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Epidemiological conditions to support dengue vaccine introduction in Asia using data from traditional and novel sources

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Declaration

I declare that:

a) the work in this thesis is my own;
b) that I made a substantial contribution to each of the underlying manuscripts, with my and others' contributions to each described below;
c) all co-authors have been informed of my intention to use our co-authored work;
d) this work has not been submitted for any other degree or professional qualification.

13nd May 2021

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Publications submitted in support of PhD by Publication

1. Nealon J, Taurel A, Capeding MR, et al. Symptomatic Dengue Disease in Five Southeast Asian Countries: Epidemiological Evidence from a Dengue Vaccine Trial. PLoS Negl Trop Dis. 2016;10(8):e0004918. doi:10.1371/journal.pntd.0004918

2. Wahyono TYM, Nealon J, Beucher S, et al. Indonesian dengue burden estimates: review of evidence by an expert panel. Epidemiol Infect. 2017;(May):1-6. doi:10.1017/S0950268817001030

3. Garg S, Chakravarti A, Singh R, et al. Dengue serotype-specific seroprevalence among 5- to 10-year-old children in India: a community-based cross-sectional study. Int J Infect Dis. 2017;54:25-30. doi:10.1016/j.ijid.2016.10.030

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10. Nealon J, Bouckenooghe A, Cortes M, et al. Dengue Endemicity, Force of Infection, and Variation in Transmission Intensity in 13 Endemic Countries. J Infect Dis. March 2020. doi:10.1093/infdis/jiaa132

Abstract

Dengue, an *Aedes* mosquito-borne flavivirus, is the most common vector-borne viral infection worldwide. Infections result in around 100 million (95% credible interval: 67 - 136 million) clinical episodes and 10.5 million (95% uncertainty intervals: 4.1 – 22.7 million) hospitalizations annually, mostly within the Asia Pacific region. There are four closely related viral serotypes, all of which cause severe disease. First infections are often mild/asymptomatic but subsequent infections with heterologous serotypes more frequently result in severe episodes and hospitalisations. National surveillance systems are not designed to estimate the full disease burden and the value of preventive measures is therefore poorly defined.

In 2014 the first dengue vaccine, CYD-TDV, was licensed having demonstrated significant efficacy against all four serotypes in study participants aged 2 – 16 years. Subsequent analyses after 5 years' follow-up revealed a more complex vaccine profile with superior protection in individuals seropositive for dengue, but an elevated risk of hospitalized dengue in seronegative vaccine recipients. Population-level benefit-risk is therefore dependent on epidemiological criteria and WHO recommends its use only under certain conditions or following confirmation of individual serostatus.

This thesis describes a body of epidemiological research designed to improve understanding of these conditions. Studies were intended to fill important knowledge gaps identified following discussions with local vaccine policymakers and experts. My role, working within a multidisciplinary team, was to understand identified gaps and develop and implement a corresponding research agenda to fill them. I designed and contributed to protocols, provided oversight to their implementation working with local research teams, analysed and interpreted the resulting data and drafted the manuscripts included in this thesis which were published between 2016 and 2020.

These studies used different methods: we analysed existing data sources to improve estimates of symptomatic dengue disease burden; measured age-stratified dengue seroprevalence in children in India and Indonesia; estimated force-of-infection (the annual rate at which seronegative individuals acquire infection) as an indicator of endemicity in

seven Asian countries; and conducted a prospective surveillance study to plan future dengue vaccine effectiveness research. After refining these methods for dengue, we extended them to Japanese encephalitis, another mosquito-borne flavivirus.

Using data derived from active case ascertainment from the placebo arm of a paediatric clinical trial, wherein parents were contacted weekly and reminded to report to study sites in case of febrile illness in their children, we identified a crude dengue attack rate of 4.6%/year. Only 29% of these events were clinically diagnosed as dengue by study investigators, indicating that most symptomatic disease fails to satisfy existing case definitions. This active case ascertainment captured a greater proportion of symptomatic dengue than national passive surveillance systems. The ratio between these two rates ("expansion factor") can be used to estimate the full disease burden from passive surveillance reports and we calculated factors ranging from 0.5 – 31.7, depending on country and case definition.

Large seroprevalence surveys in India and Indonesia confirmed very high rates of paediatric infection: by the age of 10 years, 73% of children in India and 79% in Indonesia had been infected at least once. We also identified serological evidence for circulation of multiple dengue serotypes in both countries. We used these and other serological data to estimate force-of-infection which varied widely between countries from 1.7% (Singapore) to 24.1% (the Philippines), with significant heterogeneity within countries. The force of infection of Japanese encephalitis was much lower (varying from 0.8% in Malaysia to 5.2% in Vietnam) but this demonstration of transmission in urban areas was an important finding in areas where Japanese encephalitis vaccination is not routine. After conducting a hospital-based surveillance study to plan future dengue vaccine effectiveness studies in Malaysia, we concluded that test-negative case control studies are not feasible due to small numbers of test-negative controls; and that case control studies for dengue vaccines could be significantly biased by underlying differences between cases and controls.

In summary, these studies demonstrated intense but heterogeneous dengue transmission across multiple Asia-pacific countries. These levels of transmission are broadly compatible with recommendations for dengue vaccine introduction at the national

level but, due to heterogeneity in endemicity, more local approaches would likely be needed before implementation of mass vaccination programmes.

Lay summary

Dengue fever is a disease caused by a virus passed between people by the bites of infected “tiger” mosquitoes. The disease is often not serious, causing only mild fever, but can cause more serious illness and severe cases need to spend time in hospital and in rare cases, they can die. The mosquitoes which transmit the disease can only live in warm, humid climates, so cases are concentrated in tropical areas and most occur in Asia. There are four types of dengue caused by slightly different viruses. After infection with one virus people develop life-long immunity and so it is possible to become sick a maximum of four times in somebody’s life. The total number of cases which occurs globally is not known because many of the cases are never diagnosed.

There is one vaccine available which can prevent dengue, but the vaccine can cause people who have never had dengue before to have a higher likelihood of more severe cases when infected. The WHO therefore recommends the vaccine should only be used in populations with a high probability of having been previously infected. This thesis describes research which was conducted to help understand which areas of Asia have the most dengue and therefore would be the most appropriate places to use the dengue vaccine. I reanalysed data which had been generated from other studies and worked with study teams to collect new data to make estimates of the number of people becoming sick with dengue each year. We estimated the number of people who had been historically infected, by looking for specific antibodies indicating previous dengue infection in the blood of healthy people and conducted similar calculations for Japanese encephalitis, a similar virus causing disease in Asian countries. Finally, we conducted a study in Malaysian hospitals, to help us plan future studies to monitor how well dengue vaccines are working.

We estimated that around 5% of children in some Asian cities became sick due to dengue every year. However less than 1/3 of those cases were correctly diagnosed. Our method of actively looking for dengue identified many more cases – up to 30x more – than were reported to Asian governments during their routine healthcare activities.

We found that by the age of 10 years, 73% of children in India and 79% in Indonesia had circulating dengue antibodies indicating they had been infected with dengue before. It was possible from these and other data to calculate the proportion of children who became infected annually, which varied from 1.7% of children (in Singapore) to 24.1% (in the Philippines). Japanese encephalitis infections were much less common, but we still identified circulating virus in urban areas where it had been believed the virus was rare. While planning a study to monitor if dengue vaccines prevent disease as they should, in Malaysia, we found those studies would be easily affected by methodological problems and proposed solutions to fix those problems.

In summary, these studies demonstrated that dengue circulates very widely and that children are infected frequently across multiple Asia-pacific countries. While vaccines could help to minimize this disease, not all areas would be suitable for vaccine use without prior population testing and governments will need to consider the best methods of vaccine roll-out.

Abbreviations

Ag	Antigen
ADE	Antibody dependent enhancement
AIC	Akaike's information criterion
CC	Case control
CI	Confidence interval
CRF	Case report form
DENV	Dengue virus
DENV-1, 2, 3, 4	Dengue virus serotypes 1, 2, 3 and 4
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DSS	Dengue shock syndrome
ELISA	Enzyme linked immunosorbent assay
EF	Expansion factor
FOI	Force of infection
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IR	Incidence rate
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
NS1	Dengue non-structural antigen 1
OR	Odds ratio
PRNT	Plaque reduction neutralization test
p/y	Person-years
RDT	Rapid diagnostic test
RMSE	Root mean square error
SAGE	Strategic Advisory Group of Experts
TN	Test negative
VCD	Virologically-confirmed dengue
VE	Vaccine effectiveness
WHO	World Health Organization

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Self-critical review

Introduction

Dengue is the most common vector-borne viral infection worldwide, and the neglected tropical disease responsible for the highest number of deaths and life years lost.[1,2] A mosquito-borne flavivirus, infections result in around 100 million clinical episodes, and likely results in 10.5 million hospitalizations annually.[3–5] Approximately 75% of the at-risk population resides within the Asia Pacific region where the primary vectors (*Aedes aegypti* and *Ae. albopictus)* and dengue virus have become widely dispersed over recent decades following social, environmental, and demographic changes resulting in cyclical outbreaks every 3-5 years.[6–8] The disease is caused by one of four closely related dengue viral serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) all of which circulate in tropical Asian countries.[9] Infection results in a wide and unpredictable range of clinical presentations from mild/asymptomatic flu-like illness, progressing to acute, febrile, and severe/haemorrhagic disease and rarely, death.[6,10] Disease prevention efforts with mosquito control have been largely unsuccessful and recent decades have witnessed increased disease frequencies and expanded ranges of transmission.[5,11,12] Dengue is now endemic in over 120 countries worldwide, with almost half of the global population at risk.[1,5]

Population-level risk factors for high disease frequencies include urbanization, high population density and presence of *Aedes* mosquito vector breeding sites.[13] At the patient level, disease severity depends upon the presence of heterologous antibodies from a previous infection, viral characteristics, and the age and genetic background of the infected individual.[14] Until 2009, the World Health Organization (WHO) recommended classifying clinical episodes as classical dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS).[6] While this classification remains in clinical use in some countries, a new system was proposed by WHO in 2009 primarily to improve triage and clinical management, and to capture warning signs of potentially severe dengue episodes.[6,15]

An important feature of pathogenesis, particularly important for the design of vaccination strategies, infection with one serotype provides only transient cross-protection against the others and second infections have a higher likelihood of being symptomatic and/or severe.[16,17] This phenomenon predisposes individuals to more severe disease during chronological and/or immunological windows of sub-neutralizing antibody concentrations, probably by a mechanism known as antibody dependent enhancement (ADE), or other mechanisms, arising from infection with heterologous viral serotypes.[16–19] Following a given infection the likelihood of suffering mild or more serious symptoms is therefore a complex function of ecological and immunological factors with time-varying risk windows, determined by the underlying transmission intensity. Immunological interaction with other flaviviruses may also affect disease severity and complicate diagnosis.[20,21]

In most scenarios, national surveillance systems which provide the majority of dengue epidemiological data underestimate disease burden due to the non-specific clinical presentation of dengue; unavailability and limitations of confirmatory diagnostic tests; and health system issues that result in incomplete reporting.[3,5,22,23] More reliable estimates are required to guide disease control programs, allow rational allocation of resources, and to assess the impact of new interventions such as dengue vaccination. Accordingly, estimating the full disease burden was one of the WHO's three objectives in the 2012 *Global Strategy for Dengue Prevention and Control 2012–2020*.[5]

Several methods used to improve the accuracy of dengue burden estimates including capture-recapture studies, expert consensus-based approaches, statistical and/or cartographic models incorporating dengue occurrence data or their covariates, regression methods incorporating independent variables from neighbouring countries, and derivations from seroprevalence data.[3,24–26] Most influentially, a 2013 study by Bhatt *et al.* used a cartographic modelling approach combining demographic and epidemiological data, adjusted for clinical severity and determinants of dengue incidence to estimate a global burden of 390 million (95% credible interval: 284–528 million) infections in 2010, of which 96 million (67–136 million) were symptomatic.[3] About 70% of the global burden of apparent infections were in Asia, with India contributing 34% of the total. Overall, this analysis may overestimate the number of dengue infections in some countries, such as

in Hong Kong where >300,000 episodes were estimated in the absence of notified episodes, and underestimate it in others: in the USA, the study predicted zero dengue transmission whereas local transmission occurs along the US-Mexico border and in Florida.[27,28]

While case fatality rates are low, morbidity is high and no specific treatment exists. A vaccine could therefore have significant economic as well as public health value. Dengue vaccine clinical development has been ongoing for many decades and, despite the availability of other flavivirus vaccines, has been complicated, most importantly by the requirement for vaccines to provide balanced immunity to all four serotypes.[29] In 2014 the first dengue vaccine was licensed (Dengvaxia®; Sanofi Pasteur) indicated for vaccination of individuals aged from 9 – 60 years in most countries.[30,31] Important additional information about this vaccine was published in 2015 indicating its superior efficacy in children seropositive for dengue,[32] and an elevated risk for hospitalized dengue in seronegative vaccine recipients.[33] These findings further complicate the decision to introduce dengue vaccination into public health programmes because vaccine performance and safety are products of complex epidemiological indicators which, in endemic countries in which the vaccine could provide most value, are often poorly defined. Important data gaps include: age-specific seroprevalence data and sub-national heterogeneity in endemicity; the frequency, severity and cost of symptomatic episodes; and the relative economic benefits of dengue vaccination compared with other healthcare interventions.

As with any new vaccine, post-licensure monitoring of safety and effectiveness will be needed but, because effectiveness monitoring of a dengue vaccine has never been conducted, robust study designs have not been validated. Case control studies have been widely used for vaccine effectiveness (VE) monitoring, including for vaccines against influenza;[34] Japanese encephalitis;[35] whooping cough,[36] and pneumococcal pneumonia.[37] The test-negative design is a variant whereby suspected cases with negative laboratory results – and who are therefore considered absent of the outcome of interest – are used as controls. This design has been used extensively for evaluating the effectiveness of influenza vaccines[38–41] and other vaccines.[42–44] Theoretical work has

been conducted to understand potential biases associated with these study designs,[39,45,46] but never for dengue. Given the clinical and epidemiological specificities of dengue, uncertainties exist in terms of clinical presentation and diagnosis, healthcare practice and underlying epidemiology exist which warrant a preparatory study.[47]

Japanese encephalitis virus (JEV) is another mosquito-borne flavivirus, transmitted by *Culex* mosquitoes (of which *Culex tritaeniorrhynchus* is the most important) , distributed across east, south-east and south Asia existing in zoonotic cycles with pigs and ardeid birds in which humans are incidental, dead-end hosts.[48,49] Most infections are asymptomatic or mild and are unlikely to be diagnosed as Japanese encephalitis (JE) due to a combination of low level of clinical suspicion and infrequent use of laboratory confirmation.[50,51] Symptomatic episodes are severe including encephalitis and neurological disorders, convulsions, prolonged seizures, respiratory abnormalities and spasms.[50] In hospitalized individuals around 30% will die, and around 50% of survivors will suffer severe residual neurological disease.[50,52,53] Several licensed vaccines with excellent efficacy [of 80% – 99% following complete schedules] are available and vaccination is recommended both for those living in and traveling to endemic areas.[54] Under-recognition of disease contributes to under-vaccination and the full range of transmission exceeds the geographic areas where vaccination is routine.[48,54]

A 2011 JE global disease burden estimate reviewed existing literature and extrapolated based on country archetypes to estimate 67,900 incident symptomatic cases per year across affected countries.[55] A revised estimate in 2020 incorporated age-stratified notification rates, force of infection (FOI) estimates and vaccination coverage in a modelling study to estimate 100,308 cases (95% CI: 61,720–157,522) after considering the protective benefits of vaccination.[56] These relatively modest case numbers mask the contribution of JE to long-term encephalitis complications,[57] which contributed to up to 1 million DALYs per year.[51] The disease also causes peaks in morbidity and mortality over a short period of time during focal outbreaks, which result in long-term disability and suffering, for example in Northeast India.[58]

Evidence of the range and intensity of transmission is limited and, for the reasons mentioned above, difficult to ascertain from surveillance. Serological assessments to

measure JEV infection frequency – and minimize bias – were conducted, including in naïve US servicemen, in East Asia in the 1940s – 1960s[59,60] and, more recently, have been performed to understand levels of seroprotection.[61] Following our work on dengue FOI estimation, upon realizing that JEV and dengue serology data were both available from an age-stratified paediatric population, we adapted the analytical methods for dengue to describe JE endemicity and the implications of serological cross-reaction on such assessments.

The economic value of a vaccine is broadly equal to the burden of disease, including the societal aspects and long-term sequalae, that vaccination would prevent.[62] This thesis describes a body of work which contributes to understanding of dengue and JE frequency, severity and infection history and, therefore, the burden of vaccine-preventable disease.

Thesis structure and scope

Dengue vaccine development has been ongoing for several decades. As the reality of a dengue vaccine became closer several years ago, policymaking organizations began to consider the necessary and/or ideal conditions for dengue vaccine use in endemic areas, and the information needed to direct vaccination programmes to populations who would benefit most. Many of those consultations focused on dengue epidemiological conditions supporting vaccine use which, in developing Asian countries, had previously been relatively poorly defined.[23]

Notable policy guidance included: an expert meeting in late 2010 of the "Dengue vaccine-to-vaccination" (Dengue v2V) Asia-Pacific group in Singapore;[63] recommended 'points for consideration' for dengue vaccine introduction, published in 2016, by the Dengue Vaccine Initiative;[64] and conclusions of a WHO-initiated call for mathematical modellers to estimate the long-term public health impacts of dengue vaccination, which reported results in 2016.[65] These reports documented several key areas for epidemiological research:

1. Documenting complete morbidity and mortality data to quantify and monitor public health impacts of vaccination, including proportions of inpatient and severe cases; age, geographical and serotype distributions
2. Dengue seroprevalence estimates to identify areas suitable for vaccination and which would provide the greatest public health impact
3. Protocols supporting the conduct of vaccine-effectiveness studies with appropriate case definitions and study methods.

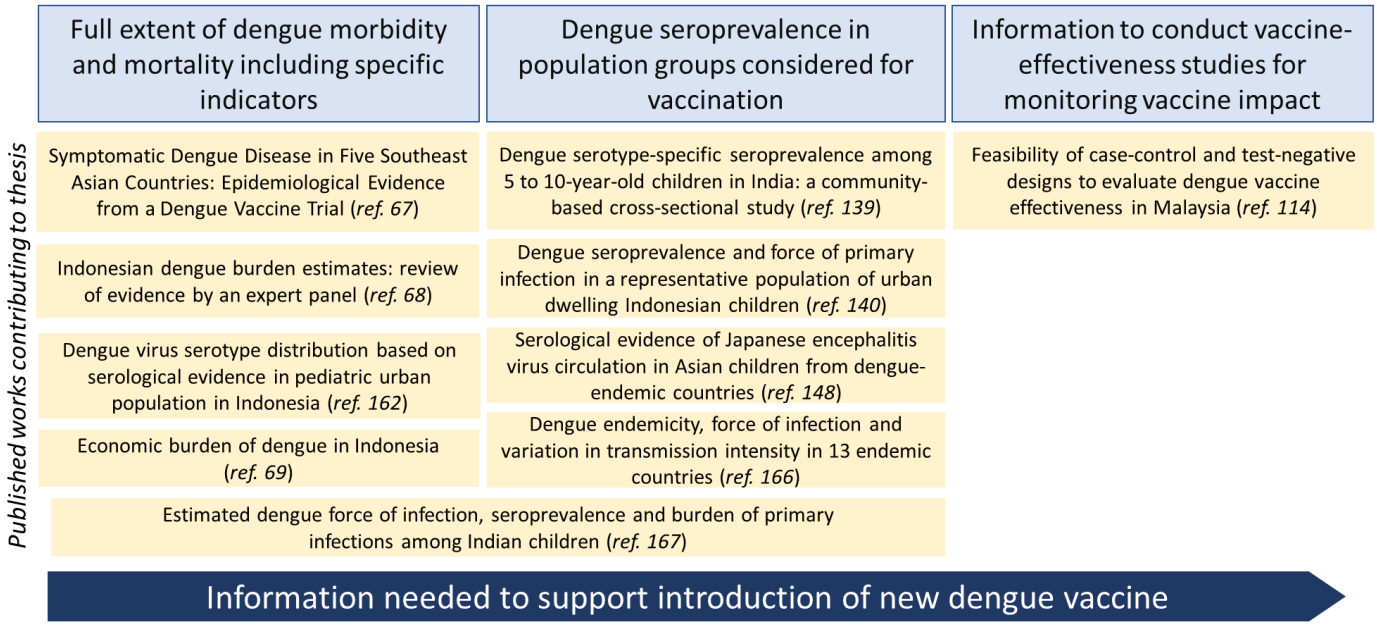
This thesis is comprised of a series of published works which were conducted to respond to the topics outlined above, as described in figure 1. Having developed these methods for dengue, we applied some to JE, a closely related endemic flavivirus, in a study which shed light on the applicability and limitations of these methods. The component papers contribute to discussions around dengue vaccine introduction and effectiveness monitoring, across a spectrum of disease severity. The focus of the thesis is on the Asia Pacific region, and therefore includes modifications of some published figures and tables

which originally included data from Latin America. Broadly aligned with the themes above, the thesis is divided into four chapters followed by discussion; with the individual manuscripts in the portfolio supporting one or more chapters of the thesis:

1. Symptomatic dengue disease burden estimation in Asia
2. The feasibility of different dengue vaccine effectiveness study designs
3. Dengue seroprevalence, serotype distributions and levels of endemicity suitable for vaccine introduction
4. Using serological data to infer dengue and JE infection rates and estimate seroprevalence at a given age
5. Discussion, conclusions and recommendations for future research

The thesis describes a range of studies, methods and results which directly and indirectly contributed to decisions around the introduction of the world's first dengue vaccine in the countries which are most-severely affected by the disease.

Figure 1: *Identified epidemiological priorities for dengue vaccine introduction; and publications within this thesis which contribute to each priority.*



Ethical approvals and civil society consultations

All studies described in this thesis underwent local governmental and institutional ethics review in the countries where the studies were conducted as indicated in each manuscript and clinical trial identifiers are included in appendix 1. Ethics committees provided review of informed consent and assent forms which were signed by study participants, parents or legal guardians before participation, in compliance with the regulations of each country.

Participant informed consent forms always clearly identified the study sponsor (Sanofi Pasteur) and described the analyses which would be performed with biological specimens and data. In addition, participants were asked for consent to use their blood specimens for unspecified further research (but never human genetic testing) and they were free to decline this permission but remain enrolled in the studies. Participants were also informed that their anonymized personal data may be transferred overseas and communicated with health authorities in other countries.

Accordingly, we did not consult with ethics committees when conducting reanalyses described in this thesis which were covered by the conditions above, partly because these studies were expected to result in a large number of secondary publications due to its numerous secondary and exploratory objectives included in the protocols. However the environment with respect to data privacy and legal definitions of “anonymous” data within the European General Data Protection Regulation may have changed slightly since these studies were planned and additional consultations with ethics committees may be advisable in case of future analyses. For prospective studies whose primary papers contribute to this thesis, consent was explicitly requested to use data for analyses to improve understanding of flavivirus epidemiology, and the ethical approvals for those studies are included in appendix 2.

Studies were conducted in accordance with the latest revision of the Declaration of Helsinki, the Guidelines for Good Epidemiology Practices,[66] and local regulatory requirements.

We did not involve patient and public representatives or civil society at large in the design or interpretation of the research presented within this thesis. All data were generated and

interpreted with local investigators from study cites and sites who, in addition to their scientific inputs, contributed local cultural expertise.

