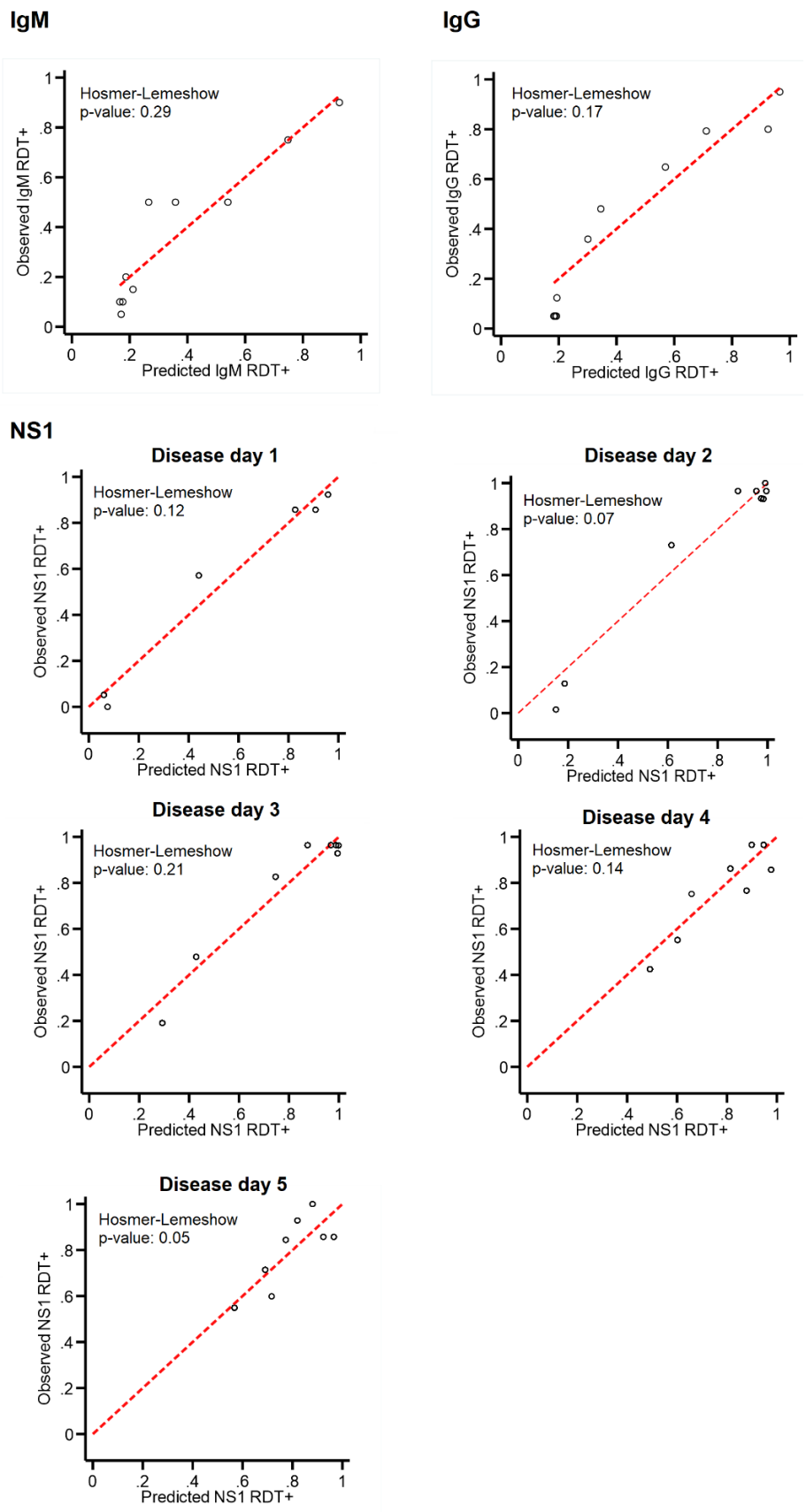
Demographics	Dengue patients					
	Indonesia		Vietnam		Philippines	
	%	n	%	n	%	n
Age						
0-5	17.0	34	16.9	206	16.8	4654
6-15	43.5	87	33.4	406	44.5	12367
16-30	23.0	46	31.7	386	28.1	7795
≥31	16.5	33	18.0	219	10.7	2975
Disease day						
1-2	19.0	38	35.4	431	25.8	6562
3-4	51.0	102	58.3	710	62.6	15932
5	30.0	60	6.2	76	11.7	2974
Serotype						
DENV-1	12.5	25	24.4	196	9.9	2632
DENV-2	12.5	25	30.0	241	10.5	2787
DENV-3	12.5	25	0.5	4	17.1	4535
DENV-4	12.5	25	11.5	92	3.4	889
PCR-	50.0	100	33.6	270	59.1	15651
DENV immune status						
Primary	27.5	55	14.7	156	19.3	4388
Post-primary	54.5	109	48.7	517	60.7	13826
Historical	10.0	20	21.9	232	13.0	2964
Negative	8.0	16	14.8	157	7.0	1599



S1 Fig. Box-plots displaying disease day stratified patterns in serotype-specific viremia (Ct value) among primary and post-primary dengue cases from across the Philippines (N: 28,326).