Chapter 1: Symptomatic dengue disease burden estimation in Asia

Published works contributing to this chapter

1. Nealon J, Taurel A, Capeding MR, *et al*. Symptomatic Dengue Disease in Five Southeast Asian Countries: Epidemiological Evidence from a Dengue Vaccine Trial. *PLoS Negl Trop Dis* 2016; 10(8):e0004918.[67]
2. Wahyono TYM, Nealon J, Beucher S, *et al.* Indonesian dengue burden estimates: review of evidence by an expert panel. *Epidemiol Infect*. 2017; (May):1–6.[68]
3. Nadjib M, Setiawan E, Putri S, *et al.* Economic burden of dengue in Indonesia. *PLoS Negl Trop Dis*. 2019; 13(1):e0007038.[69]

Introduction: symptoms, severity and surveillance

National dengue surveillance systems in Asian countries are designed for dengue outbreak response and detection, and to monitor changes in epidemic activity over time.[5] They monitor the fluctuation of case numbers and dengue serotypes over time but – because dengue symptoms are non-specific, confirmatory diagnostics are under-utilized, and reporting may not be complete – they under-estimate the full burden of disease.[22,23] More precise data are required to guide disease control, allow rational allocation of resources, and assess the impact of new interventions such as dengue vaccination. Accordingly, "estimating the true disease burden" constitutes one of the WHO's three objectives in the Global Strategy for Dengue Prevention and Control 2012–2020.[5] Important progress has been made. Notably, prospective cohort studies utilizing active surveillance, which yield more complete estimates of symptomatic disease than passive surveillance systems,[70] have been conducted in association with tropical medicine research institutes for many years in Thailand,[71] Cambodia,[72] Indonesia,[73] the Philippines,[74] and Vietnam.[75] These studies are important because they provide high-quality and granular data which have subsequently been incorporated into other comparative, regression-based or expert-informed analyses of disease burden at the country or regional levels.[76–79]

The first global dengue burden estimates derived from formal quantitative methods were published in 2013. Bhatt *et al*. used data from these cohort studies and other geo-localized dengue occurrence data and incorporated them with a subset of environmental

and socioeconomic covariates into a cartographic model estimating dengue incidence on a fine spatial scale, adjusted for clinical severity.[3] They estimated a global burden of 390 million (95% credible interval: 284–528 million) infections in 2010, of which 96 million (67–136 million) were symptomatic. This was followed in 2016 by another global estimate from the Global Burden of Disease group of 58.4 million (95% uncertainty interval: 23.6 – 121.8 million) symptomatic cases per year.[80] The difference in results can be accounted for by methods – Bhatt *et al*. extrapolated from incidence captured in cohort studies whereas GBD models used verbal autopsy data and estimated case numbers based on case fatality rate of milder cases. Confidence/uncertainty intervals of the estimates overlap, and the exercises provide overall confidence in a global symptomatic disease burden in the range of 50 – 100 million cases/year. Both estimates provide country-specific data but their relevance to local policy is hindered by complex methods, inaccuracies in some geographies induced by global assumptions, and unclear relation to local environments which local policymakers know well.

A relatively straightforward estimation of disease burden can be made by multiplying the incidence rates (IRs) of disease captured from routine surveillance systems by a multiplication or expansion factor (EF) which describes the level of under-estimation of symptomatic cases.[81,82] In Cambodia, Thailand, and the Philippines, individual studies calculated EFs for dengue of between 7.2 and 9.1.[78,83] A review using data from all WHO regions found dengue EFs in Asia of up to 126, with significant variation among countries and over time resulting from differences in underlying epidemiology, surveillance practices, and comparative study design.[81]

Dengue vaccine clinical trials are conducted with a high degree of operational integrity and produce data closely resembling those from epidemiological studies. Participants allocated to the placebo group do not receive dengue vaccine, so incidence data from these individuals can be interpreted as an observational cohort.[84] CYD14 was a phase III, observer-blinded dengue vaccine study conducted in 2011–2013 in 10,275 children aged 2–14 years in Indonesia, Malaysia, Thailand, the Philippines, and Vietnam.[30] In Nealon *et al.* 2016,[67] we used this comprehensive dataset to describe dengue incidence according to different clinical endpoints to examine the relationship between disease

frequency and severity in five Asian countries. We also made comparisons with national surveillance reports to estimate EFs for symptomatic dengue of different clinical severities, from which broader burden estimates can be inferred.

Where robust empirical data are unavailable, expert opinion-based methods have been used to estimate disease burden.[78,82] Responding to epidemiological data gaps in Indonesia, in Wahyono *et al.,* and Nadjib *et al*., we used an expert consensus method to estimate unknown parameters and combined them with subnational-level surveillance data to make national-level dengue disease burden and cost-of-illness estimates.

Aims and objectives

Aim: to improve dengue disease burden estimates, stratified by severity and setting, in Asian countries

Specific objectives:

1. Use the control arm of a large vaccine clinical trial to estimate dengue incidence and under-reporting according to different case definitions in five Asian countries
2. Apply expert opinion in a structured consultation to estimate unknown parameters needed to calculate dengue under-reporting, and apply these to estimate disease burden in Indonesia

Methods

Symptomatic disease from surveillance

In Nealon *et al.* (2016),[67] we used data generated in a randomized, phase 3 dengue vaccine trial in children aged 2 – 14 years old in Indonesia, Malaysia, the Philippines, Thailand and Vietnam.[30] We analysed data from the placebo arm which was equivalent to a 25-month epidemiological cohort study. Participants were followed up with active surveillance for episodes of fever ≥ 38°C on ≥ 2 consecutive days which were clinically diagnosed by investigators as DF or DHF according to WHO 1997 case definitions.[85] Serum samples were taken for virological confirmation of dengue (NS1 ELISA and/or RT-PCR), allowing febrile episodes to be grouped into four case definitions:

1. Clinically diagnosed dengue (CDD) as diagnosed, irrespective of the laboratory result
2. Virologically confirmed dengue (VCD), irrespective of the clinical diagnosis
3. Clinical VCD (cVCD) where a clinical diagnosis was laboratory-confirmed
4. Undifferentiated fever VCD (UF-VCD) where a laboratory-confirmed febrile episode was not diagnosed as dengue by the investigator

Site- and age-weighted incidence densities of each outcome and their 95% CIs (based on the gamma distribution) were calculated for each of three age groups (< 5 years; 5 −10 years; and > 10 years) by dividing the number of cases satisfying each case definition observed in the study by the number of person-years of observation.[86] We compared these observed rates with average annual rates reported from passive surveillance at the sub-national level (from districts, provinces, or cities) encompassing each clinical trial centre, after adjusting for population changes and demographic limitations.[87–91]

EFs and their CIs were calculated by dividing the adjusted ID captured during CYD14 for each case definition by the IRs reported by the national passive surveillance systems.[92] We additionally described hospitalization rates in participants satisfying different case definitions; and the positive and negative predictive value and sensitivity/specificity of clinical diagnoses, using VCD as the gold standard.

Indonesia-specific estimates

The Indonesian estimates generated above lack the specificity required for more granular and locally accepted health economic analyses, including the frequency of mild cases which never report to healthcare facilities. In Wahyono *et al*.[68] we used the estimates from Nealon *et al*.(2016), and other available data sources to inform a Delphi panel (a structured process which aims to achieve a convergence of expert opinion through review of iterative group estimates[93]) aiming to estimate the burden and characteristics of dengue at the national level in Indonesia.

Fourteen multi-disciplinary experts from across Indonesia were presented updated information on dengue epidemiology and sources of uncertainty, before being asked to provide their opinions on the following questions:

Q1: What percentage of dengue cases enter a healthcare facility to seek treatment?

Q2: Of all dengue cases entering a healthcare facility, what proportion are diagnosed as dengue?

Q3: Of dengue cases diagnosed in a healthcare facility what proportion are then reported in the routine Indonesian dengue surveillance system statistics?

Q4: Of dengue cases entering an Indonesian healthcare facility, what proportion are hospitalized for any duration?

Q5: Among all dengue cases entering healthcare facilities, what proportion are seen in the public sector if: a) hospitalized; b) outpatient (i.e., ambulatory)

Anonymous responses were collected, presented and discussed. Participants were invited to revise their opinions twice based on the opinions expressed. Medians of final round votes were used for analysis, and a bootstrapping resampling method (200 samples) employed to provide variability based on the theoretical non-parametric distribution of observed values, enabling estimation of medians and their 95% CIs.[94] These medians were used to estimate: 1) the total number of symptomatic dengue cases occurring in Indonesia; 2) the overall EF of total: reported cases; 3) EFs stratified by hospitalized or ambulatory cases; and 4) the proportion of cases seen in public vs private facilities. National level estimates were calculated by multiplying these EFs by the number of reported dengue cases in Indonesia, from 2006 – 2015.[95]

In Nadjib *et al*.,[69] we used Indonesian provincial passive surveillance data, adjusted by factors estimated in Nealon *et al*.(2016), and Wahyono *et al*., to make provincial- and national-level dengue burden estimates of cases requiring hospitalization, by:

$$Cases_{hosp} = C * EF_t * P_h$$

and for non-hospitalized cases by:

$$Cases_{ambulatory} = C * EF_t * 1 - P_h$$

Where C is the total number of reported cases at provincial or national level; EF_t is the overall Indonesian expansion factor estimated in Nealon *et al*. (11.5) and P_h is the estimated proportion of cases requiring hospitalization from Wahyono *et al*. (60%).

Results

Symptomatic disease from surveillance

Between June and December 2011, 3,424 children were enrolled in the placebo arm of the CYD14 study contributing 6,933 person-years of observation time. There were 3,099 febrile episodes of which 319 (10.3%) were VCD. This proportion varied in each country from 6.3% (Malaysia) to 12.3% (Indonesia). The overall crude annual VCD attack rate was 4.6%, varying from 2.2% (Malaysia) to 6.6% (Philippines); outcome frequencies according to different case definitions are provided in table 1.1.

Table 1.1 *Number and proportions of febrile episodes (in brackets) of febrile episodes satisfying different case definitions in the CYD14 control arm, June 2011 – December 2013*

Country	n	Febrile episodes	Person-years	VCD episodes*	cVCD episodes*	CDD episodes*	UF-VCD*	% of VCD diagnosed as dengue	VCD attack rate, %
Indonesia	623	357	1232	44 (12.3)	26 (7.3)	33 (9.2)	18 (5.0)	59.1	3.6
Malaysia	465	332	937	21 (6.3)	9 (2.7)	11 (3.3)	12 (3.6)	42.9	2.2
Philippines	1166	1420	2370	156 (11.0)	16 (1.1)	19 (1.3)	140 (9.9)	10.3	6.6
Thailand	392	388	792	47 (12.1)	35 (9.0)	36 (9.3)	12 (3.1)	74.5	5.9
Vietnam	778	602	1602	51 (8.5)	6 (1.0)	9 (1.5)	45 (7.5)	11.8	3.2
Totals	3,424	3,099	6,933	319 (10.3)	92 (3.0)	108 (3.5)	227 (7.3)	28.8	4.6

n = number of participants

Of the 319 VCD cases, 92 (29%) cases were diagnosed by investigators as dengue. This proportion varied widely among countries, from 10.3% (Philippines) to 74.5% (Thailand). Adjusted dengue incidence densities in the CYD14 study were considerably higher than the rates captured by the national systems and varied according to the case definition used (table 1.2). Incidence densities per 100,000 person-years (p/y) were highest for VCD

(range: 2,048 [Malaysia] to 10,960 [Philippines]), UF-VCD (range: 1,192 [Indonesia] to 10,290 [Philippines]), CDD (range: 701 [Philippines] to 4,383 [Thailand]), and cVCD (range: 261 [Vietnam] to 4,262 [Thailand]).

The IRs for all dengue cases (per 100,000 p/y) reported to national routine surveillance systems for the study locations during the study period varied by country from: 64.7 (Malaysia); 263 (Indonesia); 497 (Thailand); 509 (Vietnam); and 954 (Philippines) (Table 1.2).

Table 1.2. *Dengue incidence rates from routine surveillance systems and adjusted incidence densities (95% CIs in italics) of disease according to different case definitions from active surveillance. Cases/100,000 person-years.*

Country	Average IR from surveillance	Incidence densities captured from active surveillance, by case definition			
		VCD	cVCD	CDD	UF-VCD
Indonesia	262.9	3,017 *1,951- 4,542*	1825 *996 - 3,153*	2,479 *1,483 - 3,963*	1,192 *602 - 2,269*
Malaysia	64.7	2,048 *1,099 - 3,720*	671 *288 - 1,851*	777 *367 - 1,961*	1,377 *567 - 2,993*
Philippines	954.4	10,960 *8,673 - 13,620*	677 *262 - 1,439*	701 *281 - 1,461*	10,290 *8,055 - 12,890*
Thailand	496.7	5,938 *4,273 - 8,059*	4,262 2,914 - 6,055	4,383 *3,015 - 6,194*	1,676 *808 - 3,065*
Vietnam	509.2	2,784 *1,813 - 4,238*	261 *94 - 1,083*	840 *156 - 2,371*	2,523 *1,580 - 3,964*

These different rates gave rise to EFs of 5.5−31.7 for VCD, 0.5−10.4 for cVCD, and 0.7−12.0 for CDD (Table 1.3).

Table 1.3. *Expansion factors for VCD, cVCD, and CDD over the active phase of the CYD14 study.*

Country	VCD [95% CI]	cVCD [95% CI]	CDD [95% CI]
Indonesia	11.5 [7.4, 17.3]	6.9 [3.8, 12.0]	9.4 [5.6, 15.1]
Malaysia	31.7 [17.0, 57.5]	10.4 [4.5, 28.6]	12.0 [5.7, 30.3]
Philippines	11.5 [9.1, 14.3]	0.7 [0.3, 1.5]	0.7 [0.3, 1.5]
Thailand	12.0 [8.6, 16.2]	8.6 [5.9, 12.2]	8.8 [6.1, 12.5]
Vietnam	5.5 [3.6, 8.3]	0.5 [0.2, 2.1]	1.7 [0.3, 4.7]

Overall, 126 (4.1%) of the acute febrile episodes in the cohort were hospitalized, varying between countries from 1.2% (Vietnam) to 8.8% (Thailand). Hospitalization rates varied by case definition: 61 (19.1%) of the 319 VCD episodes; 62 (57.4%) of the 108 CDD cases; and 24 (96.0%) of the 25 VCD cases diagnosed as DHF, were hospitalized. These proportions varied between countries. The median duration of clinical symptoms was 5.0 days [min; max: 2.0; 38.0] for cases of UF-VCD; 6.0 [2·0; 38·0] for VCD and 8.0 [2.0; 31.0] for CDD.

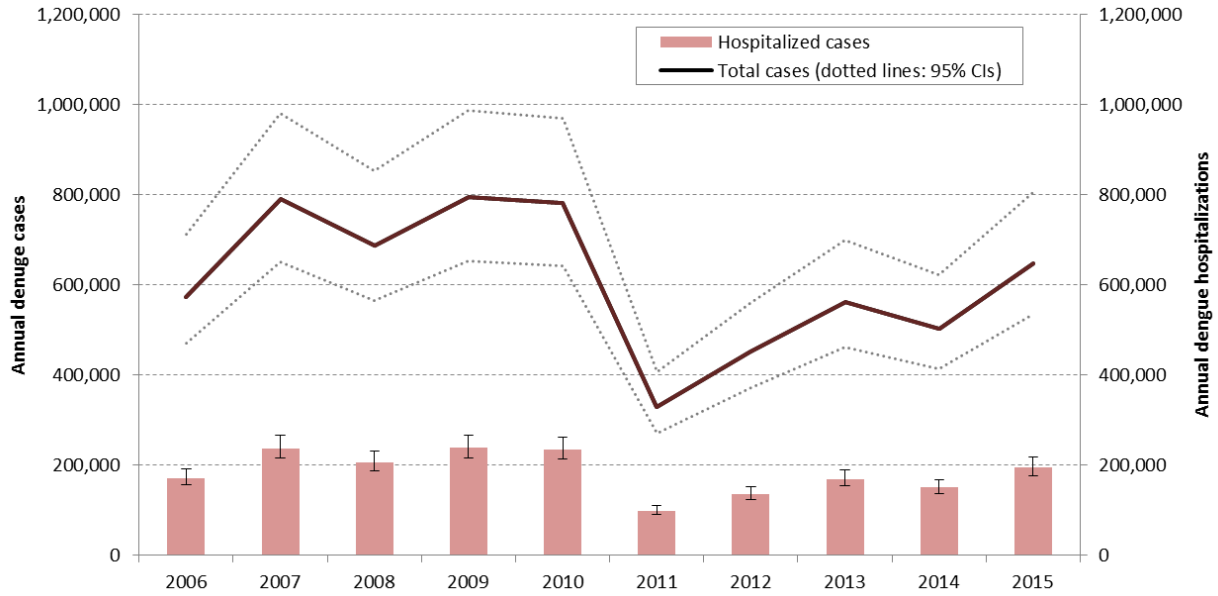
The positive predictive value (PPV) of clinical diagnosis, using VCD as the gold standard, was 85.2% (95% CI 77.1−91.3) and the negative predictive value (NPV) was 92.4% (91.4−93.3). The sensitivity of clinical diagnosis was 28.8% (95% CI 23.9−34.2) and the specificity was 99.4% (99.1−99.7).

Indonesia-specific estimates

Delphi panel participants estimated that of all symptomatic Indonesian dengue episodes, 57.8% (95% CI: 46.6–59.8) enter healthcare facilities to seek treatment; 39.3% (32.8–42.0) are diagnosed as dengue; and 20.3% (95% CI 16.1–24.3) are subsequently reported in the surveillance system. These estimates gave an overall EF of 5.0; hospitalized EF of 1.6; and ambulatory EF of 34.0 which, when combined with passive surveillance data, equates to an annual average (2006–2015) of 612,005 dengue cases, and 183,297 hospitalizations (figure 2). In the ten years from 2006 – 2015 we estimated a cumulative total of 3,537,238 (CI: 2,854,797 – 3,657,332) cases entering health

facilities; 2,476,067 (CI: 1,986,082 – 2,577,322) dengue diagnoses and 1,832,969 (CI: 1,665,785 – 2,052,687) hospitalizations, 1,164,543 of which are seen in the public sector.

Figure 2: *Estimated annual number of dengue cases and hospitalizations in Indonesia following adjustment of surveillance reports with EFs, and their 95% confidence intervals (CIs), 2006 - 2015*



In Nadjib *et al*., a cost-of-illness study, a slightly different method was used, incorporating empirical under-reporting estimates from our clinical trial reanalysis.[67] Final estimates of the disease burden combined the value from expert opinion that 60% of dengue cases were hospitalized; with our empirical observation of 11.5-fold under-reporting to generate final expansion factors for hospitalized (EFH; 7.65) and ambulatory dengue (EFA; 45.90). Provincial level totals aggregated to a national Indonesian symptomatic dengue burden of 898,475 hospitalized and 596,391 ambulatory cases in 2015.

Conclusions and critical assessment

We estimated the incidence rate of symptomatic dengue episodes in 5 Asian countries according to case severity and other characteristics, after adjustment for disease under-reporting in passive surveillance data. We found that systematic laboratory confirmation of febrile episodes gives rise to approximately 3.5-times more cases than clinical confirmation according to existing case definitions and practices. When comparing with rates reported from surveillance systems, active surveillance captures up to 30-times as many episodes. Because most passive disease surveillance systems in Asia rely almost entirely on clinical diagnosis,[23,96] a substantial proportion of symptomatic dengue disease is unrecognized and therefore unreported. Of dengue diagnoses, most were virologically confirmed, resulting in a PPV of clinical diagnosis of 85.2%. However, the sensitivity of a clinical diagnosis was 28.8%, reflecting the considerable proportion of VCD which was not clinically diagnosed as dengue. This is likely because local DF or DHF reporting case definitions had not been satisfied, even when investigators suspected dengue as the underlying aetiology of mild or non-specific febrile episodes. These episodes are unlikely to result in hospitalization or death but can be important for public health both from an overall health economic perspective, and because mild cases have been shown to contribute significantly to transmission.[97]

In Indonesia specifically, our results confirm previous reports that dengue is significantly under-reported and provide granularity which was previously lacking. However, the overall magnitude of under-reporting (5-fold) was modest in comparison with other studies. Notably, Undurraga *et al*. estimated under-reporting factors of 7.6 from a literature review and regression-based extrapolation to neighbouring countries;[25] Toan *et al*. found factors of 36 – 126, comparing published cohort studies with passive surveillance data on WHO websites;[81] Kosasih *et al*. empirically measured a factor of 43 from a factory-based cohort;[98] and we identified a factor of 11.5 from the control arm of a clinical trial.[67] Global dengue burden modelling studies estimated national burdens from which Indonesian EFs of 57 and 106, respectively, can be derived[3,80] and in 2019, O'Reily *et al*. combined the estimates above to generate national-level estimates of 7.8 million cases, approximately 10-times as many cases as our estimates.[99]

The magnitude of these under-reporting differences observed in Indonesia and elsewhere is difficult to reconcile and highlight a clear difference between studies extrapolating from local data or experience; vs those which use mathematical models, applying incidence estimates across a broad geographical scale. For example, O'Reily *et al.* used a model incorporated data from prior studies including ours, describing dengue i) occurrence, ii) incidence and iii) FOI to generate estimates of endemicity on a 5x5km cartographic scale, and adjusted the likelihood of suffering dengue of a given case definition by the population of the entire country. Modelling initiatives also allow the application of milder case definitions – corresponding to events which cannot easily be identified in real-life studies – thereby increasing sensitivity. Bhatt *et al*. use a case definition of apparent dengue with "sufficient severity to modify a person's regular schedule, such as attending school".[3] The authors liken this to a definition used in cohort studies but our clinical trial required fever of ≥38°C for inclusion, a definition which is likely more specific. The GBD study used what is probably a more severe case definition for mild cases corresponding to "infectious disease, acute episode, moderate" with a mean duration of 6 days but with no definition of fever.[80]

In contrast, the local experts participating in our Delphi panel, mostly clinicians, are likely familiar with more severe, less common presentations of medically-attended dengue. Global models are of undoubted global policy value but make strong assumptions which are not applicable to every geography and contrast with our local approach comparing age-specific rates from the provincial or lower administrative levels. O'Reily *et al*. estimated 1.1 million (220,000 - 2.9 million) hospitalized cases implying that – even of dengue cases requiring hospitalization – only 12% are reported in national statistics.[99] Considering the level of clinical suspicion applied to dengue and the frequent use of confirmatory diagnostics in hospitalized settings in Indonesia, this scale of under-reporting seems inplausible.[100] Complex modelling methods reporting extremely high disease burdens are also of questionable local policy relevance because they differ so dramatically from local realities. Allocation of resources is decided based on competitive healthcare priorities and adoption of these model-based burden estimates would require resource reallocation to prevent dengue, a decision which would be irrational without comparable models for other healthcare priorities.

Ours was the first study using local, age-stratified incidence and passive surveillance data to allow precise incidence estimates according to different case definitions and improve understanding of the full spectrum of dengue disease. Using identical protocols and laboratory methods in different countries provides confidence the results are comparable. Local experts from all included countries were included as study authors, improving our ability to interpret the results and their policy-relevance. However, we included only ~3,400 children across five countries, a sample size inadequate to generate precise estimates of more severe, less frequent outcomes observed, and a strict definition of fever was applied, resulting in a relatively specific case definition. Generalizability is also limited because sites were chosen for their historically high reported dengue burdens; results from lower-endemic areas may differ.[101] We also cannot exclude the impact of these ongoing clinical studies on case reporting or under-ascertainment; if active studies increased the proportion of cases captured in surveillance this would bias underestimation estimates towards the null.

In summary, these analyses were performed to inform policy and strengthen evidence for public health decisions, including financing for dengue control efforts such as vaccination. They enrich available evidence indicating that passive surveillance systems greatly underestimate dengue burden and emphasize that burden estimates are highly sensitive to case definitions.

Critical reflection

The initial work (Nealon *et al.* 2016) in this chapter was driven by my realization that available data from a clinical trial could be used to generate high-quality EFs, according to different case definitions, and therefore make granular descriptions of under-reporting and burden estimates. Whilst multipliers like this had been calculated before including for influenza[102] and dengue,[25] I believe this had not been done before from clinical trial data, and the approach was subsequently replicated in Latin America[103] and then from influenza trials[104] which has validated the approach. The advantage of using clinical trial data is the rich data set, originating from rigorous active surveillance, which can be readily exploited for epidemiological analysis. Lack of external validity is the limitation; sites are chosen for specific reasons and representativeness is not a priority. Study protocols may

not always reflect real-life treatment realities: for example in our study dengue diagnosis as a proportion of VCD was lowest in Philippines, but investigators indicated this is simply because cases had not satisfied the strict case definitions in the protocol, rather than due to uncertainty about the diagnosis. The sophistication of the techniques we employed is inferior to those used by global modelling consortia who have published on the topic. Their incorporation of serological data is an important difference, giving rise to incidence rates several times higher than observed in local medical practice and these global data are therefore highly sensitive to assumptions of the frequency of symptomatic infections, and the associated case definitions.

In Indonesia, the expert panel approach required extensive planning and organization, and the integrity of the Delphi process depends on the expertise of panellists. Practicing physicians who are local experts are not always experts on epidemiological research methods and the requirement to spend adequate time to explain, in local language, cannot be over-stated. Some estimation parameters are also challenging even for experts to guess and this is an inherent weakness of the approach.

My contribution to both of these papers was as the lead researcher. After conceiving the ideas for the studies, I worked with a team to design protocols and analysis plans, designed spreadsheets for data collation and analysis, contributed to statistical analyses and drafted both manuscripts. This involved close relationships with local collaborators who provided local surveillance data for analyses; and for the multi-country study,[67] individual co-authors were responsible for collating data from their respective countries. The Delphi panel meeting in Indonesia was organized by the local team but design of the meeting agenda, creation of most presentation materials, questions and analysis plan were led by me, with support from co-authors. In both papers, co-authors provided valuable contributions to discussions, provided review and feedback and local interpretation and context. More complex statistical analyses (e.g., bootstrapping to generate confidence intervals) were conducted by a statistician. In terms of learning, most analyses were conducted in MS Excel and large spreadsheets quickly became unwieldly, difficult-to-follow and carried the risk of introducing errors. A major learning was on the requirement to use clean, well-organized and well-designed templates which are properly

labelled and organized. The tables and results in Nealon *et al.* 2016 are lengthy and can be confusing; they could have been simpler, with some data relegated to supplementary materials. Because we used multiple data sources including national surveillance data for these analyses, permissions from ministries of health and many individuals and institutions was needed which took significant time. For this and to enhance understanding of the results in the context of the healthcare systems from which they originated, local collaborations were essential.

Chapter 2: The feasibility of different dengue vaccine effectiveness study designs

Published works contributing to this chapter

Nealon J, Lim W-Y, Moureau A, *et al*. Feasibility of case-control and test-negative designs to evaluate dengue vaccine effectiveness in Malaysia. *Vaccine*. 2019;37(39):5891-5898.

Introduction: dengue vaccine effectiveness studies

The previous chapter discussed dengue disease burden, with reference to vaccine introduction. A number of dengue vaccines are in clinical development and after their introduction in public vaccination programmes, evaluations of their real-world performance will be needed.[105–107] Post-licensure dengue VE studies have not been published and, given the clinical and epidemiological specificities of dengue, challenges may be expected which warrant preparatory studies.[47] These include the ability to recruit dengue patients satisfying relevant case definitions; availability of laboratory capacity to confirm infection with adequate specificity and sensitivity; and the practical infrastructure to identify and recruit suitable control participants.

There are several methods for monitoring VE which may be suitable for dengue. A workshop of international experts took place in 2014 to discuss the underlying principles; participants agreed that case-control (CC) and test-negative (TN) designs should be considered for this purpose.[108] CC studies are established methodologies for assessing associations between vaccine exposure and infectious disease outcomes including for influenza;[34] Japanese encephalitis;[35] whooping cough,[36] and pneumococcal pneumonia[37] and have been used to evaluate dengue risk factors.[109,110] The TN design is a variant of the CC study whereby suspected cases with negative laboratory results – and who are therefore considered absent of the outcome of interest – are used as controls, and has been used extensively for evaluating the effectiveness of influenza vaccines[38–41] and other vaccines.[42–44] The TN design has advantages in reducing bias in control recruitment, and has been used to understand dengue risk factors.[111]

In Malaysia, dengue outbreaks occur nationwide with increased risk in urban and peri-urban areas. Peaks in disease transmission often coincide with rainy season but cases

occur year-round, and reported case numbers have doubled since 2010.[112,113] In anticipation of vaccine introduction, we assessed the feasibility of conducting CC and TN studies for dengue VE evaluation, in Malaysia.[114] Because the vaccine was not yet in use, we used both study designs to measure associations between socio-demographic and environmental risk factors and dengue outcomes. We considered hospitalized/severe dengue as policy-relevant and specific endpoints, so we conducted hospital-based dengue surveillance for a period of one year, matching cases to control participants who were hospitalized for a non-dengue condition. Feasibility was assessed based on the ability to recruit adequate numbers of suspected cases, laboratory-confirmed cases and controls to demonstrate VE under a hypothetical scenario. Associated logistical, clinical, and laboratory aspects were documented, and we described biases and challenges which could result. Finally, we proposed modifications to improve the design of future dengue VE studies.

Aims and objectives

Aim: Assess appropriate case definitions, operational processes and study designs for future dengue VE evaluations.

Specific objectives:

1. To identify the risk factors for hospitalized and severe dengue using CC and TND study designs
2. To evaluate the sensitivity and specificity of different dengue case definitions and laboratory tests to enhance the design of dengue VE studies

Methods

Study sites and participant enrolment

Enhanced surveillance for suspected dengue cases was conducted starting October 2016, for a period of 12 consecutive months, among hospitalized patients at three large tertiary care hospitals operating within the Malaysian Ministry of Health system. The hospitals are all located in urban/peri-urban areas and are centres of excellence for dengue, treating several thousand hospitalized cases, annually.

Participants were enrolled following screening from medical and paediatric wards during weekdays and within working hours by study coordinators. Eligibility was assessed based on clinical history, physical examination and following discussions with attending physicians. Typically in Malaysia, individuals are suspected of dengue based on clinical signs and symptoms and, at these referral centres, it is likely that most participants had already received either IgM/IgG and/or non-structural antigen 1 (NS1 Ag) rapid diagnostic tests (RDT) and/or a previous clinical diagnosis of dengue at primary care clinics or hospital emergency departments. Children suspected of dengue are typically admitted, whereas adults will be hospitalized only following a poor or worsening clinical condition.

Participants were classified according to the following case definitions:

- *Suspected dengue*: patients on whom the attending clinician makes a diagnosis of probable dengue according to clinical history, physical examination and results of routine diagnostic tests which may have been used.
- *Virologically-confirmed dengue (VCD)*: suspected dengue cases virologically confirmed by the central laboratory using dengue RT-PCR and/or NS1 Ag ELISA.
- *Severe dengue*: a patient presenting with fever of 2-7 days plus any of the following: severe plasma leakage, severe haemorrhage or severe organ impairment, as derived from raw clinical data, based on WHO 2009 case definitions.[6]
- *Controls (for case control study)*: patients who were identified in hospital wards by study staff, hospitalized for conditions not likely to be dengue, age- and geographically matched to VCD cases.
- *TN controls*: suspected dengue cases who tested negative for dengue by both RT-PCR and NS1 Ag.

Suspected dengue cases aged 9 to 25 years (reflecting the population most likely targeted by public vaccination campaigns), acutely ill and suspected of dengue infection; admitted to the study hospital within 5 days (extended to seven days, nine months into the study) of fever onset and resident in the hospital catchment area were invited to join the study. For each laboratory-confirmed dengue case, study teams attempted to identify two matched controls hospitalized in the same hospital as cases with no suspicion of

dengue infection, with a final diagnosis other than dengue and admitted within one month (before or after) of laboratory confirmation of the case. Controls were individually age-matched to cases in three age groups: 9 to 12 years; 13 to 17 years; and 18 to 25 years; and geographically-matched based on the catchment areas of district health offices. The sample size was based on a hypothetical effectiveness objective comparing the odds ratio (OR) of having a virologically confirmed, hospitalized dengue episode between vaccinees and non-vaccinees. Assuming 70% of suspected cases test positive, 50% vaccine coverage within the target population and 50% VE, the TN design would require 223 cases and 96 TN controls. A CC design would require 88 cases and 352 controls (with a case:control ratio of 1:4) or 110 cases and 220 controls (with a 1:2 ratio).

Data collection and laboratory analysis

A questionnaire was administered to all participants, completed by legal guardians for children, including socio-demographic information, reported dengue histories of participants and household contacts, other risk factor data (e.g., household and neighbourhood vector control practices; time spent outdoors) and flavivirus (dengue, Japanese encephalitis or yellow fever) vaccination history. Final discharge diagnoses based on routine clinical practice were retrieved from electronic medical records. For suspected cases, dengue was confirmed by RT-PCR and NS1 Ag ELISA in a central study laboratory. The results of routinely-performed dengue diagnostic testing, which could include RDTs and ELISAs detecting IgM, IgG and NS1 Ag, before or during hospitalization, were also recorded.

Statistical analysis

Socio-demographic characteristics of VCD cases and controls were compared for both designs. Univariate logistic regression models were used to estimate associations between confirmed dengue and risk factors using the CC (in which only participants with at least 1 matched control were included, by conditional logistic regression) and TN study designs. Variables with a P-value <0.2 on univariate analysis were included in a final multivariable model and were backward-selected to remain in the model at a P-value of <0.05.

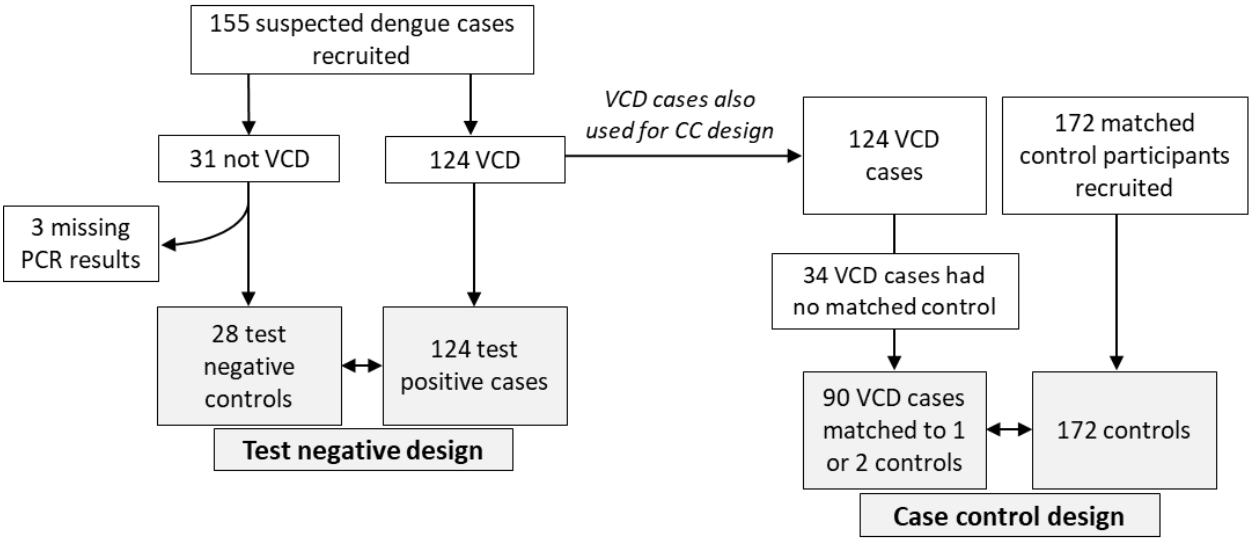
Dengue discharge diagnoses were compared with WHO 2009 case definitions, including severity assessment, as derived from participants' clinical data.[6] The sensitivity and specificity of each diagnostic test used in routine practice were calculated using RT-PCR and/or NS1 Ag ELISA positive test results as the reference standard, with confidence intervals computed using the normal approximation method.

Results

Characteristics of study participants

We recruited 327 participants. There were 155 suspected of dengue within 5 days of fever of whom 18 were aged 9-12 years; 48 were aged 13-17 years, and 89 were aged 18-25 years (study flow chart in figure 3). The planned sample size was not met in any age group. Many suspected cases were ineligible to participate because they were not aged 9 – 25 years old; had experienced onset of fever >5 days previously; because parents were unavailable to provide informed consent; and/or birth certificates – prerequisites for study participation – were not available.

Figure 3: *Study flow chart for a dengue VE feasibility assessment in Malaysia. VCD = virologically confirmed dengue.*



Of the 155 suspected dengue cases, 124 (80%) were VCD. Three participants had missing RT-PCR results and we therefore recruited 28 TN controls. To match 124 confirmed dengue cases in a 1:2 ratio, 248 controls were required but only 172 matched

controls were recruited and some cases lacked controls: 90 cases were matched with 1 or 2 controls. Time between case and matched control recruitment was on average 74 days. Table 4.1 compares the characteristics of control participants recruited for the CC study and TN controls. Significant differences between groups were observed in the sex distribution; and proportion of participants reporting family history of dengue within the past month.

Table 4.2 summarises the socio-demographic characteristics of study participants. Differences in baseline characteristics between cases and controls were observed in the sex distribution: of VCD cases, 43 (34.7%) were female in comparison with 98 (57.0%) controls and 6 (21.4%) TN controls. There were also differences in reported recent dengue history in the household (32 [25.8%] cases; 6 [21.4%] TN controls and one [0.6%] case control) and reports of recent neighbourhood fogging (68 [55.3%] VCD; 11 [40.7%] TN controls and 64 [37.2%] case-controls).

Table 4.1 *Comparison of control and test-negative control study participants' characteristics. Numbers (%; SD for Mean age). P-values derived from chi-squared test except for mean age, which was calculated using Student's t-test*

Participant characteristic	Controls (case control study)	Test-negative controls	p-value
N	172	28	-
Mean age, years (SD)	19 (4.3)	18 (3.7)	0.247
Sex			
M	74 (43)	22 (79)	<0.001
F	98 (57)	6 (21)	
Site			
Ipoh, Perak	59 (34)	5 (18)	
Selayang, Selangor	62 (36)	14 (50)	0.190
Sungai Buloh, Selangor	51 (30)	9 (32)	
Education level			
No formal or primary	14 (8.1)	2 (7.1)	
Secondary	114 (66)	23 (82)	0.204
Tertiary	44 (26)	3 (11)	
Type of dwelling			
Individual house	127 (74)	17 (61)	0.152
Apartment/ flat/others	45 (26)	11 (39)	

Number of family members in household			
≤ 3	31 (18)	7 (25.0)	0.534
4 – 5	54 (31)	11 (39)	
6 – 7	59 (34)	7 (25)	
≥ 8	28 (16)	3 (11)	
Household member diagnosed with dengue within the past month?			
No	171 (99)	22 (79)	<0.001
Yes	1 (0.6)	6 (21)	
Participant previously diagnosed with dengue?			
Yes	19 (11)	6 (21)	0.123
No	153 (89)	22 (79)	
Average time spent outdoors, daily (hours)			
< 4 hours	19 (11)	3 (11)	0.216
4 hours <= time < 8 hours	122 (71)	16 (57)	
>= 8 hours	31 (18)	9 (32)	
Insecticidal fogging in neighbourhood in the past month?			
No	108 (63)	16 (59)	0.725
Yes	64 (37)	11 (41)	

Utility of case-control and test-negative design for risk factor identification

In the CC study, two variables remained significant in the final model: respondents who reported a recent household dengue contact (OR: 54; 95% CI: 7.3 – 397; P<0.001) and those reporting neighbourhood insecticidal fogging in the last month (OR=2.1; 95% CI: 1.3 – 3.6; P = 0.005) were associated with an increased risk of hospitalized dengue as compared to participants without these risk factors. In the TN analysis, no risk factors were identified. This might be partially a result of the number of controls (n=28), resulting in imprecise estimates. No risk factors associated with severe VCD could be calculated as the number of severe dengue cases was too small (n=7).

Routine laboratory diagnosis of dengue

The most commonly used dengue confirmatory test in routine practice was the NS1 Ag RDT, in 148 (95.5%) of 155 suspected cases, followed by the IgM RDT (110; 71.0%) and the IgG RDT (109; 70.3%). The IgM ELISA, IgG ELISA and NS1 ELISA were used in 45 (29.0%), 31 (20.0%) and 2 (1.3%) participants, respectively. The NS1 Ag RDT correctly identified 108 of the 118 VCD cases on which the test was used, a sensitivity of 91.5%

(95% CI 86.5 – 96.6%). However, 14 of 27 negative samples were incorrectly classified as positive, giving a specificity of 48.1% (29.3 – 67.0%). IgM rapid tests correctly identified 8 out of 87 VCD cases, a sensitivity of 9.2% (3.1 – 15.3%) and specificity of 81.0% (64.2 – 97.7%; correctly identifying 17 of 21 negative cases). The IgM ELISA had a sensitivity of 47.2% (30.9 – 63.2%; 17/36 VCD cases positive) and specificity of 25.0% (0 – 55.0%; 2/8 negative cases correctly identified). The NS1 Ag ELISA misclassified both VCD cases on which it was used as dengue negative.

Table 4.2 *Numbers (%; SD for mean age) of participants with selected socio-demographic characteristics and risk factors recruited as VCD cases, controls and test-negative controls. P-values are in bold font with ORs below, vs the reference category.*

	Case-control design			**Test-negative design**		
	VCD cases^	**Controls**	**P-value** OR (95% CI)	**VCD cases**	**Test-negative controls**	**P-value** OR (95% CI)
N	90	172		124	28	
Mean age, years (SD)	18 (4.3)	19 (4.3)		18 (4.2)	18 (3.7)	
Sex			**0.003**			**0.181**
M	57 (63)	74 (43)	Ref	81 (65)	22 (79)	Ref
F	33 (37)	98 (57)	0.4 (0.3;0.8)	43 (35)	6 (21)	1.9 (0.7;5.2)
Site[#]			-			**0.1697***
Ipoh, Perak	31 (34)	59 (34)	-	45 (36)	5 (18)	Ref
Selayang, Selangor	32 (36)	62 (36)	-	44 (36)	14 (50)	0.3 (0.1;1.1)
Sungai Buloh, Selangor	27 (30)	51 (30)	-	35 (28)	9 (32)	0.4 (0.1;1.4)
Education level			**0.270**			**0.110***
No formal or primary	5 (5.6)	14 (8.1)	Ref	10 (8)	2 (7.1)	Ref
Secondary	58 (64.4)	114 (66)	4.4 (0.5;37)	76 (61)	23 (82)	0.7 (0.1;3.2)
Tertiary	27 (30.0)	44 (26)	5.6 (0.6;50)	38 (31)	3 (11)	2.5 (0.4;17)
Type of dwelling			**0.135***			**0.589**
Individual house	58 (64)	127 (74)	Ref	82 (66)	17 (61)	Ref
Apartment/ flat/others	32 (36)	45 (26)	1.5 (0.9;2.6)	42 (34)	11 (39)	0.8 (0.3;1.8)
Number of family members in household			**0.596**			**0.224**
≤ 3	11 (12)	31 (18)	Ref	14 (11)	7 (25)	Ref
4 – 5	32 (36)	54 (31)	1.8 (0.8;4.2)	44 (36)	11 (39)	2 (0.7;6.1)
6 – 7	31 (34)	59 (34)	1.6 (0.7;3.7)	45 (36)	7 (25)	3.2 (1;11)
≥ 8	16 (18)	28 (16)	1.7 (0.7;4.3)	21 (17)	3 (11)	3.5 (0.8;16)

Household member diagnosed with dengue within the past month?			**<0.001*[z]**			**0.630**
No	62 (69)	171 (99)	Ref	92 (74)	22 (79)	Ref
Yes	28 (31)	1 (0.6)	54 (7.3; 397)	32 (26)	6 (21)	1.3 (0.5; 3.4)
Participant previously diagnosed with dengue?			**0.589**			**0.202**
Yes	12 (13)	19 (11)	Ref	15 (12)	6 (21)	Ref
No	78 (87)	153 (89)	0.8 (0.4;1.8)	109 (88)	22 (79)	2 (0.7;5.7)
Average time spent outdoors, daily (hours)			**0.213**			**0.280**
< 4 hours	17 (19)	19 (11)	Ref	23 (19)	3 (11)	Ref
4 hours <= time < 8 hours	57 (63)	122 (71)	0.5 (0.2; 1.1)	77 (62)	16 (57)	0.6 (0.2; 2.3)
>= 8 hours	16 (18)	31 (18)	0.6 (0.2; 1.4)	24 (19)	9 (32)	0.3 (0.1; 1.4)
Insecticidal fogging in neighbourhood in the past month?			**0.0049*[z]**			**0.174***
No	39 (43)	108 (63)	Ref	55 (45)	16 (59)	Ref
Yes	51 (57)	64 (37)	2.1 (1.3; 3.6)	68 (55)	11 (41)	1.8 (0.8; 4.2)

[#] Study site not included in CC design because controls were matched to cases based on site. [^] Includes only cases with ≥1 matched control. * Variables included in multivariate model. [z] Variables retained in final multivariate model. Ref = reference category. Columns totals may vary due to lack of responses; or not equal 100% due to rounding

Conclusions and critical assessment

We aimed to assess feasibility in recruitment, logistics and laboratory confirmation of a traditional CC or TN design to evaluate dengue VE in Malaysia. Rather than identifying risk factors, the primary objective was to assess biases, stemming primarily from the methods of control recruitment and misclassification of disease and vaccine status, and describe these limitations to inform the design of future studies.[115] Primarily due to low levels of case and control recruitment stemming from our design choices (hospitalised dengue is rare in teenagers and younger adults) it is likely that protocol changes would be required before embarking on a dengue effectiveness evaluation. Key challenges and possible solutions are provided in table 4.2.

The exposures under assessment were a selection of socio-demographic and behavioural risk factors whose distribution was similar in cases and controls, and were therefore not identified as risk factors. Two risk factors were identified with the CC method: living with household members recently diagnosed with dengue (OR: 54), and neighbourhood insecticidal fogging conducted in the last month (OR: 2.1). Reporting bias may have contributed to these findings: because these risk factors were captured by questionnaire, suspected cases – attuned by their hospitalization with diagnosed dengue – perhaps recalled higher frequencies of dengue history or neighbourhood prevention than controls. This is a weakness of our approach to examine exposures ascertained through self-reporting. Reported rates of household dengue/recent fogging were higher in TN controls who had been diagnosed with suspected dengue than case-controls, providing some evidence for reporting bias. TN designs can offer logistical advantages and reduce confounding by healthcare seeking behaviour, but these advantages could be offset by reporting or other biases resulting in biased VE estimates.

We recruited a lower-than-expected number of suspected dengue cases and controls, partly because Malaysia suffered fewer dengue cases in 2017 than in preceding years.[116] But many hospitalized dengue cases were ineligible for study inclusion for other reasons and recruitment was challenging. For each VCD case we also failed to recruit two matched controls. Our study enrolled adolescents, teenagers and young adults, a healthy demographic unlikely to be hospitalized; and the logistics of identifying suitable controls

within large hospitals was challenging. Perhaps the age- and geographical matching used here should be relaxed in the future; or alternative methods of control selection such as recruitment of community-based controls, could be considered.

For the TN study, recruitment of controls was low because a higher-than expected (80% vs. 70%) proportion of suspected cases was VCD. This may be due to clinical expertise and familiarity with dengue in Malaysia and/or frequent use of RDT and subsequent decisions to admit based on their results. Indeed, 95.2% of VCD cases had received an NS1 Ag RDT as part of their routine care; and 87% of VCD cases had a positive NS1 Ag RDT result. The frequency of pre-admission testing and subsequent hospitalization are likely influenced by epidemic activity, availability of RDTs at health facilities and hospital congestion, effects which have been shown to introduce bias to TN studies of influenza vaccines.[45] Probably, a TN study would only be efficient if a higher proportion of suspected cases tested negative, perhaps by using a less specific case definition, and/or enrolment at an earlier stage of the treatment pathway, for example in the clinic before RDTs are used. We also failed to recruit large numbers of severe dengue cases, representing an important study bias, confining analysis to milder cases and prohibiting effectiveness estimation against severe outcomes which may be of particular relevance for policymakers and against which vaccine performance may differ.

Rates of confirmatory diagnostics used in routine clinical practice were variable and of inadequate sensitivity/specificity to conclude on infection status. This was most concerning for the NS1 Ag RDT which is the most-commonly used dengue diagnostic in Malaysia and displayed specificity much lower than reported elsewhere.[117] This study was not designed specifically to assess diagnostic test performance but nonetheless, the observation deserves additional investigation, for example via clinical assessment of discordant cases; or programmatic evaluation of RDTs in the field.

Potential biases identified for future VE studies

CC studies are vulnerable to a number of biases, most notably due to challenges in control selection.[115,118] Our approach was to use hospitalized controls, matched to cases and recruited within a similar time window. To minimize bias, controls should represent the exposure time at risk in the population from which cases arise; and should be selected

independently of the exposure of interest.[119] Biased VE estimates would arise if the decision to vaccinate was associated with other exposures associated with infection risk, such as family dengue history or community fogging which were elevated in cases over controls in our study, a problem with our design choice of using socio-demographic variables collected by questionnaire as exposures. This effect would likely underestimate VE. Due to resource constraints in batching and shipping samples there was an average of 74 days between case and control recruitment which may affect the comparability of these groups. Rather than awaiting laboratory confirmation it may be beneficial to recruit controls immediately following suspected case enrolment to better match on exposure risk which may vary over time. Consideration of these and other related biases deserves further assessment when patterns of dengue vaccine distribution after launch are better-understood, including by verifying the accuracy of patient-reported data with family members or public health authorities to limit recall bias. We similarly observed gender differences between cases and controls, perhaps due to differences in sex distribution between dengue wards and the wards used for control identification (e.g., gynaecology/orthopaedic surgery). This may constitute a bias because the sex-distribution of dengue in Malaysia is not equal, with males slightly over-represented,[112] or if sex is associated with health-seeking behaviour. Matching controls to cases based on sex may be advisable in the future.