S3 Table. Sensitivity (95%CI) of NS1 RDTs in capturing DENV1-4 infections stratified by immune status among patients from Vietnam (N:1,217).

DENV Serotype	N	NS1 RDT+		
			Sensitivity	
		n	%	[95%CI]
DENV-1	152	139	91.4	[87-95.9]
Primary	54	54	100.0	
Post-primary	98	85	86.7	[80-93.5]
DENV-2	256	239	93.4	[90.3-96.4]
Primary	74	73	98.6	[96.0-100]
Post-primary	182	166	91.2	[87.1-95.3]
DENV-3	15	13	86.7	[69.5-100]
Primary	5	5	100.0	
Post-primary	10	8	80.0	[55.2-104.8]
DENV-4	90	81	90.0	[83.8-96.2]
Primary	11	11	100.0	
Post-primary	79	70	88.6	[81.6-95.6]



S2 Figure: Hosmer–Lemeshow plots used to assess the logistic regression model fits for IgM, IgG and NS1 RDTs. P-values >0.05 infer good model fit.

Chapter 7. Discussion

7.1 Summary of Research Findings

The findings in this thesis demonstrate how routine immuno-epidemiological characterization of dengue case reports can be enhanced to strengthen surveillance operations in endemic countries. A key objective in the 2012-2020 WHO 'Global strategy for dengue prevention and control' highlights the importance of integrating contrasting surveillance operations to better describe the burden of dengue to assist in allocating and evaluating control interventions [1]. In the Philippines, laboratory dengue epidemiological surveillance, alongside typical epidemiological surveillance, is currently in operation [2,3]. Case reports are collated by a centralised surveillance system, coordinated by the Philippine epidemiological Bureau, while additional cross-sectional surveys of case reports, from across the country, are conducted by separate laboratory surveillance at the RITM [2]. The research described in this thesis sought to investigate how laboratory data from suspected dengue case reports could be utilised to 1) Determine the individual DENV immune status (primary vs post-primary) of the reporting population (**Chapter 3**). 2) Monitor dengue transmission intensity over space and time (**Chapter 4**). 3) Describe ZIKV transmission dynamics and immunological interactions with DENV (**Chapter 5**). 4) Investigate whether rapid tests can be utilised to determine immune status at the point-of-care (**Chapter 6**). A summary of the thesis key findings and recommendations are highlighted in Figure 1.

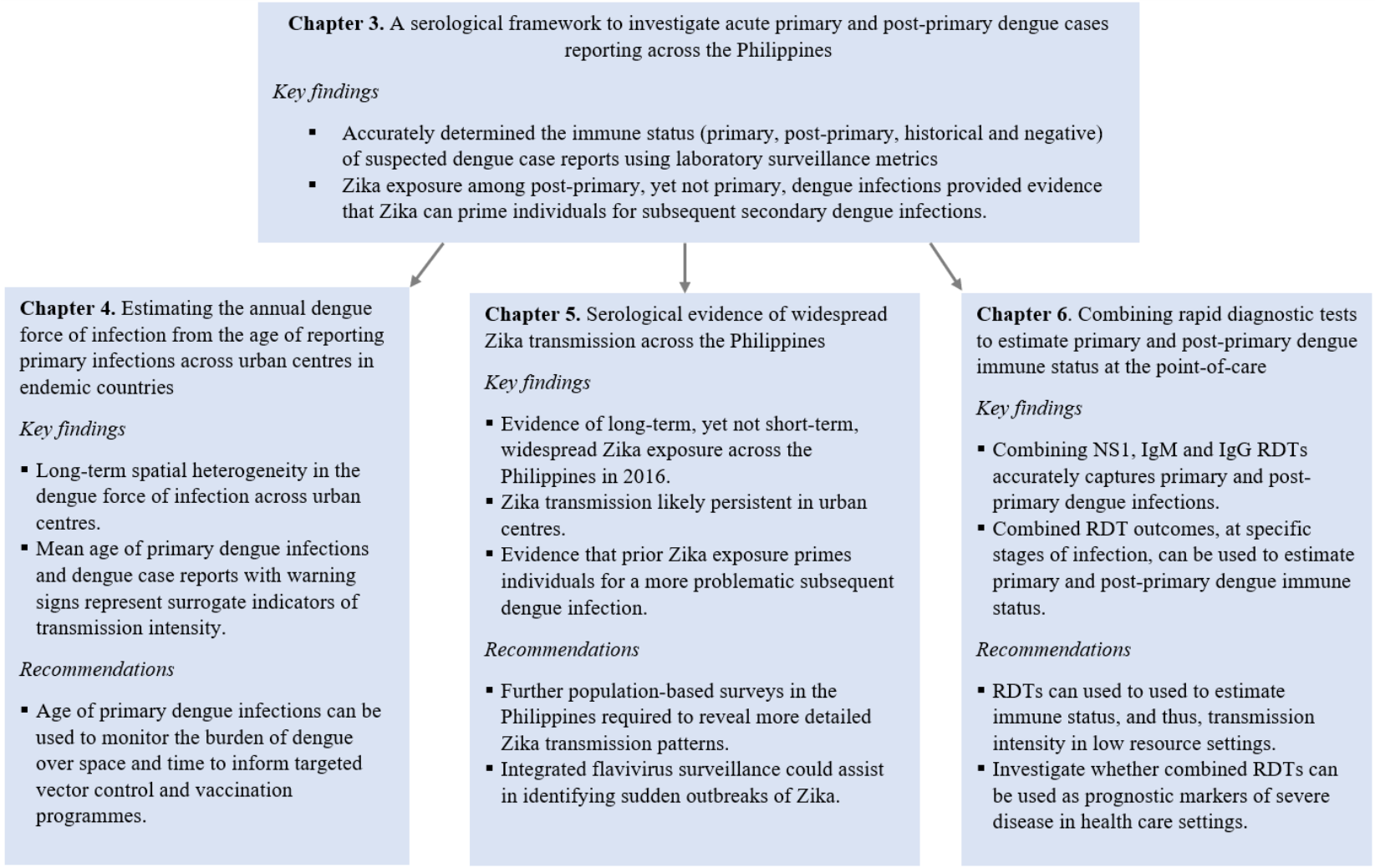


Figure 1. Summary of the key thesis research findings and recommendations.

In **Chapter 3**, assayed serum samples, obtained from reporting dengue case reports, were analysed to generate and validate an algorithm capable of categorising DENV immune status. An analytical pipeline summarising this immune status algorithm is shown in Figure 2. By combining individual molecular and serological metrics and utilising IgG cut offs/IgG:IgM ratios at appropriate stages of infection, suspected dengue case reports were categorised as primary, post-primary, historical and negative for DENV. The novel algorithm proved superior to existing commercial practise according to the WHO gold standard using paired sera [4] and captured early-stage, non-immunogenic primary infections that would otherwise have been missed using solely serological methods [5–7]. In addition, we identified evidence of long-term, IgG exposure to ZIKV among post-primary, rather than primary DENV cases, suggesting other flaviviruses can prime individuals for a secondary-like infection upon their first exposure with DENV, a finding also suggested in [8–10]. Among those who reported with suspected dengue, yet were not active DENV infections, we were able to estimate the force of infection according to the accumulation of long-term IgG exposure with age using catalytic modelling. This revealed a high infection burden across the country, which corresponds to findings in [11–13], although misrepresented the vast spatio-temporal heterogeneity in dengue burden at lower administrative levels.