This study was conducted in three sites in Malaysia over only one year. Results should be generalized only in the context of local epidemiology and treatment practices. Our difficulty in recruiting matched hospitalised controls resulted in low statistical power which may have prevented identification of risk factors, an effect difficult to describe because strong socio-demographic risk factors for hospitalized dengue are unknown. Practical limitations also led to lower-than-possible recruitment.

This was the first detailed and quantitative assessment of the feasibility of a dengue VE study. Conducted in a dengue-endemic setting including practical constraints encountered in a busy hospital, we concluded it is likely the TN design would not be efficient for a dengue VE study unless a less-specific case definition was applied, enabling recruitment of higher numbers of TN controls. The CC method, with adjustments

to methods of control recruitment, may be feasible: we recruited 124 confirmed dengue cases, an approximate minimum sample size. However, there is a risk of important biases and a full bias assessment after vaccination patterns are better understood would be needed. These findings will inform the design of dengue vaccine effectiveness studies in coming years.

Critical reflection

I contributed to study conception and outline protocol development, which was led by a multidisciplinary research group. I was then the primary contact with local study teams in Malaysia, working with them to identify study sites in Malaysia, finalize the protocol and statistical analysis plan, develop operational guidelines and refine the protocol based on local hospital working practices. Statistical analyses were conducted by the study statistician. I coordinated meetings to interpret study results in the context of local hospital practices and drafted the manuscript.

This study was a component of a dengue vaccine risk management plan, and high-level governmental cooperation and collaboration was necessary to conduct a surveillance project in multiple government hospitals. We agreed to modify the study protocol to include participants with ≤ 7 days' (instead of ≤ 5 days') fever due to low recruitment. This new definition did not correspond to WHO case definitions, increased workload and resulted in ten additional suspected cases, two of whom tested positive for dengue. Probably, this modification was not necessary. We identified low performance of routine diagnostic tests, a finding which could cause concern. The study was not powered or designed to address this research question and it would be prudent to carefully consider such analyses in future.

Table 4.2. – *Requirements for a dengue vaccine effectiveness study; challenges encountered and potential remedies.*

Study requirement	Challenge encountered	Potential remedies
Sufficient sample size and characteristics of cases	Few hospitalized suspected dengue cases	– Increase number and/or range of study sites (e.g., include emergency department) – Assess and improve enrolment mechanisms – Assess local ethics administrative requirements and incorporate mechanisms to ease enrolment
	Few severe dengue cases	– Recruit retrospectively using stored serum samples and/or medical records – Assess and improve enrolment mechanisms
Sufficient number of case-controls and test-negative controls	Few case-controls recruited	– Consider community-based control recruitment (family members; neighbours; etc.) – Assess logistics of hospital-based recruitment during site selection – Relax matching criteria based on expected exposure status
	Few test-negative controls recruited due to high confirmation rates in suspected cases	– Enrol suspected cases prior to use of rapid tests – Recruit from primary health centres or otherwise earlier in the patient pathway
Exposure history (e.g., exposure to risk factors under study) of controls representative of source population of cases	Duration between case and control recruitment may introduce bias in exposure (during a vaccination campaign; or if vaccination increases during an outbreak)	– Enrol controls immediately after identification of suspected cases – Consider community-based control recruitment (family members; neighbours; etc.) – Improve laboratory test turnaround time
	Females over-represented as controls in CC design which could bias results if vaccination rates are unequal	– Match controls on sex – Recruit from alternative hospital wards
Controls have similar outcome risk (e.g., reporting to study site with hospitalized dengue) as cases	Severity of conditions suffered by case-controls may have differed from hospitalized dengue	– Assess impact of using different control populations – Use test-negative design after assessing misclassification bias arising from imperfect confirmatory diagnostics

Chapter 3: Dengue seroprevalence, serotype distributions and levels of endemicity suitable for vaccine introduction

Published works contributing to this chapter

1. Garg S, Chakravarti A, Singh R…Nealon J. Dengue serotype-specific seroprevalence among 5- to 10-year-old children in India: a community-based cross-sectional study. *Int J Infect Dis*. 2017;54:25-30.
2. Prayitno A, Taurel A, Nealon J, *et al.* Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children. *PLoS Negl Trop Dis*. 2017;11(6):e0005621.
3. Sasmono RT, Taurel A-F, Prayitno A…Nealon J. Dengue virus serotype distribution based on serological evidence in pediatric urban population in Indonesia. *PLoS Negl Trop Dis*. 2018;12(6):e0006616.

Introduction: seroprevalence as an indicator of endemicity

As discussed in the previous chapters, estimates of symptomatic dengue burden are affected by case definitions, health-seeking behaviour and health system specificities, in addition to underlying infection rates. This results in disparities between reported and estimated case numbers of up to >100-fold.[81] Serological data, by which antibodies to a specific pathogen are detected in circulating blood, present a relatively unbiased view of historical infection at the individual level. In unvaccinated individuals antibodies are a consequence of natural infection and since age reflects duration of exposure, median age of first exposure is an indicator of population level infection frequency which will increase in areas of lower endemicity where infections are less likely to have occurred by a given age.[24] Because many first dengue infections are asymptomatic, the age of individuals' first-infection is rarely determined but this information can be estimated from cross-sectional serological surveys of healthy individuals conducted across countries, continents or time-points enabling comparisons of historical transmission intensity within specific populations to plan the optimal age of vaccine introduction.[120]

There are four dengue virus serotypes all of which can cause severe disease. Individual studies have identified more severe or variable clinical episodes following infection with different serotypes [121–125] or according to the order of infection.[126] However, many of these study results are specific to individual points in time and geography and there is no clear

relationship between infecting serotype and disease severity.[127] Nonetheless, the distribution of serotypes affects the modelled performance of dengue vaccination due to serotype-specific differences in vaccine effectiveness; and also because second infections, which have a higher likelihood of being severe, are possible only when heterologous serotypes are circulating.[65] Individual or multiple serotype-specific infection history can be determined by plaque reduction neutralization test (PRNT) assays which measure neutralizing antibody responses to individual viral serotypes, though interpretation of those data is not straightforward.[128,129]

India and Indonesia are the world's second and fourth most populous countries with a combined population of over 1.6 billion, spread across over 19,000 islands, belonging to religious and linguistic groups in different stages of economic development. Both are dengue-endemic and while reliable burden estimates in each country are highly variable, Bhatt *et al*. estimated 7.6 and 32.5 million cases in Indonesia and India, annually, which translates to 41.8% of the global total.[3] In both countries, the geographical distribution of disease is evolving. Historically considered an urban disease in India, dengue has spread to rural areas in recent decades. Whereas individual serotypes were typically isolated during outbreaks in the 1960s, more recent reports describe circulation of all four virus serotypes, without clear geographical boundaries, and many urban areas are now considered hyper-endemic.[130] In Indonesia, dengue is most common in urban areas but since the 1980s incidence in rural areas, has gradually increased.[131] While routine laboratory confirmation is uncommon, all four serotypes have been identified during dedicated studies in Yogjakarta, Makassar, Surabaya and Jakarta, including during outbreaks, and it is likely that all four serotypes circulate across many islands.[73,132,133]

At the time of these studies (2011 – 2014), both countries suffered from a lack of dengue seroepidemiological data; there had been no studies conducted nationwide or using representative sampling methods to describe variations in transmission intensity. In India, limited studies had been confined to the Andaman and Nicobar Islands,[134] Hyderabad,[135] and Chennai;[136] and in Indonesia older data were available from Bandung[73] and Yogjakarta.[137] We therefore performed the first multi-site seroprevalence surveys conducted in these countries. We used gold-standard diagnostics to document infection

history in healthy children to understand dengue endemicity at the sub-national level and provide data to facilitate planning of dengue vaccination strategies.

Aims and objectives

Aim: Describe dengue seroprevalence and serotype distributions in India and Indonesia.

Specific objectives:

1. Conduct a multi-site cross-sectional survey to measure serotype-specific dengue seroprevalence at multiple Indian sites
2. Conduct a nationally representative, multi-site cross-sectional survey to measure serotype-specific dengue seroprevalence in Indonesia.

Methods

Common to both India and Indonesia

Eligible children were identified using specific methods described below, and enrolled following collection of written informed consent from parents/guardians and assent from older children. Socio-demographic characteristics, significant medical histories and information on reported infection history were collected by interview with parents. 5 mL blood samples were drawn from children by trained laboratory technicians, nurses or clinical investigators and left at room temperature for 1–2 hours before centrifugation; serum samples were divided into aliquots and frozen at -20°C or below until analysis at central laboratories. Dengue IgG antibody levels were assessed using commercially available ELISA kits according to manufacturers' instructions (Focus diagnostics and/or Panbio Diagnostics, both of which have reported sensitivity >98% and specificity ≥80%[138]). The titre of dengue serotype-specific neutralizing antibodies of dengue IgG positive samples was determined by the PRNT test based on 50% or greater reduction in plaque counts ($PRNT_{50}$).[128]

For the interpretation of $PRNT_{50}$ titres, participants were classified as follows:

- Naïve, if antibody titres <10 (1/dil) for the 4 serotypes

- Monotypic, if antibody titres ≥10 (1/dil) for only one serotype or if titres ≥10 (1/dil) for different serotypes with a single serotype having a high titre (>80 (1/dil) titre, and ≥5 times higher than other titres)
- Multitypic, if antibody titres ≥10 (1/dil) for different serotypes without a single predominant titre.

Descriptive statistics reported baseline characteristics and dengue IgG and $PRNT_{50}$ results. Associations between dengue positivity and socio-demographic covariates were assessed using chi-squared tests or t-test (for age); followed by multiple logistic regression to identify risk factors associated with serostatus in each country. Characteristics specific to each study are described below and summarized in table 2.1.

Table 2.1. *Comparison of dengue seroprevalence studies conducted in India*[139] *and Indonesia.*[140]

	India (DNG10)	**Indonesia (DNG26)**
Number, characteristics of sites	8, peri-urban/urban	30, urban
Sampling strategy	Convenience sample across wide geographical area	Population representative across country
Recruitment sites	Households and schools	Neighbourhood association meetings
Participant age (years)	5 – 10	2 – 18
Target recruitment per site	323	107
Rationale for sample size	Assuming 30% seroprevalence for the overall study population, with precision of 5% and 95% confidence	National estimate in each of four age groups, adjusted for clustering, assuming 25%, 45%, 55% and 65% seroprevalence, 5% precision, 95% confidence
Enrolled participants	2,609	3,210
$PRNT_{50}$ confirmation of IgG-positive sera	All dengue positive samples	Sub-set of 780 dengue positive samples
Japanese encephalitis testing?	Yes	No
Primary analysis	Crude percentage	Adjusted for clustering with random effects
Dengue seroprevalence, age 10	73%	79%
Proportion of dengue positives multitypic	49%	51%

India study features

A convenience sample of eight private (three) or government (five) medical colleges at six geographically distinct locations was selected: a) to provide a wide geographical distribution across India; b) to represent rural and peri-urban areas; and c) based on the site's recognized ability to conduct epidemiological research. Overall, two sites were selected in each of New Delhi and Hyderabad, and one site each in Kalyani (West Bengal), Wardha (Maharashtra), Mumbai, and Bangalore. Participants were invited to participate either during routine health worker household visits; or at informational meetings held at schools. In addition to dengue, samples were tested for JEV.

Indonesia study features

Sites were selected based on cluster survey methods used by WHO.[141] Based on the probability of a subdistrict being selected being proportional to its population size, 30 urban subdistricts were selected using demographic and geographical data. The main health centre (*puskesmas kecamatan*) in each selected subdistrict was used as the study centre for each site. Participants were enrolled following informational sessions at monthly neighbourhood meetings and a maximum of one eligible child from each household was invited to join the study.

Results

India

In India, 2591 participants (52.6% girls) were included and blood sampled with a mean age of 7.9 years. Laboratory results for dengue were available for 2558 participants; overall, dengue IgG prevalence was 59.6%, ranging between 23.2% and 80.1% according to study site (Table 2.2) and increased from 40.7% in 5-year-olds to 73.4% in 10-year-olds (figure 4). Participants from one site (Kalyani, West Bengal in Eastern India) experienced notably lower dengue exposure than others.

In multivariable logistic regression analysis, household water storage (OR 5.0, 95% CI 3.5–7.1) and indoor piped water from the public water supply (OR 1.5, 95% CI 1.2–1.9), increasing participant age (OR 1.4, 95% CI 1.3 – 1.4 per year of age) and living in

temporary/non-fixed accommodation* compared to a fixed house (OR 1.5, 95% CI 1.2–2.0) were also associated with dengue status. Being resident in Kalyani was associated with decreased exposure (OR 0.18, 95% CI 0.10–0.31), while participants from Wardha (OR 1.5, 95% CI 1.1–2.1) and Mumbai (OR 2.5, 95% CI 1.7–3.7) had an elevated risk, in comparison with Delhi. A reported history of dengue or a family history of dengue was reported by 15 (0.6%) and 48 (1.9%) of participants, respectively.

$PRNT_{50}$ data were available for 1,512 participants; overall, 89.2%, 92.2%, 93.9%, and 84.9% had antibody titres ≥ 10 (1/dil) for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. Despite the young age of study participants, 48.7% expressed a multitypic profile. Overall, JE IgG prevalence was 13.6%.

Table 2.2. *Age and site-stratified dengue IgG prevalence (%) from India*[139]

Age (years)	***5***	***6***	***7***	***8***	***9***	***10***	**Overall**
Kalyani	15.0	14.5	26.5	26.3	27.9	50.0	**23.2**
Hyderabad (2)	28.3	48.1	61.4	74.6	77.6	77.6	**58.2**
Hyderabad (1)	38.9	52.6	55.6	73.0	70.0	58.3	**58.6**
New Delhi (1)	39.0	51.0	62.1	65.0	76.0	70.8	**60.2**
Bangalore	61.2	60.6	58.8	70.9	62.8	63.6	**62.5**
New Delhi (2)	48.9	63.5	67.2	69.2	71.9	76.1	**66.5**
Wardha	46.3	51.0	63.0	81.8	81.1	89.3	**69.0**
Mumbai	51.6	68.4	79.0	90.2	88.3	96.7	**80.1**
Sites combined	**40.7**	**50.9**	**58.6**	**67.4**	**70.8**	**73.4**	**59.6**

Indonesia

In Indonesia, 3,210 participants were enrolled from 30 clusters spread from west to east across the country. After exclusions, 3,194 participants, 48% male, were included in the analyses, 672 aged 1–4-years-old, 861 aged 5–9, 886 aged 10–14 and 775 aged 15–18-years-old. Seroprevalence ranged from 26.4% (95% CI: 15.8–37.1) in those aged 1-year-

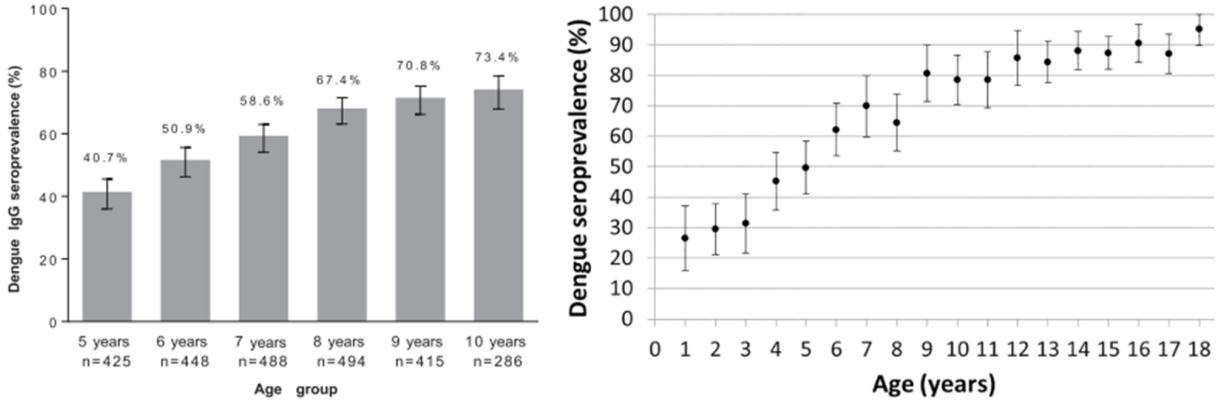
* Hindi used on case report form: *Anishchit/Asurakshit Aavaas*

old to 95.3% (89.8–100) in the 18-year-old participants (figure 4). The median age at seroconversion was 4.8 years. The overall nationwide seroprevalence was 69.4%, with a minimum of 34.6% and a maximum of 87.9% observed per site, and the seroprevalence per age group was 33.8% (26.4–41.2) in the 1–4 year-old group, 65.4% (59.1–71.7) in the 5–9-year-old group, 83.1% (77.1–89.0) in the 10–14-year-old group and 89.0% (84.0–94.1) in the 15–18-year-old group.

Increasing age and female gender were associated with dengue serological status, with the odds of seropositivity 22 times higher in participants aged >15 vs those aged 1 – 4 ($p<0.0001$) and values of 71.1% (95% CI: 65.9–76.3) in females versus 67.4% (62.4;72.5) in males ($p=0.018$). In the multivariate model, two variables remained associated with dengue serologic status, the participant age group (OR increasing to 21.9 in the 15 – 18 year age group vs 1 – 4 year olds; $p<0.0001$) and the number of cases diagnosed in the household since the participant was born (OR for >1 vs none, 2.96; $p=0.0004$).

Multitypic profiles were observed in 50.9% of the participants, a proportion which increased with age. Amongst monotypic samples, the highest proportion was reactive against DENV-2, followed by DENV-1, and DENV-3, a trend which was most clearly observed in the two youngest age groups.

Figure 4: *Changing dengue seroprevalence with age in India (left) and Indonesia (right)*



Conclusions and critical assessment

We conducted the first multi-site dengue seroprevalence studies in India and Indonesia to understand dengue infection risk in two of the world's most populous countries contributing >40% of the world's dengue burden.[3] Overall, 60% of 5 – 10 year old Indian and 69% of 2 – 18 year old Indonesian children had been infected at least once and most children had been infected by the age of 5 years (Indonesia) and 6 years (India). Children expressed neutralizing antibody responses to >1 serotype suggesting most had experienced multiple infections, and our results confirmed that all four serotypes circulate widely in both countries. These studies did not describe symptomatic disease episodes but clearly demonstrate both countries represent hyper-endemic environments in which symptomatic episodes are common and severe, and in which effective vaccines would provide a significant public health benefit.[16]

We conducted a risk factor analysis in both countries, attempting to identify socio-demographic variables associated with positive serostatus. Increasing age was strongly associated with serological status but other explicit socio-demographic risk factors were not observed. For example, in India, having household water supply and storing household water were both associated with higher odds of dengue positivity. It is likely these findings were confounded (e.g., both could be indicators of socio-economic status) or were artefacts arising from multiple hypothesis testing. Overall, these data imply that strong individual-level risk factors within communities are absent, perhaps unsurprisingly for a mosquito-borne disease, and that community-level prevention (e.g., reducing vector densities to low thresholds) are needed to reduce outbreak risk.

In both countries, rates of reported dengue history were much lower than the infection rate, but more markedly in India where only 0.6% of participants reported a previous episode. While only 30 – 50% of primary cases are symptomatic,[65] the size of the disparity implies a lack of health seeking, lack of recognition of the disease, misclassification of dengue as another febrile illness, or lack of reporting. This may be a consequence of a broader de-prioritization or under-appreciation of the disease in India: while the observed seroprevalence in our study was comparable to that reported in other highly endemic

countries of South and Southeast Asia,[140,142–144] the officially reported average annual incidence of disease is approximately 100-times lower than other countries.[145] We should assume that dengue burdens reported in the routine surveillance system represent only a fraction of symptomatic episodes.

The major limitation of these studies concerns representativeness and, therefore, generalizability. In India, we chose 8 sites with geographical spread across the country, but sites were not representatively or randomly sampled. In Indonesia, we applied a representative sampling method to choose urban sites across the country. We also adjusted results for the effects of clustering within sites with a random-effects model, providing results and confidence intervals which describe national level seroprevalence within urban areas. In both countries, participants were enrolled through community meetings which could have resulted in study populations which do not fully represent the communities from which they were drawn and results were not adjusted to reflect demographic or other characteristics of those who declined to participate. Participant age was different in India and Indonesia due to differences in available resources. This allowed enrolment of the full paediatric age group in Indonesia in whom differences in seroprevalence were considered likely, but neither study was powered to identify sub-national variation in seroprevalence. Since publication of our data, a more comprehensive Indian dengue serosurvey was conducted which tested 12,300 randomly-selected participants from 240 urban and rural clusters.[146] Overall lower dengue seroprevalence was observed, with higher seroprevalence in urban than rural areas. This suggests the peri-urban and urban sites included in our study may represent areas of heightened dengue transmission and are not representative of the country as a whole. Additionally, our study did not recruit randomly, and it is possible participants at higher risk of dengue were over-represented in our study. Five of our eight sites were government facilities and we did not compare seroprevalence between these and the private institutions involved in the study.

Flavivirus IgG assays are also susceptible to providing false-positive results when testing sera from individuals previously infected with or vaccinated against a related flavivirus, a

phenomenon which could theoretically inflate the positivity rates observed here.[21,147] We confirmed ELISA results by dengue PRNT to minimize this risk and >97% were confirmed positive, but it remains possible that the $PRNT_{50}$ assay also suffers from cross-reaction.[148] We used ELISAs as a screening assay and have no way of assuring their sensitivity: PRNT may have identified an even higher number of exposed children. Within the PRNT, cross-reaction between serotypes is likely for samples expressing high neutralizing titres and the algorithm applied to define serological profiles is rather subjective.[149] While these PRNT data from cross-sectional studies provide valuable insights on the circulation of multiple serotypes, longitudinal studies are needed to provide additional data on infection frequency, serotype dynamics and implications of disease symptomology and severity.[71,150]

In summary these studies both confirmed a high level of dengue infection which is compatible with a high burden of symptomatic disease. More granular assessments of infection rates across age groups and geography would further strengthen vaccine decision-making, and analyses to support this objective are discussed in the following section.

Critical reflection

For the Indian study, my participation in the study began after raw data had been collected and I was therefore not involved in study design. I jointly developed the analysis plan, selected the data tables for inclusion in the paper and performed logistic regression modelling to identify risk factors, under the supervision of a statistician. I also interpreted the results, decided on the manuscript outline and content and drafted the manuscript. For the Indonesian study, I contributed to development of the protocol and study design, conducted meetings with study partners and local officials to plan conduct of the study, responded to ongoing queries from the local study team and was involved in all aspects of the data interpretation. I contributed to the manuscript, which was drafted by the first authors.

Major lessons from these studies included the difficulty of selecting a representative sample during field research. Even in Indonesia where we dedicated significant time and

resources to this, our sample was not representative due to the final selection of houses close to healthcare centres. It is possible that translating an English language protocol to Bahasa Indonesian language and then training staff at multiple sites introduced inconsistencies in field implementation. Our statistical methods were not corrected for multiple-testing and I suspect the environmental risk factors for dengue serostatus were confounders for socio-economic status – a variable we did not include in our statistical models.

Chapter 4: Using dengue and Japanese encephalitis serological data to infer flavivirus infection rates

Published works contributing to this chapter

1. Prayitno A, Taurel A, Nealon J, *et al*. Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children. *PLoS Negl Trop Dis*. 2017;11(6):e0005621.
2. Bhavsar A, Tam CC, Garg S, …Nealon J. Estimated dengue force of infection and burden of primary infections among Indian children. *BMC Public Health.* 2019;19(1):1116.
3. Nealon J, Taurel A-F, Yoksan S, *et al*. Serological Evidence of Japanese Encephalitis Virus Circulation in Asian Children from Dengue-Endemic Countries. *J Infect Dis*. 2019;219(3):375-381.
4. Nealon J, Bouckenooghe A, Cortes M, *et al*. Dengue Endemicity, Force of Infection, and Variation in Transmission Intensity in 13 Endemic Countries. *J Infect Dis.* March 2020; jiaa132

Introduction: force of infection

The previous chapters discuss symptomatic and serological evidence of dengue infection, and how those data contribute to understanding of dengue epidemiology. Whilst symptomatic disease data are often more relevant for policymaking, seroprevalence data are less influenced by testing and reporting practices, providing relatively unbiased information on historical infections within a population. However, data from dedicated serosurveys are rare, generalizing results from one site to another is unreliable and estimates often offer limited precision around specific age groups. This is a particular problem when considering immunization, because the efficacy of the world's first dengue vaccine (CYD-TDV, Dengvaxia®, Sanofi Pasteur, Lyon, France) is dependent on an individual's infection history.[33] The WHO Strategic Advisory Group of Experts (SAGE) guidelines recommend vaccination only after individual screening for dengue antibodies or, if this is not feasible, where seroprevalence in 9 year-olds exceeds a threshold of 80% in sub-national areas of high endemicity.[31] Dengue seroprevalence is therefore an important determinant of the public health impact, cost effectiveness and acceptability of dengue vaccination, but few countries have these data available at the sub-national level.

Estimates of age-stratified seroprevalence can be made if underlying information on the rate of infection within a population is available. Assuming transmission intensity is constant over time, the rate at which seroprevalence increases with age can provide a measure of FOI, or the rate at which susceptible (seronegative) individuals acquire infection.[151] FOI can be applied under assumptions of constant or varying endemicity, incorporating uncertainty to estimate seroprevalence at a given age, to complement empirical seroprevalence measurements.[152,153]

Dengue FOI is also an important determinant of the characteristics of symptomatic disease. Following first infection – which is normally mild or asymptomatic[65] – homologous antibodies to the infecting serotype are protective, probably for life.[154] But after a certain duration of time, their presence increases the risk of symptomatic and severe disease following infection by a heterologous serotype.[16–18] The risk of suffering a symptomatic or severe episode is therefore a function of time since previous infection and, accordingly, the age-distribution of symptomatic/severe dengue disease is dependent on FOI, with more intense transmission resulting in a younger median age of symptomatic/severe cases.[155] Infection frequency may be constant or may be shaped by individual events such as outbreaks; or changes in human behaviour which affect the risk of exposure.[156]

Most JEV infections are asymptomatic, with only 1:25 – 1:1000 resulting in symptomatic disease.[157] Laboratory confirmation practices are variable and even severe JE cases may not be reported to public health authorities leading to highly uncertain disease burden estimates.[55] Sero-epidemiological data can therefore provide useful information on the range and frequency of transmission which is more common in rural areas. A well-documented challenge to this approach – in areas where multiple flaviviruses co-circulate or where vaccination is common – is cross-reactivity, particularly in IgG antibodies, between members of the flavivirus family.[21,147,158] Neutralizing assays including the PRNT are more specific;[159] for JEV, a PRNT titre ≥1:10 dilution by $PRNT_{50}$ is considered protective but a more stringent threshold, $PRNT_{90}$, may be preferred for epidemiological studies of historical exposure, reducing the risk of background serum cross-

reactivity.[160,161] To our knowledge, no previous study had used serological data to estimate the JEV infection rate.

This chapter describes a series of studies which estimated FOI of dengue from seroprevalence studies in Indonesia and India; and from a global analysis which included 7 Asian countries. Using related methods we also estimated the ages of median, 70% and 80% seroconversion; assessed variations in transmission intensity by participant age; and estimated the number of children infected. We also provided the first estimates of JEV FOI from urban areas of 4 endemic Asian countries and explored immunological interactions which can affect these measurements.

Aims and objectives

Aim: Estimate the infection rate of dengue and Japanese encephalitis in endemic countries

Specific objectives:

1. Use dengue seroprevalence data to estimate dengue FOI across sites in multiple Asian countries
2. Estimate the age at which 50%, 70% or 80% of children had experienced their first dengue infection
3. Make the first estimates of JE FOI from serological data in 4 Asian countries

Methods

Data sources

Underlying data sources have been described in detail in previous sections and in original manuscripts. Briefly, we analysed data from clinical trials or epidemiological studies which measured dengue and/or JE serological status in healthy, asymptomatic children. All data and blood samples originated from baseline measurements of clinical trials before any vaccine or placebo were administered, or from dedicated cross-sectional seroprevalence surveys. Participants were enrolled directly by investigators who recruited patients under their care or following informational events at primary health care centres, schools or community centres, depending on the local health care system and community

organization. Dengue or JEV exposure for each participant was ascertained either by $PRNT_{50}$ with a lower limit of quantitation titre of 10 (reciprocal dilution)[128] and/or IgG ELISA.[139,162] As the youngest participant was aged 0.6 years we assumed declining maternal antibodies had no impact on our analyses.

Statistical analyses common to all studies

For each analysis, data were combined into a single database including participant exact age; serological status and information on geographical origins of participants at the national and/or sub-national levels. FOI was estimated using catalytic models in which seroprevalence is assumed to increase with age and seroconversion is irreversible by:

$$P_a = 1 - e^{-\lambda a}$$

P_a is the seroprevalence at age a years, λ represents FOI, or the annual risk of seroconversion among initially seronegative individuals. The parameter λ can be estimated through a generalized linear model with complementary log-log link:[153,163]

$$ln(-ln(1 - p_a)) = ln(\lambda) + ln(a)$$

where the logarithm of each participant's age is included as an offset with a coefficient constrained to 1 and the model constant is the log FOI. This model implicitly assumes that FOI is constant throughout the included age range and that transmission intensity is stable over time.

FOI estimates were derived for each country/site considered in the analysis. Uncertainty was described through generation of exact binomial 95% CIs; inter cluster-level variance in countries including >1 site was accounted for by generating robust standard errors, assuming sites were independent clusters. This underlying method was followed for all studies; with the specificities described in table 3.1, and below.

Bhavsar et al., (India)

This study used data from a previously-described dengue seroprevalence study in children aged 5 – 10.[139] Dengue FOI was estimated for the country as a whole and for individual cities after pooling data from the two sites in both Delhi and Hyderabad,

assuming that their populations were exposed to a similar risk of infection because of their geographical proximity (within a few hundred meters). We additionally estimated the age at which seroprevalence equalled 50% and 70%. Model goodness of fit was assessed by the Hosmer-Lemeshow test with predicted probabilities grouped into deciles.[164]

Based on Indian 2011 census data[165] and estimated annual seroconversion rates, we then estimated the number of children aged <11 years experiencing a primary dengue infection in 2011, assuming constant FOI from 2002 to 2011, according to:

$$\sum_{a=1}^{11} \delta_a (p_a - p_{a-1})$$

Where, δ_a represents the total size of the Indian population aged *a* years; and p_a is the proportion of the population seropositive by age *a* years.

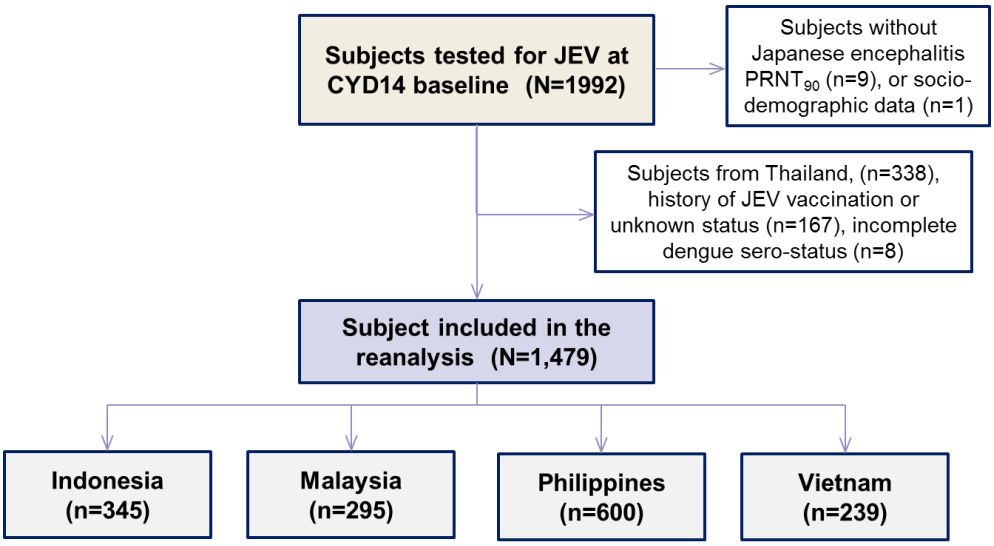
Nealon et al., 2019 (multi-country Japanese encephalitis)

Data were from a phase 3 dengue vaccine trial (CYD14) in children aged 2 – 14 years old in Indonesia, Malaysia, the Philippines, Thailand and Vietnam.[30] In an immunological subset of approximately 20% of participants, serum was collected after enrolment but before any study vaccine administration. Neutralizing antibody against JEV was measured by $PRNT_{50}$.[128] Individuals with a history of JEV vaccination were removed from the analysis to ensure serological status was a consequence of natural infection. Participants from Thailand were excluded because >95% of children were vaccinated leaving a sample too small for meaningful analysis (n=15).

Because we observed that JEV seroprevalence varied according to DENV serostatus, neutralizing JEV titres achieving 90% plaque reduction ($PRNT_{90}$) were subsequently calculated to explore the impact of increasing specificity of the assay by decreasing the background serum cross-reactivity from other flaviviruses.[161] JEV seroprevalence was calculated according to 1) $PRNT_{50}$ and 2) $PRNT_{90}$; overall; and separately for DENV seropositive and seronegative populations. We considered JEV serostatus in the DENV-naïve population could not have been affected by cross-reactive flavivirus antibodies and

therefore treated the resulting FOI estimate as a minimal estimate of annual infection risk. The study flow chart is in figure 5.

Figure 5: *Study flow chart from Nealon et al. (2019), describing the population included in JEV seroprevalence study.*[148]



Nealon et al., 2020 (dengue, seven countries)

We analysed data from cross-sectional, Sanofi Pasteur dengue vaccine clinical trials or epidemiological studies which collected dengue serological data from healthy, asymptomatic, unvaccinated individuals in 13 countries collected over six years. While the original study included sites from Latin America only results from the seven Asian countries, which are aligned with the topic of this thesis, are described here. The analysis was restricted to participants aged 7 months to <19 years on the day of blood sampling; to dengue-endemic areas; and to participants with conclusive dengue serological results. (figure 6). A country-level analysis was followed by a site-level analysis, in which sites with <10 participants; and those which enrolled only participants aged <2 years, were excluded.

To describe possible changes in FOI within large age strata, we also generated age-varying FOI estimates for specific age groups using linear piecewise models. We fitted log-binomial models with two age terms:[153]

$$-\ln(1 - P_a) = \lambda_1 a_1 + \lambda_2 a_2$$

For each study site, we determined the optimal age-varying FOI model by sequentially varying the age breakpoint for each whole year of data with at least 2 adjacent data points (e.g., for countries with data starting in 3-year old children the first possible breakpoint was age 5) and identifying the model with the lowest value for Akaike's information criterion (AIC). For each country, we determined whether constant or age-varying models fit the data better by ten-fold cross-validation, taking a random 10% of the sample, and selected the model (constant or age-varying) with lower root mean squared error (RMSE).

Seroprevalence by age, per country, was estimated from the resulting models. Graphs of estimated constant and age-varying seroprevalence were developed for each country, overlaid with observed seroprevalence and their 95% CIs grouped by year.

We estimated the age at which 50% and 80% (p = 0.5 or 0.8) of children seroconverted in each country and site from the optimal model, using the following formula in case of constant FOI or if the threshold was reached before the breakpoint:

$$a = \frac{-\ln[p]}{\lambda}$$

or if after the observed breakpoint for age-varying models, by:

$$a_2 = \frac{-\ln[p] - \lambda_1 a_1}{\lambda_2}$$

Figure 6: *Study flow chart, modified from Nealon et al. (2020), multi-country dengue FOI study*[166]

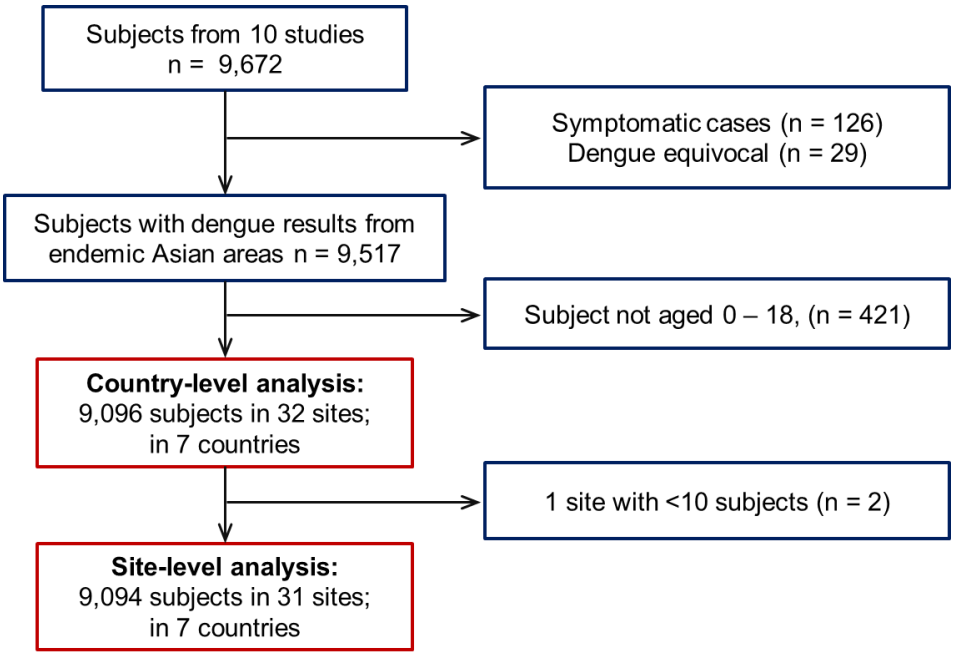


Table 3.1. *Comparison of endemicity studies which captured force-of-infection.*

	Indonesia, dengue[140]	**India, dengue**[167]	**Multi-country JE**[148]	**Multi-country, dengue**[166]
Flavivirus	Dengue	Dengue	Japanese encephalitis	Dengue
Asian countries included	Indonesia	India	Indonesia, Malaysia, Philippines, Vietnam	India, Indonesia, Malaysia, Philippines, Singapore, Thailand, Vietnam
Data source	Nationally-representative seroprevalence survey	Multi-site seroprevalence survey	Multi-country clinical trial	Seroprevalence surveys + clinical trials
Number of participants included	3,194	2,556	1,479	9,096
Timing of sample collection	Oct 2014 – Nov 2014	Jan 2011 – Oct 2012	June – Dec 2011	Feb 2009 – Nov 2014
Recruitment methods	Community meetings	Community/ school-based	Mostly school-based meetings	Mostly school-based meetings

Geographical stratification?	No	Yes	Yes	Yes
Participant age (years)	2 – 18	5 – 10	2 – 14	2 – 18
Method of testing	IgG ELISA	IgG ELISA	$PRNT_{50}$	Mixed
Age at seroprevalence thresholds	50%	50% and 70%	No	50% and 80%

Results

Prayitno et al., (Indonesia)

In Indonesia overall, we estimated that 13.1% of naïve children experience their first dengue infection, annually.[140] There was no clear trend in changes in FOI with age but FOI seemed to be higher in the younger study participants.

Bhavsar et al., (India)

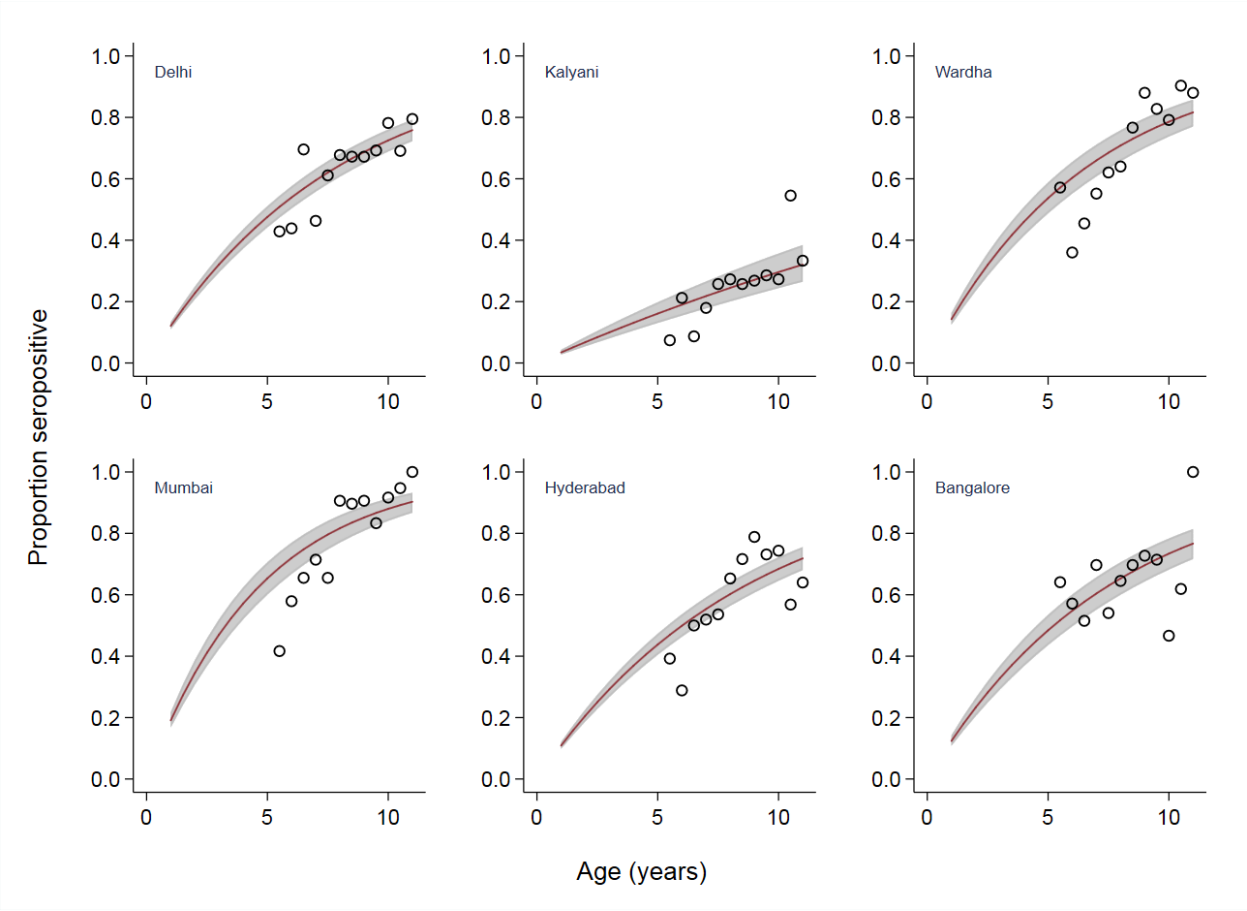
The analysis included data from 2,556 participants, between 301 and 649 children per site. The overall annual FOI for all sites combined was 11.9% (95% CI 8.8 – 16.2), varying across sites from 3.5% (2.8 – 4.4) in Kalyani to 21.2% (18.4 – 24.5) in Mumbai (table 3.2; figure 7). The ages by which 50% and 70% of children were first infected were lowest in Mumbai, 3.3 and 5.7 years respectively. In the study population overall, 70% of children were estimated to have been infected at least once by the age of 10.1 years and the median age of infection in all sites except Kalyani varied between 3.3 and 6.0 years. Model goodness of fit indicated differences between predicted and observed seroprevalence in Wardha (P = 0.03), Hyderabad (P = 0.01) and for India overall (P = 0.01).

In 2011, India had a population of ~260,000,000 children aged <11 years. We estimated that in 2011 17,013,527 (95% CI 14,518,438 – 19,218,733) children aged up to 10 years – 6.54% of the total population within this age group – were infected with dengue for the first time.

Table 3.2. *Annual FOI, goodness-of-fit statistics; and the ages by which 50% and 70% of children seroconverted from multiple Indian cities. 95% CI in brackets*[167]

Site	Annual FOI, %	Goodness of fit Chi^2 statistic; P- value	Age of 50% seroconversion, years	Age of 70% seroconversion, years
Kalyani	3.5 (2.8 - 4.4)	4.72; 0.79	> 11	>11
Hyderabad (2 sites)	11.5 (11.2 – 11.8)	20.9; 0.01	6.0 (5.9 – 6.2)	10.4 (10.2 – 10.7)
Delhi (2 sites)	12.9 (11.1 – 15.0)	2.96; 0.94	5.4 (4.6 – 6.2)	9.3 (8.0 – 10.8)
Bangalore	13.2 (11.5 - 15.3)	10.2; 0.25	5.2 (4.5 – 6.0)	9.1 (7.9 – 10.5)
Wardha	15.4 (13.4 - 17.7)	16.9; 0.03	4.5 (3.9 – 5.2)	7.8 (6.8 – 9.0)
Mumbai	21.2 (18.4 - 24.5)	12.8; 0.12	3.3 (2.8 – 3.8)	5.7 (4.9 – 6.6)
All sites combined	11.9 (8.8 - 16.2)	20.4; 0.01	5.8 (4.3 – 7.9)	10.1 (7.4 – 13.7)

Figure 7: *Estimated seroprevalence (red lines), 95% confidence intervals (shaded areas) and observed seroprevalence (circles) from Indian sites in Bhavsar et al.*[167]



Nealon et al., 2019 (multi-country Japanese encephalitis)

The mean age of study participants in each country was 8.2 years in Indonesia, 8.3 years in Malaysia, 8.2 years in Philippines and 7.6 years in Vietnam. By $PRNT_{50}$, overall JEV seroprevalence was 46.1% in Indonesia, 22.4% in Malaysia, 45.7% in Philippines and 47.5% in Vietnam. Seroprevalence increased with age, reaching >70% in the 13 – 14 years old children in Indonesia, Philippines and Vietnam; and 40% in Malaysia (table 3.3). When stratified by DENV serostatus, JEV seroprevalence was 54.4% in Indonesia, 41.0% in Malaysia, 55.3% in Philippines and 59.4% in Vietnam in DENV seropositive individuals; and 8.1% in Indonesia, 5.8% in Malaysia, 10.8% in Philippines and 30.7% in Vietnam in DENV seronegative individuals. By JEV $PRNT_{90}$, seroprevalence was considerably lower: 1.7% in Indonesia, 2.4% in Malaysia, 3.7% in Philippines and 11.3% in Vietnam.

FOI estimates based on seropositivity using the $PRNT_{50}$ threshold revealed an annual infection rate within DENV positive participants of 9.1% (95% CI: 7.7; 10.7) in Indonesia, 5.4% (95% CI: 4.1; 6.9) in Malaysia; 9.3% (95% CI: 8.2; 10.6) in Philippines, and 11.1% (95% CI: 8.8; 13.8) in Vietnam. In DENV seronegative participants FOI was considerably lower: 1.4% (95% CI: 0.5; 3.0) in Indonesia, 0.8% (95% CI: 0.4; 1.4) in Malaysia, 1.8% (95% CI: 1.0; 2.9) in Philippines and 5.2% (95% CI: 3.6; 2.3) in Vietnam. The goodness of fit statistics were respected for all models (Pearson test P-value >0.05; deviance test P-value >0.05) except for the DENV positive population in Vietnam.

Table 3.3: *Number of participants included by age (N) and Japanese encephalitis seroprevalence (%) by $PRNT_{50}$ according to DENV serostatus in four Asian countries.*[148]

	Indonesia				**Malaysia**				**Philippines**				**Vietnam**			
Dengue status	*Positive*		*Negative*		*Positive*		*Negative*		*Positive*		*Negative*		*Positive*		*Negative*	
Age	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
2	13	23%	11	9%	1	0%	21	0%	21	19%	18	22%	3	100%	7	14%
3	19	37%	10	0%	9	11%	12	0%	33	30%	24	8%	9	56%	11	46%
4	18	56%	13	8%	12	42%	12	0%	33	30%	20	10%	14	43%	8	38%
5	27	33%	6	0%	6	50%	25	8%	35	40%	17	6%	13	15%	22	9%
6	14	21%	2	0%	8	75%	10	0%	41	51%	14	7%	12	33%	10	30%
7	21	52%	2	50%	4	0%	9	11%	29	52%	6	0%	8	75%	4	25%
8	14	43%	3	0%	9	22%	10	10%	20	55%	5	20%	11	72%	8	38%
9	14	36%	3	0%	9	44%	9	11%	31	61%	4	25%	9	67%	6	50%
10	23	52%	3	0%	8	25%	4	0%	21	67%	1	0%	13	54%	7	43%
11	13	69%	2	50%	11	36%	8	0%	29	45%	-	-	12	75%	4	25%
12	51	75%	6	17%	30	57%	20	5%	58	64%	12	0%	28	71%	13	39%
13	34	74%	1	0%	24	42%	14	14%	68	75%	8	25%	6	100%	1	100%
14	22	73%	-	-	8	38%	2	50%	51	80%	1	0%	-	-	-	-
Total	**283**	**54%**	**62**	**8%**	**139**	**41%**	**156**	**6%**	**470**	**55%**	**130**	**11%**	**138**	**59%**	**101**	**31%**

Nealon et al., 2020 (dengue, seven countries)

After exclusions, our dataset included 9,096 participants in seven country-level analyses and 9,094 in 31 site-level analyses with a mean of 293 participants per site. Under the

assumption of constant FOI, dengue FOI varied between countries from a low of 1.7% (95% CI: 1.4 – 2.2) in Singapore, increasing to 24.1% (21.8 – 26.5) in the Philippines (table 3.4). In most countries, constant and age-varying models predicted similar seroprevalence at most ages; constant models fit data better in four out of seven countries (figure 8). The highest FOI estimates occurred in very young Filipino children, with an annual seroconversion risk of 43% up to the age of two years. Estimated dengue seroprevalence increased with age in all scenarios, except for the age-varying Singapore model where estimated seroprevalence declined at age 4 years. At the site level, the age-constant FOI was >10% per year at 23 of 31 sites and constant models fit observed data better at 25 of 31 sites.