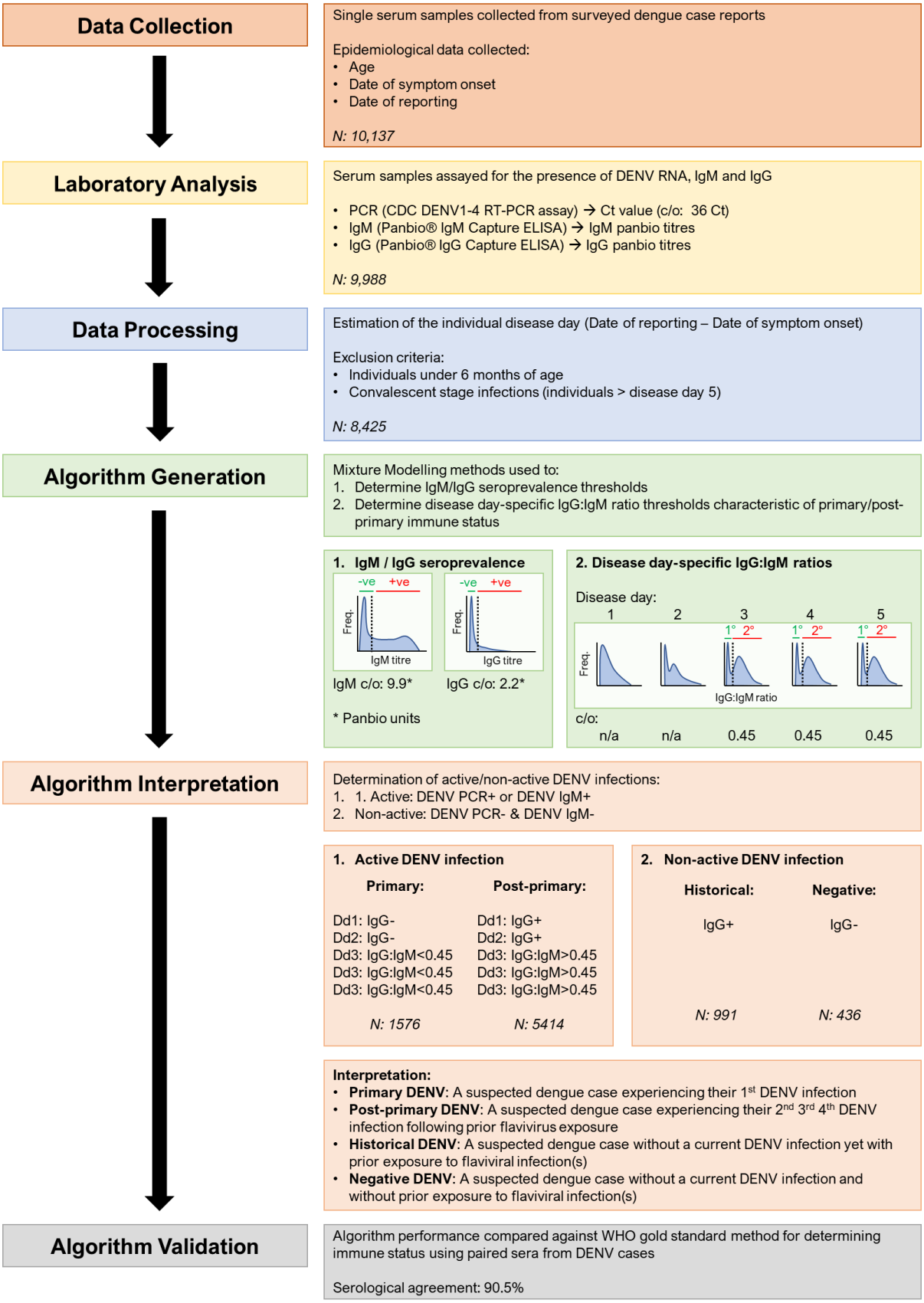


Figure 2. An analytical pipeline summarizing the process of determining the primary and post-primary immune status of suspected dengue cases described in chapter 3. 1°: Primary DENV. 2° Post-primary DENV. c/o: cut off. Dd: disease day. n/a: non applicable.

Across dengue-endemic urban centres in the Philippines, catalytic models revealed prominent spatial heterogeneity in the dengue FOI between 2014 and 2018 which has been shown previously in China [14] and Colombia [15]. City aggregated FOI estimates were then compared to both laboratory and non-laboratory dengue aggregated metrics to identify alternate surrogate indicators of transmission intensity that could be easily estimated by routine surveillance operations (**Chapter 4**). Across cities, the mean annual age of those experiencing their first (primary) DENV infection, and case reports with warning signs, correlated best with force of infection estimates according to catalytic models of age seroprevalence. In high transmission cities, the mean age at which primary DENV infections and suspected dengue cases with warning signs reported was younger compared to low transmission cities. Regression modelling was then employed to predict dengue FOI according to these averaged metrics and revealed prominent spatio-temporal heterogeneity in the transmission intensity across cities. Interestingly, no positive association was identified between long-term dengue incidence estimates and force of infection, which could be attributed to measures representing disease and infection, respectively.

In **Chapter 5**, the algorithm used to categorise DENV immune status in **Chapter 3** was utilised to investigate Zika transmission dynamics, and its immunological interactions with DENV, across the Philippines. After assaying a subset of sampled dengue case reports for anti-ZIKV IgM and IgG, no evidence of short-term ZIKV exposure was identified, although a subset of those reporting with post-primary, historical or negative DENV infections experienced evidence of long-term exposure to ZIKV. Moreover, long-term exposure to ZIKV accumulated with age among the sample population in urban, opposed to non-urban, areas across the country implying widespread persistent transmission in cities. Interestingly we found ZIKV IgG levels increased by day of disease among post-primary, yet not primary, infections suggesting ZIKV-induced ADE mechanisms when a DENV infection is preceded by a ZIKV infection. A finding coupled by the fact post-primary cases with ZIKV IgG exposure were more likely to experience adverse clinical symptoms compared to post-primary cases without ZIKV exposure, similarly identified in [10]. Despite this, the substantial IgG cross-reactivity between dengue and Zika confounded these observations as those with elevated ZIKV IgG nearly always had elevated DENV IgG and those with adverse clinical symptoms tended to experience higher levels of DENV IgG.