Table 3.4. *Constant and age-varying FOI estimates for each of 7 Asian countries included in Nealon et al. (2020).*[166]

Country	Constant FOI (95% CI)	Age-varying FOI (95% CI)	Corresponding age ranges (yrs)	Better fit*
India	11.9 (8.7 - 16.2)	10.7 (6.8 - 14.4) 20 (10.7 - 29.4)	5 – 6 7 – 18	Constant
Indonesia	14.7 (12.8 - 16.9)	15.1 (13.1 - 17.1) 4.1 (-3.9 - 12.1)	1 - 13 14 – 18	Constant
Malaysia	8.6 (6.7 - 10.9)	12.2 (11.2 - 13) 4.6 (-0.2 - 9.5)	2 - 3 4 – 14	Constant
Philippines	24.1 (21.8 - 26.5)	42.6 (35.4 - 49.7) 13.5 (10.1 - 16.9)	0 - 1 2 – 14	Age-varying
Singapore	1.7 (1.4 - 2.2)	6.6 (3.9 - 9.2) -0.8 (-2 - 0.3)	2 - 3 4 - 18	Age-varying
Thailand	14.8 (13.7 - 16.0)	16.4 (14.9 - 17.8) 8.7 (6.5 - 10.9)	2 - 6 7 - 14	Age-varying
Vietnam	11.4 (10.2 - 12.8)	17.3 (16.4 - 18.1) 5.4 (3.8 - 6.9)	2 - 3 4 - 18	Constant

According to the best-fitting model, the estimated age at which 50% of children had seroconverted was <10 years in six of the seven countries in our analysis; the youngest was in the Philippines (1.6 years; 1.4 – 3.1). In Singapore, a seroprevalence of 50% was not reached within the range of our observed data, by age 18. An 80% seroprevalence threshold was reached at the age of 8.7 years in the Philippines, at 11.0 years in Indonesia, 12.7 years in Thailand, 13.5 years in India, 14.1 years in Vietnam and 18.8 years in Malaysia.

Thirty-one sites were included in the site level analysis. We estimated 80% of children had been infected by age 18 years (e.g., within the range of our observed data) at 24 (77%) sites and by age 9 years, at 8 (26%) sites (figure 9). The youngest estimated age at which 80% of children seroconverted was 5.3 years, observed in Southeast Sulawesi in Indonesia. At least 50% of children were estimated to have seroconverted by the age of 18 at 29 (94%) sites. Seroprevalence at age 9 years was also high at other sites, notably across Indonesia. Within countries, there was substantial heterogeneity between sites in the age at which 80% of children seroconverted (figure 9).

Figure 8: *Observed seroprevalence in seven Asian countries by age (circles) and 95% CIs (spikes) and estimated seroprevalence assuming constant FOI (solid line) and 95% CIs (shaded area) or age-varying FOI (dotted lines).*[166]

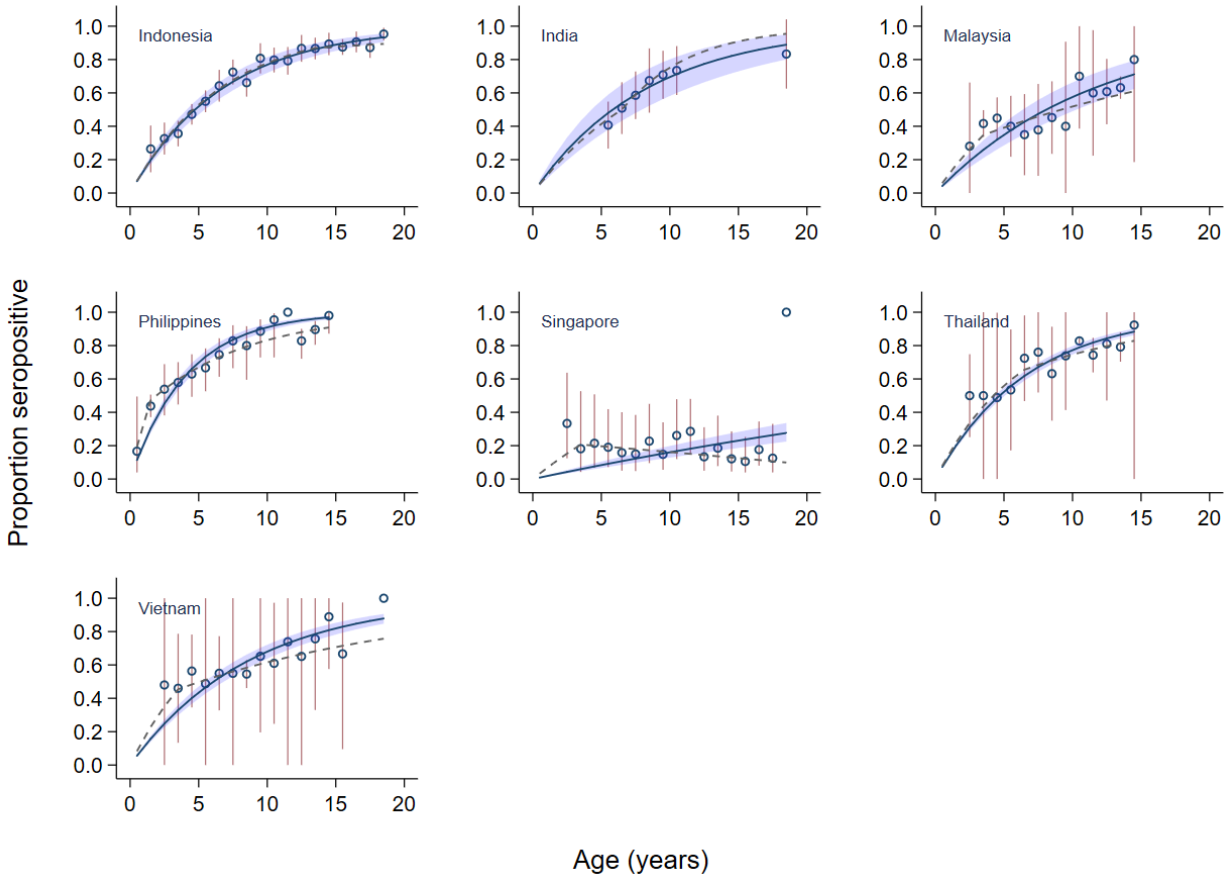
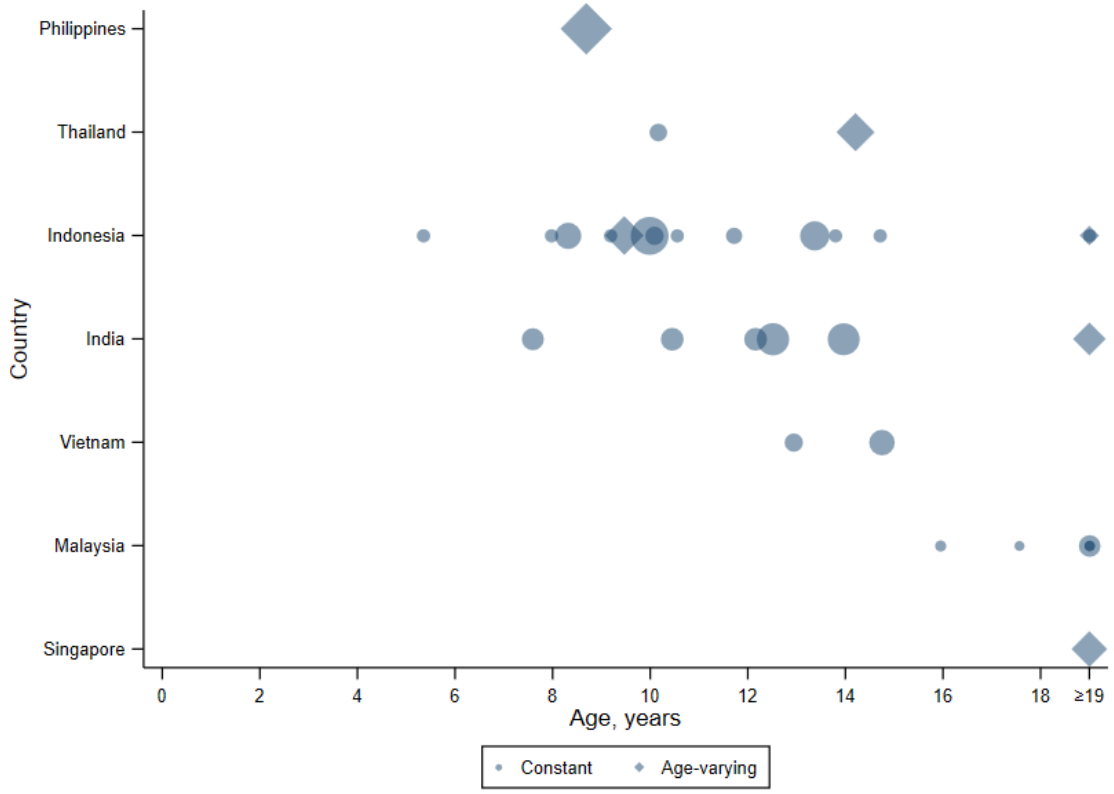


Figure 9: *Age of 80% seroconversion by site, estimated from best-fitting constant (circles) or age-varying (diamonds) models. Bubble size corresponds to frequency weights. ">19" signifies estimates were outside the range of our data.*[166]



Conclusions and critical assessment

We analysed data from over 9,000 children from 13 countries to describe dengue and JEV transmission intensity in endemic countries. Study participants were in age groups likely to seroconvert, providing the necessary variation in seroprevalence to estimate FOI. Serological status was confirmed with gold-standard diagnostics and consistent analyses were used to make comparisons across countries and sites.

Across the age ranges sampled, children at most sites were at high risk of dengue infection, with FOI exceeding 7% in all countries except Singapore. Countries with higher levels of dengue transmission included the Philippines, Indonesia and Thailand, in which ≥14% of seronegative children were infected each year. In these countries, intense dengue exposure results in a steep reduction in dengue-naïve individuals early in life.

While these data describe the force of primary – i.e., first – dengue infections, these transmission intensities would translate, at the population level, to a significant burden of secondary infections which are more likely to be symptomatic and severe. Malaysia and Singapore had lower transmission than other Asian countries, which could be an indication of improvements in dengue control measures.

Age-varying models were developed to assess whether clear variation in infection risk was observed as children aged. Strong evidence for this variation was lacking; seroprevalence estimates from constant and age-varying models were broadly similar and differences in cross-validation errors from different models were small. However in age-varying models transmission intensity was more frequently (six out of seven countries) higher in younger children, perhaps indicating their increased exposure to infectious mosquito bites. In Singapore, FOI declined for a significant proportion of the study sample (children aged >3 years old), a finding which is biologically counter-intuitive. This is possibly a consequence of intensive and effective vector control activities and behaviour which minimizes exposure to infectious bites (e.g., use of air-conditioning) resulting in low seroprevalence throughout childhood. Singapore also tends to experience severe, cyclical epidemics, and a recent large outbreak could result in higher seroprevalence in younger than older children. For example there was a large outbreak in late 2005, approximately 4 years before study participants were bled and if young children were disproportionately infected, this could give the impression of declining FOI.[168] These models assume constant FOI across included age groups, an assumption which was violated for Singapore, where more flexible modelling approaches incorporating more granular variations in epidemiology would be more appropriate.

According to WHO guidelines, an overall population benefit of dengue vaccination with CYD-TDV dengue vaccine can be expected in very high transmission settings, as defined by seroprevalence of ≥80% in participants aged 9 years of age or above, noting that such areas are rare.[31] Here, we estimated that 26% of sites met this criterion, one in India, six in Indonesia and at the only site included from the Philippines. These data represent transmission levels when blood samples were drawn, several years ago, but indicate several sites may benefit from dengue vaccination at the population level.

Another objective of this analysis was to understand variability in endemicity and we identified substantial variability within the same country: for example, in Indonesia, the median age of first infection varied between sites from 2.3 to 10.1 years; and in Malaysia this varied from 6.9 to 12.5 years. We did not have data from multiple sites in all countries, but this implies site-specific seroprevalence assessments would be needed prior to dengue vaccine introduction without prior sero-testing. Subnational data would also be useful to prioritize areas where vaccination would be most efficient.[31] However, few observed seroprevalence data points fell outside the confidence intervals of our estimated seroprevalence and statistical approaches such as this, accounting for uncertainty, could be considered complementary to empirical seroprevalence studies in endemic countries.[169]

For JE, our study documented serological evidence of JEV circulation in urban and peri-urban areas of Indonesia, Malaysia, Philippines and Vietnam, countries with differing epidemiology and JEV risk. The WHO vaccine-preventable disease monitoring system reports an annual average for these countries over recent years varying from 35.2 cases (Malaysia) to 310.6 cases (Vietnam).[170] In contrast, we calculated minimal estimates that 0.8% - 5.2% of children were infected annually and, even after correcting for a low proportion of symptomatic infections, these infection rates imply a substantial level of under-reporting of symptomatic cases. These urban regions were previously considered of low JEV risk and have no JEV vaccination programs in place; measures to improve disease awareness, increase use of confirmatory diagnostics and surveillance enhancements may be justified in response.

To our knowledge, this was the first time JEV FOI has been estimated from serological data. JEV seroprevalence varied according to DENV status, likely a consequence of cross-reactive antibodies raised following DENV infections. Indeed, these sites were originally selected for inclusion in dengue clinical trials due to their high levels of dengue endemicity.[67] We therefore estimated JEV FOI for individuals with no previous DENV exposure, resulting in minimal JEV exposure estimates and strong evidence for JEV circulation within these study populations, findings which should lead to consideration of expanding JEV vaccination national immunization programmes to these urban areas. The

estimated JEV FOI in DENV-exposed individuals was considerably higher and the true infection rate is likely somewhere in between.

These analyses suffer from several limitations. Perhaps partly because of small numbers of dengue positive/negative study participants in each age group, JEV seroprevalence did not increase monotonically, and relatively small numbers of JEV positive subjects could have been influential on overall results. Phenomena including focal outbreaks, migration from rural areas or transmission during visits to those areas could have inflated seroprevalence in specific age groups and incorrectly implied ongoing local transmission in rural areas from which study participants were enrolled. JE vaccination rates were close to zero except in Vietnam, where a national JE vaccination programme exists in some parts of the country. Vaccinated individuals were excluded from our analysis and because humans are dead-end, incidental JE hosts, this vaccination coverage would not have provided indirect protection that would influence serostatus in the population as a whole.

It is well-known that flavivirus genera share epitopes inducing cross-reactive antibodies leading to difficulty in differentially diagnosing flaviviral infections.[21,147] More recent or secondary infections generate broader, heterotypic, cross-reactive responses and – because these sites were chosen due to their high level of DENV endemicity – we considered anti-DENV antibodies would be more likely to cross-react with JEV virus in the PRNT than the reverse. Our additional observation that JEV seroprevalence by $PRNT_{50}$ in DENV naïve children was higher than corresponding rates derived from JEV $PRNT_{90}$ implies that $PRNT_{90}$ is overly specific, excluding true positive samples, for epidemiological studies such as this.

These studies estimated FOI from increasing seroprevalence as a function of age, but during outbreaks or where transmission is irregular the entire population may be concurrently exposed irrespective of their age, violating assumptions of constant infection risk as individuals age.[171] FOI estimates are dependent on the underlying data, so limitations in laboratory assays and recruitment biases described in the previous section are also applicable here, most importantly that participants were recruited for the purposes of clinical research from areas of high dengue endemicity and, are not nationally

representative and likely represent populations with higher-than-average dengue exposure.

The models used for FOI estimation impose certain constraints; notably, the power of age-varying models to detect a meaningful breakpoint is partly dependent on the age range of the data which varies between countries. We used AIC, RMSE and also the Hosmer Lemeshow test to assess model fit and identified optimal (constant vs age-varying) models for each country. These tests are somewhat subjective and, in many cases, both models fit the data well and we identified only weak evidence for age-varying effects.

All four dengue serotypes circulate in most of these countries[9] and we calculated only total, (or average), dengue FOI, assuming this is relatively stable over time, without more granular or serotype specific variation. From our samples, over 90% tested by $PRNT_{50}$ showed evidence of infection with >1 serotype (possibly due to cross-reaction rather than true infection), and calculation of meaningful serotype-specific FOI estimates was therefore not possible without making unreliable assumptions from PRNT titres. Because total FOI has been shown to approximate the sum of serotype-specific FOI[155] we considered the approach was reasonable to represent long-term average dengue exposure rates, and more complex modelling activities would be needed to further understand serotype-specific transmission dynamics.

Nonetheless, these data provide one of the largest flavivirus seroprevalence analyses performed. They present new information regarding flavivirus endemicity in different Asian countries, can be used to inform future studies, guide public health decision-making including the benefits/risks of vaccination and inform health economic analyses.

Critical reflection

My contribution to these studies was variable. For the Indonesian paper, they were confined to advising on the approach and providing critical comments and review of the first draft. But for the other three papers, I was the study lead and was responsible for identifying the research question, designing the analysis plan, generating the statistical code, conducting analyses (with the exception of Nealon *et al*.,[148] where this was

performed by a co-author) and drafting the final manuscripts, with the exception of the Indian paper.[167] For this analysis I also developed the statistical model to estimate the number of Indian infections. Throughout this process I received academic guidance for these papers from co-authors with expertise in epidemiology, serology and clinical research. Key learnings were of the importance of working with experts: there was a mistake in the FOI estimates reported in Prayitno *et al*.[140] which required an corrigendum and without strong expert guidance it would not have been possible to publish some of these manuscripts, particularly Nealon *et al*.,[166] where the analyses and interpretation for multiple countries was most complex.

Chapter 5. Discussion and recommendations for future research

In the preceding chapters I summarize a body of epidemiological research conducted with the overarching objective of informing policies around dengue vaccine introduction in Asia. It can be broadly divided into two sections. The first discusses symptomatic disease and related burden estimates, including from a case-control study conducted to prepare for dengue VE studies (chapters 1 and 2); and the second describes dengue endemicity and heterogeneity based on serological assessments, and includes applications to JEV, a closely-related flavivirus (chapters 3 and 4).

The first section identified a substantial burden of symptomatic disease: nearly 1/20 children had suffered a dengue episode each year and eight percent were assessed as DHF, almost all hospitalized, representing an important public health burden of severe disease episodes in some of the most populous countries in the world. Systematic laboratory confirmation of febrile episodes gave rise to approximately 3.5-times more cases than clinical diagnosis alone, and the rates of disease captured using active surveillance were up to 30-times higher than from passive national surveillance reports. The hospitalized case-control study described in chapter 2 was a template for future VE studies but we identified limitations which could challenge their validity, most notably through challenges in recruitment of adequate numbers of control participants. In chapters 3 and 4, serological methods identified that children receive intense early dengue exposure with as many as 1/4 infected each year in the most endemic areas. However a site-level analysis revealed heterogeneity within countries which would prevent national-level dengue vaccine introduction aligned with WHO recommendations without prior serotesting. Site-specific seroprevalence assessments or subject level serotesting would be needed to ensure that appropriate populations were vaccinated. Applying similar methods to JEV, another flavivirus, we estimated 0.8% – 5.2% of children had been naturally infected annually in urban areas where transmission is thought to be low and vaccination rare.

Strengths and novelties

Uniform surveillance and diagnostics

Several of the underlying studies described in this thesis used data derived from placebo-controlled clinical trials. As a consequence, very similar or identical protocols were applied in several countries concurrently using uniform case definitions and gold-standard laboratory confirmation which allowed us to combine data from multiple sites and countries into uniform databases of flavivirus data suitable for epidemiological analysis. These allowed us to conduct the published estimates of dengue according to different case definitions (VCD, cVCD, and CDD) providing a quantitative comparison of different case definitions and more complete understanding of the full spectrum of dengue disease according to clinical and laboratory criteria. The strict definition of fever ≥38°C as an inclusion criterion makes these estimates reproducible but would not capture episodes of milder febrile illness. This work has been cited by subsequent researchers, notably in Asian disease burden and health economic assessments of dengue interventions for which robust incidence rate estimates are needed.[99,172–175]

Incorporation of sub-national data and local expertise

When comparing active and passive surveillance, rather than comparing with national data our studies accessed age-stratified, sub-national surveillance data from provinces or districts in which study hospitals were located therefore improving the accuracy of under-reporting estimates. All studies included local experts as team members and authors, improving our ability to interpret data in the context of local healthcare practices, and enhancing their policy-relevance.

Comparison of serological responses to multiple flaviviruses

It is well-known that flavivirus genera share epitopes inducing cross-reactive antibodies which can confuse the interpretation of serological tests where >1 virus circulates.[21,147] The work in Nealon *et al*., 2019[148] compared immunological profiles for JEV and dengue by both $PRNT_{50}$ and $PRNT_{90}$, providing the first quantitative understanding of the implications of these decisions for study design. For example, our observation that JEV seroprevalence by $PRNT_{50}$ in DENV naïve children was higher than corresponding rates

derived from JEV $PRNT_{90}$ implies that $PRNT_{90}$ is overly specific, excluding specimens from some individuals who have experienced natural infection, in epidemiological studies such as this.

Limitations

Lack of national level generalizability, bias and sample size

Study sites selected for clinical trials which generated some data were urban areas of known dengue endemicity, which likely experienced higher levels of transmission than elsewhere and their generalizability is therefore limited. This was also a limitation of our seroprevalence studies in India and Indonesia: in India, data were generated from 8 sites which were geographically diverse but were not representative of the Indian population or randomly sampled. In Indonesia, we applied a probability proportional to size sampling method to choose 30 urban sites across the country but, as in India, participants were enrolled through community meetings which could have resulted in study populations which do not fully represent the communities from which they were drawn. A more comprehensive Indian dengue serosurvey, published since, identified overall lower seroprevalence suggesting the peri-urban and urban sites included in our study may represent areas of heightened dengue transmission, enforcing the requirement of more local data to make local policy decisions.[146]

Another limitation is that study protocols may not always reflect real-life treatment realities: for example in our surveillance study, dengue diagnosis as a proportion of VCD was lowest in Philippines, but investigators postulated that Filipino study staff may have applied case definitions in the protocol more strictly than at other sites, in the full knowledge that participants were dengue cases. We also cannot exclude the impact of ongoing studies in towns or hospitals on case reporting or other surveillance practices, potentially biasing results. Underlying surveillance studies were designed to assess the benefits of CYD-TDV and were not powered to measure incidence rates with precision. For example, in Nealon et al. 2016,[67] we included only ~3,400 children across five countries, a sample size inadequate to generate precise estimates of the more severe, less frequent outcomes observed. This was a consequence of the opportunistic use of secondary data for these analyses.