Lastly, as laboratory techniques have limited value in low resource settings and point-of-care diagnosis, we investigated whether dengue rapid diagnostic tests (RDTs) could be used to estimate primary and post-primary DENV immune status among reporting patients (**Chapter 6**). Testing suspected dengue patients using all three NS1, IgM and IgG rapid tests accurately captured those reporting with either primary or post-primary DENV infections. Moreover, as infection kinetics change so rapidly during a DENV infection [16], we estimated the probability of being primary or post-primary according to any RDT outcome using all three tests stratified by day of disease. Certain combinations of RDT outcomes, on certain days of infection, gave rise to clear immune status classifications. The presence of an IgG

positive RDT result was nearly always indicative of a post-primary infection, while IgG negative RDT results, particularly towards the end of the acute phase of disease, were highly suggestive of primary infections.

7.2 Opportunities for Enhanced Surveillance Operations

Given the findings in this thesis, dengue surveillance practises in the Philippines, and other dengue endemic countries, could be modified to better monitor the burden of disease and target limited control interventions appropriately. Currently in the Philippines, integrated vector control strategies are implemented by regional health authorities and targeted to areas with higher case reporting within their jurisdiction. In **Chapter 4** however, substantial discordance was identified between long-term dengue incidence and the force of infection, similarly observed in Singapore [17]. Consequently, by prioritising cities with elevated case reporting for vector control, other cities with low reporting rates, yet high levels of transmission, would be excluded from vital control interventions. Indeed, interventions are more likely to be targeted to areas with adequate surveillance systems and more disease awareness than areas of true elevated transmission intensity [18].

Instead of deploying vector control interventions to cities with higher case reporting, regional local health authorities could also target cities with an elevated dengue force of infection. These represent areas with more community transmission and would likely benefit from more IVM strategies. Moreover, routinely estimating the annual FOI among cities could aid in the evaluation of control interventions deployed in urban centres. One of the potential caveats of relying on case reporting to target and assess the impact of interventions is that community engagement programmes, often included in dengue control programmes, can heighten individual disease awareness and prompt more cases to seek care. Consequently, implemented control programmes could be associated with an increase in case reporting which would distort the true measured impact of the interventions [18]. It has already been proposed one of the major factors for increased global dengue case reporting is a consequence of improved dengue awareness in endemic countries, not just simply continued disease emergence [19,20]. Furthermore, despite the lack of randomised control trials (RCT) evaluating the impact of vector control on dengue incidence worldwide [21], two studies revealed mosquito coils and insecticide aerosols were associated with an increase, opposed to a decrease, in dengue incidence [22,23]. Investigators state heightened dengue awareness in the community may have confounded this association, however it should be noted this could be due to other factors including inadequate insecticidal activity of the interventions. It should also be noted, a cluster RCT in Nicaragua revealed a decrease in both dengue seroconversion and reported dengue incidence due to trialled community-lead vector control programmes [24]. Future studies in other dengue-endemic countries are therefore warranted to investigate whether reported dengue incidence represents a suitable surrogate indicator of transmission intensity. Furthermore, population-based dengue KAP (knowledge, attitudes and practises) surveys may

assist in identifying factors that prompt and restrict individuals from seeking care when experiencing dengue like-symptoms [25].

Routinely estimating the dengue FOI according to the mean age of those reporting with their first (primary) DENV infection is logistically more challenging than simply collating dengue case reports to characterise transmission intensity. **Chapter 3** demonstrated how centralised laboratory surveillance practises, set up across the Philippines, could be altered to characterise the immune status of reporting cases while **Chapter 4** described how the mean age of those reporting with primary infections corresponded to the dengue FOI across cities. This however relied on sophisticated laboratory procedures which are typically not feasible in regional health care settings [1]. To overcome this, **Chapter 6** explored how best to categorise the immune status of reporting cases using just simple-to-use dengue rapid tests and showed they can accurately categorise primary and post-primary DENV infections. This therefore offers regional, low resource, surveillance centres with the means to characterise immune status easily and estimate the FOI among cities in their jurisdiction. Strengthening regional surveillance operations is a key WHO operational strategy as they are the first to detect outbreaks and can coordinate the deployment control strategies more rapidly than centralised surveillance systems [1,26]. To achieve this however, NS1, IgM and IgG RDTs would need to be made accessible across all health centres in urban centres. Fortunately, these rapid tests are now widely available as combined commercial kits [27], and their widespread distribution remains an existing WHO goal [1].

It should be noted that enhanced laboratory surveillance practises alone would likely be inadequate to characterise dengue transmission intensity and inform targeted control interventions. Findings in this thesis reveal how alternations to existing laboratory surveillance operations in the Philippines can be used to monitor the annual FOI across urban centres. It remains unknown however whether the average age of those reporting with primary dengue corresponds to the FOI at finer spatio-temporal scales. Substantial intra-annual variation in dengue transmission intensity due to cyclical rainfall patterns has previously been observed in endemic countries [28–30]. Therefore, annual FOI estimates across cities would lack the sensitivity to detect sudden outbreaks of dengue. Moreover, it has previously been shown deploying vector control strategies prior to/at the beginning of an outbreak is critical to combat dengue before the disease is too widespread [31]. Consequently, it remains vital that additional systems, such as dengue outbreak warning systems, are developed and integrated into surveillance operations to ensure interventions have maximum impact [32]. Ideally, monitoring the annual FOI across cities, according to the mean age of primary dengue cases, could be used to target interventions across large administrative areas while localised outbreak warning systems could target control measures within smaller administrative units. However, this strategy would require validation prior to widespread implementation. A cluster-randomised control trial could be utilised to investigate whether this novel

surveillance strategy is both more effective and cost-effective in curbing transmission intensity compared to existing surveillance operations [21,24,33].

In addition to vector control, enhanced laboratory surveillance practises may assist in vaccination deployment. The fully licensed Dengvaxia® vaccine is recommended by the WHO for individuals aged between 9-45 years who live in dengue-endemic areas [34,35]. This criteria helps ensure those who are screened prior to vaccination are more likely to have experienced one previous DENV infection, as dengue-naïve recipients are thought to be at higher risk of developing severe symptoms upon a subsequent DENV infection [36,37]. In **Chapter 4**, the age at which individuals report with their first infection varied among dengue-endemic cities across the Philippines. In some highly endemic cities, the average age of reporting primary DENV infections was below 9 years, therefore, these cities might benefit from screening younger age groups prior to vaccination. Assessing the age range for vaccination screening has previously been suggested in [35,38] and is thought could maximise vaccine safety and effectiveness. In addition, patients who report with primary infections themselves might represent suitable vaccine recipients and could be asked to return for vaccination to help prevent patients from experiencing a potentially more dangerous secondary DENV infection in the future.

Lastly, centralised laboratory surveillance systems in the Philippines could be altered to monitor the transmission dynamics of other flaviviruses, including Zika. **In Chapter 5**, assaying those reporting with suspected dengue-like symptoms for ZIKV antibodies revealed widespread exposure to ZIKV across the country which potentially primes individuals for more adverse DENV infections. Together these findings justify further, population-based, epidemiological investigations into Zika transmission dynamics across the Philippines and indeed other dengue-endemic countries with the appropriate environmental conditions to support Zika transmission [39–41]. More specifically, as **Chapter 5** suggested Zika transmission was more persistent in urban, opposed to rural, areas of the Philippines, future Phylogenetic studies could be employed to characterise the historical spread of ZIKV across the country. Such studies have already revealed DENV outbreaks spread outwards from urban centres to rural areas [42,43], which could explain why ZIKV transmission is more established in urban areas as it is spread by the same vector [41]. This might provide evidence IVM strategies are better focused across urban centres to prevent transmission spill over into rural areas during outbreaks. Lastly, given the similarity between dengue and Zika clinical manifestations [44,45], assaying suspected dengue case reports for serological evidence of ZIKV could help identify future potential outbreaks of the disease. In **Chapter 5** however, the comparatively low number of patients assayed for ZIKV compared to DENV exposure may have resulted in a lack of active ZIKV infections being identified. Future population-based serosurveys of Zika across the Philippines would be crucial for determining the appropriate number of serum samples, collected from reporting dengue cases, to detect serological evidence of the disease [46].

7.3 Future Research Questions

Integrating arbovirus serological surveillance

In the Philippines, other arboviruses apart from dengue and Zika, including Japanese encephalitis [49] and Chikungunya [50], are co-endemic across the country. These febrile infections often present with similar acute clinical manifestations and are spread by the same *Aedes* mosquito vector [51]. Therefore renewed focus, according to the WHO, has been on strengthening integrated vector management to combat the transmission of these diseases simultaneously [1]. In the Philippines, current immuno-epidemiological surveillance for monitoring arboviruses, conducting in the RITM, involves assaying suspected patients using separate, disease specific serological ELISAs [2]. This approach however is costly, labour intensive and involves utilising commercial kits that have been shown to detect cross-reactive antibody responses [52,53]. Therefore, a more appropriate and cost-effective option might involve integrating arbovirus immuno-epidemiological surveillance using multiplex immunoassay platforms.