FOI modelling assuming a constant infection risk

Our endemicity analyses were confined to exploring FOI and seroprevalence as a function of age, but because dengue is a cyclical, epidemic disease, calendar time is another, and perhaps more plausible, explanation for observed variation in FOI. For example, a detailed cohort study from a single city of Iquitos in Peru has identified time-varying dengue FOI, characterized by cyclical introductions of different serotypes giving rise to outbreaks.[171,176]

Policy value in context of other health priorities

These dengue disease burden estimates were performed to inform prevention and control. But a general limitation of conducting dedicated burden assessments is the lack of comparative assessments of burden of other important diseases such as malaria, diarrhoeal diseases or bacterial pneumonia with which dengue is a competing healthcare priority in endemic areas.[177] Applying enhanced surveillance with sensitive diagnostics and detailed modelling approaches can provide granular and impressive estimates of dengue infection and disease risk. But without equivalent initiatives for other health priorities and harmonization with other development and environmental goals these data cannot easily inform local prioritization activities and therefore lack policy relevance. A solution could be to conduct studies comparing disease burdens of >1 pathogen using similar methods, rather than focussing on individual diseases which are well-funded or fashionable.[178]

Sources of uncertainty

The studies described in this thesis are subject to variability/random error and to address this we calculated confidence intervals based on standard distributions for all studies, including robust errors adjusted for clustering where appropriate. This approach is inherently narrow, ignoring systematic error/bias which cause uncertainty in the study results; an incomplete consideration of this uncertainty over-estimates confidence in the findings and therefore diminishes the value of the study to support policy.[179] As discussed previously, important biases from these studies include that data were derived primarily from geographical sites with historically high dengue transmission and are therefore not representative of entire countries; data were captured from clinical trials in which

treatment and diagnostic practices vary from routine settings; and that study participants may differ systematically from the overall population in unknown ways (because, for example, of their willingness to participate in clinical studies). Quantifying this systematic bias is complex; was not included in our analysis outside of the bootstrapping in Wahyono *et al.,*[68] and represents a major limitation when extrapolating these data outside of the study setting.

Global and regional modelled burden estimates have used various methods to incorporate uncertainty. Bhatt *et al*. expended considerable effort to derive the relationship between incidence and environmental conditions through an ensemble of hundreds of boosted regression tree models and incorporated additional uncertainty in the relationship between incidence and probability of occurrence using a Bayesian hierarchical model.[3] Stanaway *et al.* used a Bayesian posterior simulation, resampling incidence rate estimates drawing input data from plausible distributions of key predictor variables.[80] Similarly, O'Reilly *et al.* used a combination of bootstrapping and Bayesian resampling methods.[99]

This level of modelling complexity was beyond the scope of our work, but even relatively simple sensitivity analyses or resampling methods which can relatively easily implemented in statistical software would have incorporated some uncertainty and improved the quality of the resulting estimates.[180] For example in Nealon *et al.*, 2016,[67] we assumed dengue incidence from the surveillance system were known data without variability but we could have incorporated data from multiple years and employed a resampling technique such as bootstrapping to estimate a sampling distribution whose variability would have been more representative of historical transmission. These uncertainty estimates may have been even more informative than the point estimates presented. We intentionally collected local surveillance data for comparison but should also have conducted a qualitative and quantitative assessment of whether the conduct of a clinical trial nearby changed those surveillance practices. And a relatively simple description of the characteristics (in terms of profession, socio-economic status or demography) of study participants as compared to the underlying population may have alerted us to differences between those groups.

Our seroprevalence assessments assumed diagnostic performance was perfect and we did not attempt to estimate seroprevalence for populations outside of our sample but a recent study – using SARS-CoV-2 seroprevalence data – shows how multiple sources of uncertainty can be incorporated into serosurveys. Larremore *et al*., adopted a Bayesian framework to produce a posterior distribution of seroprevalence that incorporates uncertainty associated with a finite sample size; and specified a hierarchical model describing sub-populations within the sample.[181] The distribution of subpopulation prevalences was then sampled using a Markov chain Monte Carlo process and combined in a demographically weighted average to represent the uncertainty overall. A similar project for dengue seroprevalence would represent interesting future work to describe sources and consequences of uncertainty including stemming from heterogeneity in transmission and age of first infection.

Comparisons with other dengue burden estimates

Disease burden estimates and under-reporting

Nealon *et al.* 2016[67] estimated multiplication factors which, when combined with national surveillance reports, are analogous to dengue burden estimates. These factors varied between countries from 5.5 – 31.7 which allow comparison with estimates performed using different data sources, populations and methods. Undurraga *et al*. estimated under-reporting of 7.6 across southeast Asian countries with some, but not all, country-specific point estimates falling within the 95% confidence intervals of our estimates.[25] Their comparison was based on published literature of variable quality, expert opinion and regression-based extrapolation to neighbouring countries which involves relatively untested assumptions. Toan *et al*. found national-level under-reporting factors of 11 – 126 depending on country and year.[81] Their literature review included published studies of variable quality and methods and compared with passive surveillance data from WHO websites which may themselves have suffered from under-reporting, inflating under-estimation estimates. Global dengue burden modelling studies estimated national burdens from which Indonesian EFs of 57 and 106, respectively, can be derived.[3,80] As described in chapter 1, the magnitude of these under-reporting differences from mathematical models and local empirical methods is difficult to reconcile. O'Reily *et al.*

estimated Indonesian dengue burden 10X higher than our estimates implying only 12% of hospitalized dengue cases are reported in national surveillance statistics. They speculate spatial reporting bias may contribute and describe future work to account for the sensitivity and biases in the underlying data populating their model.[99]

Case definitions and their impact on burden estimates

Variability in case definitions is an important determinant of dengue disease burden estimates and their interpretation which is often obscured by binary definitions of "symptomatic" dengue. But the precise case definition which triggers diagnostic testing in the cohort studies which underpin most burden estimates are different, including: school absence and history of fever or measured oral temperature ≥38°C;[124] febrile illness detected after ≥2 days' school absence;[182] [in our study] temperature ≥38°C on ≥2 consecutive days following self-reporting by parents;[30] or fever following self-reporting at participating healthcare facilities.[137] The definitions used for passive surveillance and by various research groups, highlighting their variation, is provided in table 5.1. Symptomatic dengue incidence is defined as the rate of test positivity and can be expressed as the proportion of infections which are symptomatic, by dividing this number by the number of study participants seroconverting over the study duration. This proportion is inversely associated with severity and has been estimated at ~30 – 50% for primary infections and ~40 – 80% for second infections.[65] However a labour-intensive Cambodian cluster investigation study, which recruited study participants prior to development of symptoms, showed that in fact >90% of dengue infected-individuals display some level of symptoms,[97] demonstrating the need to clearly understand underlying case definitions when interpreting resulting burden estimates. One solution would be to adopt uniform protocols when conducting dengue cohort studies, but this is complicated by different research study objectives, diagnostic practices, local clinical and hospitalization practices and resource availability. The variability of case definitions used in modelling studies is discussed in chapter 1 and, similarly, greater uniformity in these definitions would aid in the interpretation of these data.

Table 5.1. *Dengue case definitions as recommended by WHO (in 1997 and 2009 published guidelines) and those applied in different dengue cohort studies or clinical trials.*

Case definition	Fever	Other criteria
WHO 1997, 'probable dengue fever'[85]	None	Two or more of: headache; retro-orbital pain; myalgia; arthralgia; rash; haemorrhagic manifestations; leukopenia, AND Supportive serology or occurrence at same time/place as other dengue cases
WHO 2009, 'probable dengue'[6]	Fever	Two or more of: Nausea/vomiting; rash; aches and pains; tourniquet test positive; leucopaenia; any 'warning sign'
Kamphaeng Phet cohort, Thailand[124]	History of fever or measured temperature ≥38°C	School absence
Bangkok cohort, Thailand[182]	Febrile illness	≥2 days' school absence
CYD14 dengue vaccine trial[30]	≥38°C on ≥2 consecutive days	Self-reporting by parents
Bandung factory cohort, Indonesia[73]	Fever ≥37.8°C	Absence from work as validated by factory physician
Yogjakarta cohort, Indonesia[137]	Fever	Self-reporting at participating healthcare facility

Implications for dengue vaccine introduction

WHO recommends that an overall population benefit of dengue vaccination with CYD-TDV dengue vaccine can be expected in very high transmission settings, as defined by seroprevalence of ≥80% in participants aged 9 years.[31] We found this condition, which corresponds to a constant FOI of ~18%, had been satisfied in approximately 30% of sites, suggesting that many endemic areas could benefit from dengue vaccination. But these sites are distributed throughout large, heterogeneous countries which also contain pockets of lower endemicity which would not be candidates for vaccine use. Local seroprevalence assessments would therefore be needed prior to mass dengue vaccine introduction to identify those areas where vaccination would be most efficient and WHO has published a guideline to design and conduct these studies.[169] The recommended study design and laboratory methods are complex and resource-intensive and to my

knowledge no country has yet initiated a vaccination campaign with CYD-TDV after conducting a dedicated sub-national serosurvey for this purpose.

Countries wishing to conduct mass vaccination would need to develop optimal programmes reflective of local epidemiolocal data in a manner which would be perceived as ethical and acceptable to politicians and vaccine recipients. This is not straightforward: opinion has been divided on the ethics of vaccinating populations who would receive considerable health benefits at the expense of rarer, individual-level increased risks of severe disease in small numbers of seronegative vaccine recipients.[183,184] These debates focus on thresholds recommended by WHO but a well-regarded modelling analysis concluded that individual-level harm could be averted at lower levels of endemicity.[185] Ferguson *et al*. examined the impact of vaccination at different ages and seroprevalence, concluding that population-level benefits would arise when vaccinating an age group with seroprevalence exceeding ~35% and that even individual-level risks could be averted, thanks to indirect effects conferred on the overall population, by vaccinating where seroprevalence is over ~70% in 9-year olds.[185] The Philippines is the only Asian country which decided to introduce dengue vaccination in a public programme, in 2016. This campaign was halted following safety concerns and resulted in declining vaccine confidence[186] despite our and other results indicating suitability for vaccine introduction.[74,187] Independent researchers have since concluded that the programme will have resulted in significantly more benefits than harm.[188]

An implication of our FOI estimates, whereby >8% of children in every country except Singapore were infected with dengue annually, is that a very high proportion of adults in endemic countries will have been infected with dengue more than once. This was confirmed by serological surveys providing evidence for multiple previous infections in ~50% of Indian children aged 5 – 10 years; and >60% of Indonesian children aged >15 years. These immunological profiles are important for vaccine policymaking because the most efficient deployment of CYD-TDV would likely to be in individuals previously infected only once or twice.[65,189]

CYD-TDV is currently the only licensed dengue vaccine but others are in clinical development and may exhibit different clinical profiles which are not dependent on

serostatus. The Takeda vaccine TAK-003 has reported phase 3 trial results which show efficacy of 74.8% (95% CI 68.6 – 79.8) in baseline seropositive and 67% (53.6 – 76.5) in baseline seronegative recipients after 24 months' follow up.[190] Depending on longer-term clinical results, different epidemiological criteria may be needed to guide vaccine introduction.

Proposals for future studies and initiatives

Improving understanding of the contribution of age and time

The serological analyses described within this thesis were confined to exploring FOI and seroprevalence as a function of age, but because dengue is a cyclical, epidemic disease, calendar time is another explanation for observed variation in FOI. Additional work should examine the impact of time – and particularly outbreaks – on population-level seroprevalence and multiple infections, to identify populations who would benefit most from vaccination. Such an analysis would be possible using longitudinal serological survey data. An improved understanding of monotypic vs multitypic historical exposure would also allow better targeting of dengue vaccine to populations in whom benefits would be greatest. This could be possible using cross-sectional serological titre measurements from PRNT or IgG ELISA to classify individuals according to their likely infection history[191] and could be particularly interesting using our dataset which includes data from >30 sites across seven, diverse countries.

Risk communication

CYD-TDV displays long-term efficacy for high-risk individuals and could prevent a substantial burden of paediatric disease. However policymakers, media and the public are unfamiliar with nuanced scientific positions and complex data which inform them. Many inputs of benefit/risk assessment based on seroepidemiology are highly technical and, in the context of vaccine hesitancy and alarm in the media, improvements are required in the communication of accurate, impartial and understandable information to politicians and the general public. Risk mitigation and communication, to ensure individuals are aware of benefits and risks, remains an important topic of research, increasingly important to retain public confidence in new vaccines.

Bias assessment for VE studies

We conducted a study to identify potential biases which could present in a dengue VE study but, lacking information on patterns of vaccine use, failed to address several potential biases, particularly around control selection. These are complex topics which – even for vaccines in routine use for many years such as against influenza – remain topics of research.[192] Important, interrelated biases which could be relevant for dengue VE studies include misclassification of outcome due to suboptimal diagnostic performance; potential vaccine-modified disease severity; differential healthcare seeking in vaccine recipients who perceive themselves at risk; and the effects of serostatus on disease severity and likelihood to be vaccinated. Stratified analyses of seronegative and seropositive vaccine recipients would seem justified but challenging in practice because information on prior dengue infection is typically unavailable, and affected by vaccination status. A theoretical causal assessment of potential biases in dengue VE studies, as has been recently conducted for COVID-19,[193] would be a valuable addition to the literature.

Vaccination in combination with other interventions

Another potential tool for dengue control involves the controlled release of mosquitoes transfected with *Wolbachia* bacteria which persists in wild-type *Aedes* mosquitoes and confers resistance to dengue infection and, therefore, transmission. Promising field trials in Yogyakarta, Indonesia, have recently demonstrated 73% reductions in dengue incidence in treated areas which, if expanded and replicated elsewhere could fundamentally change dengue epidemiology.[194] Other promising vector control interventions are in development[195] and dengue vaccine introduction would need to consider their impacts. For example, reduced transmission mediated by *Wolbachia* introduction could lower the proportion of seropositive individuals at a given age, thereby altering the benefit-risk profile of vaccine introduction. Recent studies have attempted to dissect the contribution of each strategy, much uncertainty currently exists, and this will remain a topic of theoretical and applied research for years to come.[196,197]

Recommendations

Dengue surveillance

Important differences in dengue surveillance systems include a) whether reporting of confirmed and/or suspected cases using various definitions is mandatory; b) the use of electronic vs paper systems; 3) the level of age group stratification reported; 4) the use of laboratory confirmation and serotype surveillance.[23] (Surveillance systems of five Asian countries also reviewed in supplementary material of Nealon *et al* 2016.[67]). These differences give rise to reporting frequencies which are not descriptive of – but can easily be confused with – disease burden. This was illustrated in this thesis with an example from India, a country with FOI of ~12% per year, similar to other highly endemic countries. However India reports approximately 100-fold fewer cases than other endemic countries, a difference which must be ascribed to surveillance differences.[145] We also observed symptomatic disease episodes which did not satisfy strict case definitions, for example from the Philippines, thereby underestimating the frequency even of recognized, symptomatic dengue cases.

Recommendations to improve and standardize dengue surveillance include:

- Increase laboratory confirmation of undifferentiated febrile episodes to identify milder dengue cases and increase reporting of outpatient cases. Because it would not be feasible to confirm all episodes, a sentinel network could be employed for this purpose to estimate a positivity rate which could inform the broader disease burden (as is currently done for influenza in many countries).
- Mandatory reporting of confirmed dengue or suspected cases satisfying case definitions including those which are relatively mild (e.g., outpatient cases). This change would be accompanied by an increased number of notifications which may become burdensome for healthcare providers and it may be necessary to simplify reporting procedures, ideally using electronic tools.
- Reporting using uniform age categories which provide sufficient granularity to detect a changing age distribution of cases in children, indicative of changing endemicity (e.g.: 0 – 5 yrs; 6 – 10 yrs; 11 – 18 yrs; 19 – 64 yrs; ≥ 65yrs).

Future VE studies

Several operational and theoretical problems with the conduct of dengue VE studies were identified in this thesis. We identified differences between cases and controls indicating that controls may not represent the exposure time at risk of cases; found it difficult to enroll adequate numbers of control participants; and possibly observed recall bias in their responses to questionnaires. Recommendations for a future VE study:

- It will be necessary to validate important exposures from reliable records rather than relying on self-reported history. This would be essential for dengue vaccination status but also applies to other potentially confounding variables (egg, proximity to dengue cases/outbreaks).
- The superiority of a case control vs TND study has not been demonstrated by the work in this thesis and future VE studies should enroll both types of controls to further assess important biases and their implication on VE.
    - For TND studies, enrolling from secondary or regional hospitals (rather than tertiary centres of excellence) may increase the number of participants who have not yet received confirmatory diagnostics and therefore increase the proportion of test-negative controls, improving study feasibility. This would require a greater number of study sites to enroll the same number of participants, which could increase costs and complexity. TND controls should be matched on age, sex and geography (e.g., district) to improve their representativeness of the exposure time at risk of cases.
    - TND studies for dengue remain untested and a full bias assessment, as has been recently conducted for COVID-19,[198] should be conducted, including assessment of the impact of spatiotemporal and laboratory practices which are unique to dengue.
    - For CC studies, enrolling community controls (e.g., neighbours) should be attempted to increase the number of eligible participants and also improve their comparability in terms of exposure risk. Controls should be matched by age and sex. This approach would increase logistical resources because

it would be necessary for study teams to physically travel to identify controls participants from their residences.

- VE study designs to identify and quantify vaccine-associated enhanced disease, expressed as a negative VE, are also needed. These are challenging because the outcome (hospitalized/severe dengue) would occur several years after the exposure (vaccination) and the risk is modified by baseline serostatus which is typically unknown. The most promising study design would be a large, prospective observational cohort, with blood samples from participants stored and, for economy, tested only if participants developed a symptomatic episode. For severe dengue which is rare, a cohort would be very large and a case control study measuring the frequency of vaccination in severe vs mild cases would estimate the odds of vaccination in severe vs non-severe dengue which could act as an indicator of harm, but it would not be possible to control for baseline serostatus with this design.
- Any future dengue VE studies should be conducted for the long term (e.g., 10 years) to account for all vaccine mediated benefits and harm and minimize the chance of conducting studies during particular windows of enhanced risk, whose duration would be determined by the underlying endemicity.

Chapter 6: Conclusions

At the onset of the body of work described in this thesis, it was anticipated that decisions around dengue vaccine introduction would be taken in light of vaccine efficacy and safety, age-stratified disease burden and related health economic considerations. These topics form the first chapters of this thesis. The availability of additional data on vaccine performance changed this decision-making framework when endemicity, indicated by seroprevalence, became a criterion for safe and effective vaccine use. Despite those thresholds having been reached in several countries, as described in the second part of this thesis, CYD-TDV remains in very limited use globally. Irrespective of public health benefits, it is likely that the theoretical risk of vaccine-mediated harm will limit any future CYD-TDV mass vaccination programmes to those that follow individual-level verification of infection history, either following serological testing or a laboratory-confirmed dengue episode. These approaches seem feasible: commercial dengue serological tests are highly specific and can therefore be used to avoid inappropriately vaccinating seronegative individuals.[199] A recent consultation described the ideal characteristics of point of care tests for pre-vaccination screening and programmatic guidance on using them, which would potentially allow wider vaccine uptake.[189] As post-licensure data accumulate on the safe and efficient use of this and other vaccines it is hoped that the impact of this highly endemic disease will begin to be blunted. The methods described in this thesis can be used to monitor the changing epidemiology of dengue and the real-life performance of interventions aimed at preventing transmission and symptomatic episodes across Asian countries.

Specific contribution to the portfolio

Crrsp. = corresponding author

Type	Author position	Title and reference	Authors	My contributions as agreed with co-authors (appendix 3)
*Original research	1st and crrsp.	Symptomatic Dengue Disease in Five Southeast Asian Countries: Epidemiological Evidence from a Dengue Vaccine Trial. *PLoS Negl Trop Dis*. 2016;10(8):e0004918.	**Nealon J**, Taurel A, Capeding MR, Tran NH, Hadinegoro SR, Chotpitayasunondh T, Chong CK, Wartel TA, Beucher S, Frago C, Moureau A, Simmerman M, Laot T, L'Azou M, Bouckenooghe A.	- Study conception and design - Data collection and analysis - Drafted manuscript as principal author - Final approval
*Short report	Joint 1st and crrsp	Indonesian dengue burden estimates: review of evidence by an expert panel. *Epidemiol Infect*. 2017 26;(May):1–6.	**Nealon J**, Tri Yunis Wahyono M, Beucher S, Prayitno A, Moureau A, Nawawi S, Adryanto AFT, Hasbullah T, Mardiati N.	- Study conception and design - Data collection and analysis - Drafted manuscript as joint principal author - Final approval
*Original research	Last	Dengue serotype-specific seroprevalence among 5- to 10-year-old children in India: a community-based cross-sectional study. *Int J Infect Dis*. 2017;54:25–30.	Garg S, Chakravarti A, Singh R, Masthi NRR, Goyal RC, Jammy GR, Ganguly E, Sharmaa N, Singh MM, Ferreira G, Moureau A, Ojha S, **Nealon J**.	- Data analysis - Drafted manuscript as principal author - Final approval
*Original research	3	Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children. *PLoS Negl Trop Dis*. 2017;11(6):e0005621.	Prayitno A, Taurel A, **Nealon J**, Satari HI, Karyanti MR, Sekartini R, Soedjatmiko S, Gunardi H, Medise BE, Sasmono RT, Simmerman JM, Bouckenooghe A, Hadinegoro SR.	- Contributed to study design - Data collection - Drafted manuscript - Final approval
Original research	Last	Dengue virus serotype distribution based on serological evidence in pediatric urban population in Indonesia. *PLoS Negl Trop Dis*. 2018; 12(6):e0006616.	Sasmono RT, Taurel A, Prayitno A, Sitompul H, Yohan B, Hayati RF, Bouckenooghe A, Hadinegoro SR, **Nealon J**	- Study conception and design - Drafted manuscript - Final approval

*Original research	Joint 1st and crrsp	Serological evidence of Japanese encephalitis virus circulation in Asian children from dengue-endemic countries. *J Infect Dis*. 2019;219:375–81	**Nealon J**, Taurel A-F, Yoksan S, Moureau A, Bonaparte M, Quang LC, Capeding M, Prayitno A, Hadinegoro S, Chansinghakul D, Bouckenooghe A	- Study conception and design - Drafted manuscript as principal author - Final approval
Original research	4th	Economic burden of dengue in Indonesia. *PLoS Negl Trop Dis.* 2019;13:e0007038.	Nadjib M, Setiawan E, Putri S, **Nealon J**, Beucher S, Hadinegoro S, Permanasari V, Sari K, Wahyono T, Kristin E, Wirawan D, Thabrany H	- Conception and study design - Data collection and analysis - Drafted manuscript - Final approval
*Original research	1st and crrsp	Feasibility of case-control and test-negative designs to evaluate dengue vaccine effectiveness in Malaysia. *Vaccine*. 2019;37(39):5891-5898.	**Nealon J**; Lim WY; Moureau A; Junus S; Linus S; Kumar S; Jeyaseelan P; Rosman A; Devi S; Radigue C; Cowling B; Ochiai L; Singh A	- Study design - Data analysis - Drafted manuscript as principal author - Final approval
*Original research	Joint 1st and crrsp	Estimated dengue force of infection, seroprevalence and burden of primary infections among Indian children. *BMC Public Health*. 2019;19(1):1116.	Bhavsar, Amit; Tam, Clarence; Garg, Suneela; Jammy, Guru Rajesh; Taurel, Anne-Frieda; Ching, Sher-Ney; **Nealon, Joshua**	- Study conception and design - Data analysis - Drafted manuscript as principal author - Final approval
*Original research	1st and crrsp	Dengue endemicity, force of infection and variation in transmission intensity in 13 endemic countries. *J Infect Dis*. March 25th 2020. doi:10.1093/infdis/jiaa132	**Nealon J**, Bouckenooghe A, Cortes M, Coudeville L, Frago C, Macina D, Tam	- Study conception and design - Data analysis - Drafted manuscript as principal author - Final approval

Crrsp = corresponding author. * = forms primary focus of thesis.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Symptomatic Dengue Disease in Five Southeast Asian Countries: Epidemiological Evidence from a Dengue Vaccine Trial

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Abstract

Dengue incidence has increased globally, but empirical burden estimates are scarce. Prospective methods are best-able to capture all severities of disease. CYD14 was an observer-blinded dengue vaccine study conducted in children 2–14 years of age in Indonesia, Malaysia, Thailand, the Philippines, and Vietnam. The control group received no vaccine and resembled a prospective, observational study. We calculated the rates of dengue according to different laboratory or clinical criteria to make inferences about dengue burden, and compared with rates reported in the passive surveillance systems to calculate expansion factors which describe under-reporting. Over 6,933 person-years of observation in the control group there were 319 virologically confirmed dengue cases, a crude attack rate of 4.6%/year. Of these, 92 cases (28.8%) were clinically diagnosed as dengue fever or dengue hemorrhagic fever by investigators and 227 were not, indicating that most symptomatic disease fails to satisfy existing case definitions. When examining different case definitions, there was an inverse relationship between clinical severity and observed incidence rates. CYD14's active surveillance system captured a greater proportion of symptomatic dengue than national passive surveillance systems, giving rise to expansion factors ranging from 0.5 to 31.7. This analysis showed substantial, unpredictable and variable under-reporting of symptomatic dengue, even within a controlled clinical trial environment, and emphasizes that burden estimates are highly sensitive to case definitions. These data will assist in generating disease burden estimates and have important policy implications when considering the introduction and health economics of dengue prevention and control interventions.

vaccine. MRC, NHT, SRH and TC have been clinical trial investigators for and received associated payments from Sanofi Pasteur. CCK has no competing interests to declare.