Multiplex immunoassays (MIAs) are high-throughput serological techniques that simultaneously detect antibody responses to a host of antigenic targets from a single serum or dried blood spot sample. Recent studies have demonstrated how MIAs can be utilised to detect antibody responses to separate flaviviruses including Dengue, Zika, West Nile virus, Yellow Fever, Tick-Borne Encephalitis and Japanese Encephalitis [54,55]. Both studies demonstrate how this approach can be used to discriminate between certain arboviral infections and describe how their antigen targets are highly immunogenic to ensure high assay sensitivity. Despite this, studies still identified cross-reactive antibody responses between ZIKV and DENV. Therefore, identifying immunogenic, arbovirus-specific antigens markers that elicit detect antibodies remains crucial. For centralised surveillance purposes, MIAs offers a range of benefits. Serum samples from patients with suspected arboviral infections could be sampled and assayed, as part of a diagnostic algorithm, to determine spatio-temporal patterns in mosquito-borne viruses across the country and assist in targeting and evaluating control interventions. This would reduce the need to orchestrate separate surveillance programs for individual arboviral infections. Moreover, determining the level of serological exposure to separate arboviruses could assist in the deployment of current and future vaccines [34,35,51].

Improving dengue prognosis

In **Chapter 6**, findings revealed how combining different types of RDTs can be used to estimate the DENV immune status of reporting patients during the acute stage of disease. In addition to benefiting surveillance operations in low resource settings, estimating the primary and post-primary immune status of patients using quick, inexpensive, and simple to use diagnostics could assist in dengue case management. According to the 2012-2020 ‘WHO global strategy for dengue prevention and control’

research focus should include improving dengue prognosis to reduce mortality [1]. Given secondary dengue is a major risk factor for progressing to severe disease outcomes [16,56], determining those with post-primary infections during the acute disease phase might assist in prioritising patients for further monitoring and treatment.

Whether utilising RDTs as severe disease prognostic markers in health care settings would reduce case mortality however requires further investigation. As shown in **Chapter 4** and elsewhere, the majority of those who report with dengue experience post-primary infections as they are more likely to experience adverse clinical symptoms and be prompted to seek care than primary infections [57–59]. Therefore, prioritising all post-primary infections that visit health facilities for further monitoring and care would likely be unviable, particularly in limited resource settings. Despite this, other factors are known to influence severe disease outcomes including age [60] serum chymase levels [61] and the persistence of NS1 [62]. In **Chapter 4**, findings revealed individuals under 10 years were at the greatest risk of severe disease. Integrating such markers into an early severe disease warning system could assist in prognosticating severe dengue. Indeed, previous studies have investigated integrated early warning systems which utilised laboratory and clinical metrics [63–65]. However, the reliance on a host of different laboratory techniques limits these approaches to high resource settings. Whether simple, and inexpensive techniques, including rapid tests, would be appropriate in the severe disease early warning system warrants further investigation.

Outbreak preparedness

According to the 2016 WHO 'technical handbook for dengue surveillance, dengue outbreak prediction/detection and outbreak response' laboratory surveillance practises can be utilised in syndromic surveillance systems to detect outbreaks. The proportion of surveyed case reports with DENV virus confirmed infections has previously been used as outbreak alarm signals in Singapore [66] and Vietnam [67]. During outbreak periods, the virus isolation rate increased as a higher percentage of those reporting experienced true DENV infections. However, given the rapidly changing infection kinetics during a DENV infection [16], not all those experiencing a DENV infection would be captured using just molecular methods. Incorporating serological markers such as IgM, which is detectable soon after the viraemic period, could improve the sensitivity of these outbreak warning systems particularly in settings where individuals delay seeking treatment. Moreover, as laboratory methods are more suitable for centralised and not regional surveillance systems [1], determining whether rapid tests can be utilised to determine the case reporting dengue positivity rate warrants future research too.

Algorithm refinement

The serological framework described in **Chapter 3** distinguishes primary from post-primary immune status among acute dengue case reports. Currently however, the algorithm is unable to differentiate secondary from, typically milder, post-secondary (tertiary and quaternary) dengue infections.

Accurately categorising those with just secondary DENV infections could have major implications for dengue prognosis. Further characterisation of antibody responses among post-primary infections could be used to further distinguish post-secondary infections, as secondary infections would likely experience excessive levels of IgG, however additional dengue Plaque reduction neutralisation tests (PRNTs) would be necessary to validate such categorisation [68,69]. Furthermore, the immune status algorithm has only been generated and validated to determine immune status among acute dengue case reports and not those who report during the later, critical stage of disease. Future longitudinal studies, investigating antibody responses over time among confirmed DENV cases could be used to better describe distinguishable antibody kinetics between primary and post-primary infections later during disease. This could be beneficial for health care settings where patients present at the later stages of disease such as hospital referrals.

7.4 Conclusions

The research presented in this thesis demonstrates how enhanced immuno-epidemiological analysis of reported dengue case reports can be utilised to improve surveillance operations in endemic countries. As dengue continues to emerge globally, its vital surveillance operations are strengthened to ensure limited control interventions are deployed appropriately. By generating and validating a novel DENV immune status algorithm and utilising it to estimate the burden of dengue over space and time, findings illustrated how this additional laboratory surveillance framework could accompany existing surveillance practises to accurately describe dengue transmission patterns. Moreover, this research described how immuno-epidemiological characterisation of dengue case reports could be used to monitor and describe co-circulating Zika transmission patterns at the sub-national levels. Lastly, results in this thesis explored methods for strengthening decentralised surveillance operations by illustrating the value of point-of-care rapid tests in determining immune status in low resource settings. Together it is hoped these strategies can lead to more informed targeting of control interventions to reverse the continued global expansion of dengue globally.

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Appendices

Appendix 5. Philippine dengue case report form

Philippine Integrated Disease Surveillance and Response

Case Report Form
Dengue (ICD 10 Code: A90-A91)

Region: ____________________ **Province**: ____________________ **Municipality/City**: ____________________

Name of DRU: ____________________ **Type**: ☐RHU ☐CHO ☐Gov't Hospital ☐Private Hospital ☐Clinic

Address: ____________________ ☐Private Laboratory ☐Public Laboratory ☐Seaport/Airport

Patient No.	Patient's Full Name	Age	Sex (F/M)	Date of Birth	Complete Address	Admitted?	Date admitted/seen/consulted	Date onset of illness	Type	Case classification	Outcome
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
Response Codes / Instructions	Indicate First name, Middle name, Last name	*Age*: Indicate D - days M - months Yr. - years *Sex*:F - Female M - Male		mm/dd/yy	Specify Street/Purok/Subdivision, House #, Barangay, Municipality/City, Province	Y - Yes N- No	mm/dd/yy	mm/dd/yy	W—with warning signs N– no warning signs S-Severe Dengue	S - Suspect P - Probable C - Confirmed	A - Alive D - Died (specify date) U - Unknown

Clinical Case Definition/Classification:
Dengue without Warning signs.

- ***Suspect***
A previously well person with acute febrile illness of 2-7 days duration plus two of the following:
Headache, Body malaise, Myalgia, Arthralgia, Retro-orbital pain, Anorexia, Nausea, Vomiting, Diarrhea, Flushed skin, Rash (petecheal, Herman's sign)
- ***Probable***
A suspect case **plus:**
Laboratory test, at least CBC (leucopenia with or without thrombocytopenia) and/or Dengue NS1, antigen test or dengue IgM antibody test (optional)
- ***Confirmed***:
  - Viral culture isolation,
  - Polymerase Chain Reaction

Dengue with Warning Signs

,A previously well person with acute febrile illness of 2-7 days duration plus
any one of the following:

- Abdominal pain or tenderness
- Persistent vomiting
- Clinical signs of fluid accumulation
- Mucosal bleeding
- Lethargy, restlessness
- Liver enlargement
- Laboratory: increase in Hct and/or decreasing platelet count

Severe Dengue

A previously well person with acute febrile illness of 2-7 days duration and any of the clinical manifestations for dengue with or without warning signs,
Plus any of the following:

Severe plasma leakage leading to
- Shock
- Fluid accumulation with respiratory distress

Severe bleeding

Severe organ impairment
- Liver: AST or ALT ≥1000
- CNS: e.g. seizures, impaired consciousness
- Heart: e.g. myocarditis
- Kidneys: e.g. renal failure

Philippine Integrated Disease Surveillance and Response

Case Report Form
Dengue (ICD 10 Code: A90-A91)

Patient No.	Patient's Full Name	Age	Sex (F/M)	Date of Birth	Complete Address	Admitted?	Date admitted/seen/consulted	Date onset of illness	Type	Case-Classification	Outcome
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
				___/___/___			___/___/___	___/___/___			
Response Codes / Instructions	Indicate First name, Middle name, Last name	*Age*: Indicate D - days M - months Yr. - years *Sex*:F - Female M - Male		mm/dd/yy	Specify Street/Purok/Subdivision, House #, Barangay, Municipality/City, Province	Y - Yes N- No	mm/dd/yy	mm/dd/yy	W - with Warning signs N– no warning signs S—Severe Dengue	S– Suspect P- Probable C – confirmed	A - Alive D - Died (specify date) U - Unknown