Author Summary

Dengue is a mosquito-borne, viral febrile disease transmitted between humans in most of the tropical and sub-tropical world. In recent years, an increasing number of cases has been widely reported. However, understanding the full disease burden remains a topic of public health research. One reason for under-reporting is that severe episodes are more likely to be captured in routine surveillance statistics, and mild episodes unreported/unrecognized. We re-analyzed data from the control arm of a dengue vaccine clinical trial in five Asian countries. The trial captured dengue incidence rates following active surveillance and virological confirmation, and we compared those with incidence rates from the passive surveillance system. As expected, the active surveillance system captured many more cases of symptomatic dengue than routine systems. Of virologically confirmed dengue in the clinical trial, only ~29% were diagnosed by investigators as dengue, indicating there is a significant disease burden excluded from existing case definitions and diagnostic practices. The analysis confirmed that dengue is under-reported, by different magnitudes, in these Asian countries. Case definition is an important determinant of burden. These findings are important when considering the health economics and public health impacts of new prevention and control tools.

Introduction

Dengue is a viral disease transmitted between humans by *Aedes* mosquitoes throughout the tropical and subtropical world. Infection may be asymptomatic, or can result in a spectrum of clinical disease including self-limiting fever with manifestations of varying severity (classical dengue fever; DF) progressing to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). [1] While this classification remains in clinical use in some countries, a new system was proposed by the World Health Organization (WHO) in 2009 primarily to improve triage and clinical management, and to capture warning signs of potentially severe dengue episodes. [1,2] Disease prevention efforts with mosquito control have been largely unsuccessful and recent decades have witnessed increased disease frequencies and expanded ranges of transmission. [3,4] Dengue is now endemic in over 120 countries worldwide, with almost half of the global population at risk. [3,5]

Approximately 75% of this at-risk population resides within the Asia Pacific region, where the primary vectors (*Aedes aegypti* and *Ae. albopictus)* and dengue virus have become widely dispersed over recent decades following a number of social, environmental, and demographic changes. [6,7] Multiple dengue virus serotypes co-circulate and the disease constitutes a leading cause of hospitalization and death in some countries. [8] In the midst of this expansion, and possibly due to it, reliable dengue disease burden estimates are uncommon. [3,9] Passive national dengue surveillance systems are designed to detect outbreak activity rather than describe burden. [10] More reliable estimates are required to guide disease control programs, allow rational allocation of resources, and assess the impact of new interventions such as dengue vaccination. Accordingly, estimating the true disease burden constitutes one of the WHO's three objectives in the 2012 *Global Strategy for Dengue Prevention and Control 2012–2020*. [3]

In most scenarios, national surveillance systems underestimate disease burden due to the non-specific clinical presentation of dengue; unavailability and limitations of confirmatory diagnostic tests; and health system issues that result in incomplete reporting. [11] Under-estimation is typically most severe in the milder manifestations of illness, and is a function both of under-ascertainment and under-reporting. [12] In recent years, several methods have been

used to improve the accuracy of historical global disease burden estimates of approximately 100 million infections/year. [3,13] These include empirical methods where overlapping data sources enable estimation of cases missed (capture-recapture studies); expert consensus-based approaches; statistical and/or cartographic methods incorporating dengue occurrence data or their covariates; regression methods to estimate unknown variables; and derivations from seroprevalence data. [9,14–16] Notably, a 2013 study by Bhatt *et al*. used a cartographic modeling approach combining demographic and epidemiological data, adjusted for clinical severity and determinants of dengue incidence, to estimate a global burden of 390 million (95% credible interval: 284–528 million) infections in 2010, of which 96 million (67–136 million) were symptomatic. [9] It has been estimated that 70% of cases and >50% of the economic burden of dengue are in Asia. [9,17]

Prospective cohort studies utilizing active surveillance can yield more accurate estimates of symptomatic disease than passive surveillance systems. [18] Resulting incidence rates (IRs), when compared with data from routine surveillance systems, can describe the extent of underestimation, expressed as multiplication or expansion factors (EFs). [12,19] In Cambodia, Thailand, and the Philippines, individual studies using these methods calculated EFs for dengue of between 7.2 and 9.1. [20,21] A review using data from all WHO regions found dengue EFs in Asia of up to 126, with significant variation among countries and over time resulting from different underlying epidemiology, surveillance practices, and comparative study design. [19]

Dengue vaccine clinical trials are conducted with a high degree of operational integrity and produce data closely resembling those from active epidemiological studies. Subjects allocated to the control group do not receive dengue vaccine, so incidence data from these individuals can be interpreted as an observational dengue cohort. [22] CYD14 was an observer-blinded dengue vaccine study conducted in 2011–2013 in 10,275 children aged 2–14 years in Indonesia, Malaysia, Thailand, the Philippines, and Vietnam. [23] Each of these countries conducts passive routine dengue surveillance, sometimes using different case definitions and different reporting, laboratory, and diagnostic practices. [10,11] (described in S1 File).

Dengue epidemiological data from CYD14 and its Latin American sister, CYD15, were recently described by L'Azou *et al*., allowing comparison across countries of data collected using standardized, active methods. [24] Here, we exploit the comprehensive dataset to further explore dengue incidence in the CYD14 control group according to different clinical endpoints (in addition to the primary clinical endpoint of the efficacy trial) to examine the relationship between burden and severity in five Asian countries. We also made comparisons with national surveillance reports to estimate EFs for symptomatic dengue of different clinical severities, from which broader burden estimates can be inferred.

Materials and Methods

Ethics statement

This was a secondary analysis using records from a vaccine clinical trial. The original clinical trial which generated the data (ClinicalTrials.gov number NCT01373281) underwent ethics committee approval of the protocol, amendments, consent, and assent forms. [23] Parents or legal guardians provided informed consent before participation, and written assent was obtained from older children, in compliance with the regulations of each country. All data were analyzed anonymously.

CYD14 study design and data

CYD14 (CT.gov identifier NCT01373281) was an observer-masked, randomized, controlled, multicenter, phase 3 dengue vaccine trial in Indonesia (3 study centers), Malaysia (2 study

centers), the Philippines (2 study centers), Thailand (2 study centers), and Vietnam (2 study centers) and has been described previously. [23] There was ethics committee approval of the protocol, amendments, consent, and assent forms. Parents or legal guardians provided informed consent before participation, and written assent was obtained from older children, in compliance with the regulations of each country. Briefly, children aged 2–14 years were randomly assigned to receive three injections of a recombinant, live-attenuated, tetravalent dengue vaccine (CYD-TDV), or placebo, at 0, 6, and 12 months. Participants were followed up actively for a total of 25 months and episodes of fever $\geq$ 38°C on $\geq$ 2 consecutive days were recorded and clinically diagnosed as DF or DHF based on 1997 WHO guidelines (Fig 1). Concurrent

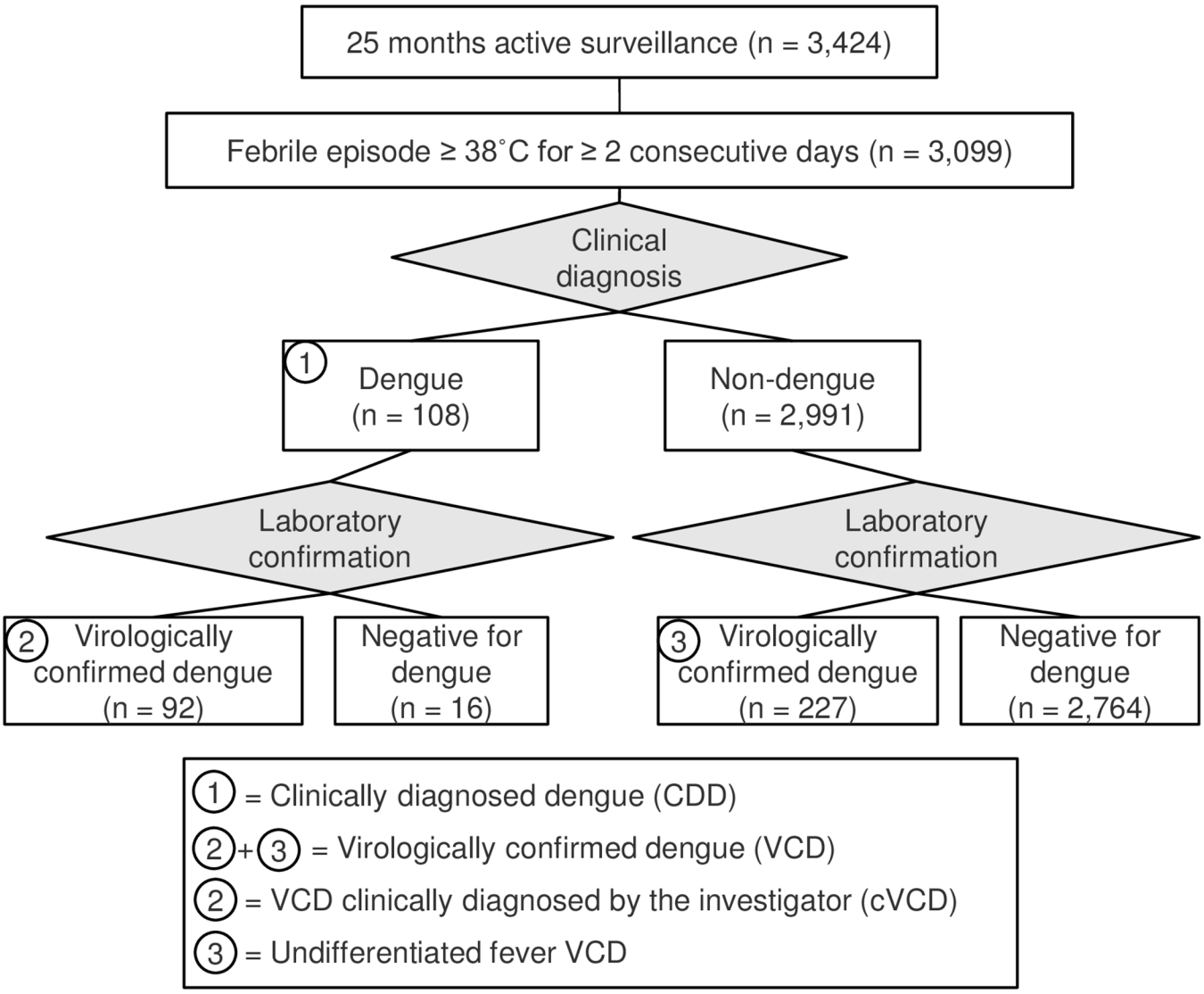

Fig 1. CYD14 study flow chart and source of each case definition. Control arm subjects were actively followed for 25 months to detect episodes of fever $\geq$38°C for $\geq$ 2 consecutive days. Febrile episodes were recorded and clinically diagnosed as dengue based on 1997 WHO guidelines, or an alternative etiology. Irrespective of clinical diagnosis, serum samples were taken for virological confirmation of dengue by detection of NS1 antigen by immunoassay and viral RNA by RT-PCR. A positive result for either laboratory test was considered confirmatory of dengue. Clinically diagnosed dengue (CDD): all episodes that were clinically diagnosed as dengue, irrespective of virological confirmation. VCD: all virologically confirmed dengue episodes, irrespective of clinical diagnosis. cVCD: all VCD episodes that were also clinically diagnosed as dengue. UF-VCD: all VCD episodes that were not clinically diagnosed as dengue.

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with and irrespective of clinical diagnosis, serum samples were taken for virological confirmation of dengue by detection of NS1 antigen by ELISA and dengue viral RNA by RT-PCR. A positive result for either laboratory test was considered virological confirmation of acute dengue infection. This allowed febrile individuals to be grouped into four case definitions according to their clinical diagnosis and laboratory results: 1) clinically diagnosed dengue (CDD) was diagnosed by the investigator as dengue, irrespective of the laboratory result; 2) virologically confirmed dengue (VCD) was a dengue virological laboratory confirmation, irrespective of the clinical diagnosis; 3) clinical VCD (cVCD) was clinically diagnosed by the investigator as dengue and accompanied by laboratory confirmation of dengue infection; 4) undifferentiated fever VCD (UF-VCD) was laboratory confirmation of dengue infection but was not diagnosed as dengue by the investigator. Detailed case report forms were completed for each febrile episode, including whether subjects required hospitalization. This manuscript describes results of a secondary analysis of anonymous data from this vaccine clinical trial.

National dengue surveillance, population data and incidence rates

Sub-national passive dengue surveillance data from districts, provinces, or cities (hereafter referred to as "geographical units") encompassing each clinical trial center were retrieved from official government surveillance websites for Thailand [25] and Jakarta, Indonesia, [26] or from personal communications with trial investigators, or sub-national health authorities in Malaysia [27], Indonesia, the Philippines, and Vietnam [28]. Dengue cases of any severity were pooled for the period of time during which CYD14 was active in that country.

Age-specific population data for sites were retrieved from census or other official records for each geographical unit. [29–33] Populations at the mid-point of the study were estimated by applying national-level population growth factors. Where surveillance or census data lacked age-stratifications (Vietnam and Indonesia for census; Malaysia and Thailand for monthly age-specific surveillance data), we assumed age-distributions of populations/cases were proportional to those at the national level. Average annual IRs were calculated for each country by pooling data from all geographical areas participating in the study, expressed as cases/100,000 population/year, as:

$$Average\ annual\ incidence\ rate = \frac{\frac{N_c}{T_m}}{Population_{ji}} * 12$$

Where N_c is the number of cases reported to the surveillance system over the study observation period; T_m is the duration of the study in each country, in months; and $\overline{Population_{ji}}$ is the average population size over the study period.

Calculating CYD14 incidence densities

Site- and age-weighted incidence densities (IDs) were calculated by direct standardization for each country to correct for the fact that the age and geographic distributions of study populations were different from those in the geographic units from which they were drawn. [34] Study populations were divided into three age groups according to their age when they contributed time to the study: < 5 years; $5 - < 10$ years; and > 10 years (all were aged <15 years at enrollment). Crude age-specific IRs were calculated for each age group and each center by dividing the number of cases satisfying each case definition by the number of person-years (p/y) of observation. These crude IRs were to match the demographics from CYD14 with those of each geographical area, resulting in age- and site-adjusted IDs aligned with the census populations at the country level. [34] Results were presented as cases/100,000 p/y. Standardized 95%

confidence intervals (CIs) were calculated based on the gamma distribution using SAS 9.4 (SAS Institute, Cary, NC).[35]

Expansion factors and case definitions

Expansion factors were calculated by dividing the adjusted ID captured during CYD14 for each case definition by the IRs reported by the national passive surveillance systems at each geographical unit. For calculating 95% CIs, IRs from surveillance systems were considered known data without variability. [34]

Descriptive exploratory statistical analysis was performed on the ability of each case definition to identify symptomatic dengue cases; the proportion hospitalized; and the duration of clinical symptoms, fever, and hospitalization, for each. Using VCD as the gold standard diagnosis of dengue following a febrile episode, positive predictive value, negative predictive value, sensitivity, and specificity were calculated for clinical diagnosis of dengue disease with their 95% CIs according to the efficient-score method [36]. Data used for the analyses described above are provided in S1 and S2 Tables.

Results

VCD and CDD in the CYD14 cohorts

Between June and December, 2011, 3,424 children were enrolled in the control arm of the CYD14 study (Table 1). The demographics of the subjects have been reported elsewhere. [23] The period of observation was 6,933 p/y, during which there were 3,099 febrile episodes tested for dengue, of which 319 (10.3%) were VCD. This proportion in each country varied between 6.3% (Malaysia) and 12.3% (Indonesia). The overall crude annual VCD attack rate was 4.6%, varying from 2.2% (Malaysia) to 6.6% (Philippines). A total of 108 cases satisfied the CDD definition and 227 satisfied the UF-VCD definition (underlying data in S1 Table).

Of the 319 VCD cases, 25 (7.8%) were clinically diagnosed as DHF and 67 (21.0%) as DF, giving a total of 92 (28.8%) cases of cVCD. This proportion of VCD correctly diagnosed varied widely among the countries, from 10.3% (Philippines) to 74.5% (Thailand). In addition to the 25 clinical diagnoses of DHF in the VCD group, there were an additional 4 DHF diagnoses which were not virologically confirmed. Only one DHF diagnosis was in a subject aged <5.

Table 1. Number (n) and proportion of subjects experiencing episodes satisfying different case definitions in the CYD14 control arm, June 2011 – December 2013.

Country	Subjects injected n	Febrile episodes, n	Person-years followed, n	VCD episodes, n (% of fevers)	cVCD episodes, n (% of fevers)	CDD episodes[1], n (% of fevers)	UF- VCD[2], n (% of fevers)	Proportion of VCD clinically diagnosed as dengue[3], %	Crude VCD attack rate (%), %
Indonesia	623	357	1232	44 (12.3)	26 (7.3)	33 (9.2)	18 (5.0)	59.1	3.6
Malaysia	465	332	937	21 (6.3)	9 (2.7)	11 (3.3)	12 (3.6)	42.9	2.2
Philippines	1166	1420	2370	156 (11.0)	16 (1.1)	19 (1.3)	140 (9.9)	10.3	6.6
Thailand	392	388	792	47 (12.1)	35 (9.0)	36 (9.3)	12 (3.1)	74.5	5.9
Vietnam	778	602	1602	51 (8.5)	6 (1.0)	9 (1.5)	45 (7.5)	11.8	3.2
Totals	3,424	3,099	6,933	319 (10.3)	92 (30)	108 (3.5)	227 (7.3)	28.8	4.6

n, number of subjects or events; VCD, virologically confirmed dengue. cVCD, clinically diagnosed and virologically confirmed dengue; CDD, clinically diagnosed dengue; UF-VCD, virologically confirmed dengue not diagnosed as dengue. Crude attack rates are VCD cases/person-years followed.

[1]Total episodes that were clinically diagnosed dengue (CDD).

[2]VCD episodes accompanied by a clinical diagnosis other than dengue.

[3]cVCD/VCD (i.e. sensitivity).

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Table 2. Dengue incidence rates [and 95% CIs] from routine surveillance systems and adjusted incidence densities of disease according to different case definitions from the CYD14 study.

Country	Average IR from routine surveillance system	CYD14			
		VCD, adjusted ID	cVCD, adjusted ID	CDD, adjusted ID	UF-VCD, adjusted ID
Indonesia	262.9	3,017 [1,951–4,542]	1825 [996, 3,153]	2,479 [1,483, 3,963]	1,192 [602, 2,269]
Malaysia	64.7	2,048 [1,099, 3,720]	671 [288, 1,851]	777 [367, 1,961]	1,377 [567, 2,993]
Philippines	954.4	10,960 [8,673, 13,620]	677 [262, 1,439]	701 [281, 1,461]	10,290 [8,055, 12,890]
Thailand	496.7	5,938 [4,273, 8,059]	4,262 [2,914, 6,055]	4,383 [3,015, 6,194]	1,676 [808, 3,065]
Vietnam	509.2	2,784 [1,813, 4,238]	261 [94, 1,083]	840 [156, 2,371]	2,523 [1,580, 3,964]

Incidence rates and densities are in cases/100,000 person-years. Incidence rates for routine surveillance calculated from total number of cases over the period of study and study mid-point populations. IR, incidence rate; VCD, virologically confirmed dengue; ID, incidence density; CI, confidence interval; cVCD, clinically diagnosed and virologically confirmed dengue; CDD, clinically diagnosed dengue; UF-VCD, virologically confirmed dengue not diagnosed as dengue.

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Incidence of dengue in the cohorts compared to national routine surveillance estimates

There was significant heterogeneity in average IRs observed between countries over the duration of the clinical trial. The IRs for all dengue cases (per 100,000 p/y) reported to national routine surveillance systems for the study locations during the study period varied by country from: 64.7 (Malaysia); 263 (Indonesia); 497 (Thailand); 509 (Vietnam); and 954 (Philippines) (Table 2). Adjusted dengue IDs (per 100,000 p/y) in the CYD14 study were considerably higher than the rates captured by the national systems and varied according to the case definition used. IDs were highest for VCD (range: 2,048 [Malaysia] to 10,960 [Philippines]), and were followed by the IDs for UF-VCD (range: 1,192 [Indonesia] to 10,290 [Philippines]), CDD (range: 701 [Philippines] to 4,383 [Thailand]), and finally cVCD (range: 261 [Vietnam] to 4,262 [Thailand]). Surveillance data and corresponding incidence rates provided in S2 Table.

Expansion factors

EFs varied according to case definitions. They were 5.5–31.7 for VCD, 0.5–10.4 for cVCD, and 0.7–12.0 for CDD (Table 3). These factors varied widely but tended to be lowest in Vietnam and highest in Malaysia. The incidence rates of dengue reported to the routine surveillance system appeared to be important determinants of EF: the highest EF (31.7) was observed in Malaysia (with the lowest reported IR) and the Philippines (with low EFs) reported the highest IRs in passive surveillance.

Table 3. Expansion factors for VCD, cVCD, and CDD over the active phase of the CYD14 study.

Country	VCD [95% CI]	cVCD [95% CI]	CDD [95% CI]
Indonesia	11.5 [7.4, 17.3]	6.9 [3.8, 12.0]	9.4 [5.6, 15.1]
Malaysia	31.7 [17.0, 57.5]	10.4 [4.5, 28.6]	12.0 [5.7, 30.3]
Philippines	11.5 [9.1, 14.3]	0.7 [0.3, 1.5]	0.7 [0.3, 1.5]
Thailand	12.0 [8.6, 16.2]	8.6 [5.9, 12.2]	8.8 [6.1, 12.5]
Vietnam	5.5 [3.6, 8.3]	0.5 [0.2, 2.1]	1.7 [0.3, 4.7]

CI, confidence interval; VCD, virologically confirmed dengue CDD, cVCD, clinically diagnosed and virologically confirmed dengue; CDD, clinically diagnosed dengue.

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Table 4. Number of episodes and hospitalizations in CYD14 study control subjects experiencing acute fever, VCD, CDD, or VCD clinically diagnosed DHF.

Country	Febrile episodes		VCD		CDD		VCD clinically diagnosed DHF	
	n	Number of episodes hospitalized (%)	n	Number of episodes hospitalized (%)	n	Number of episodes hospitalized (%)	n	Number of episodes hospitalized (%)
Indonesia	357	30 (8.4)	44	20 (45.5)	33	21 (63.6)	10	11 (100.0)
Malaysia	332	20 (6.0)	21	8 (38.1)	11	7 (63.6)	1	1 (100.0)
Philippines	1420	35 (2.5)	156	17 (10.9)	19	17 (89.5)	9	10 (100.0)
Thailand	388	34 (8.8)	47	13 (27.7)	36	13 (36.1)	2	2 (100.0)
Vietnam	602	7 (1.2)	51	3 (5.9)	9	4 (44.4)	3	3 (60.0)
Total	**3,099**	**126 (4.1)**	**319**	**61 (19.1)**	**108**	**62 (57.4)**	**25**	**24 (96.0)**

n, number of subjects or events; VCD, virologically confirmed dengue, CDD, clinically diagnosed dengue; DHF, dengue haemorrhagic fever.

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Hospitalization and symptoms

Overall, 126 (4.1%) of the acute febrile episodes in the cohort were hospitalized (Table 4). For the individual countries, this proportion varied between 1.2% (Vietnam) and 8.8% (Thailand). Hospitalization rates varied according to local standard of care (i.e., laboratory and clinical diagnosis): 61 (19.1%) of the 319 VCD episodes; 62 (57.4%) of the 108 CDD cases; and 24 (96.0%) of the 25 VCD cases diagnosed as DHF, were hospitalized. These proportions varied between countries. Clinical dengue diagnosis appeared to be an important determinant of hospitalization. The median duration of clinical symptoms was 5.0 days [min; max: 2.0; 38.0] for cases of UF-VCD; 6.0 [2·0; 38·0] for VCD and 8.0 [2.0; 31.0] for CDD. Median durations of fever were 3.0 [2.0; 11.0], 3·0 [2·0; 11·0] and 4.0 [2.0; 9.0)] and hospitalization 4.0 [3.0; 6.0]; 5·0 [2·0; 9·0] and 5.0 [2.0; 9.0)], respectively.

Positive and negative predictive values, sensitivity, and specificity of clinical diagnosis

The positive predictive value (PPV) of clinical diagnosis, using VCD as the gold standard, was 85.2% (95% CI 77.1–91.3) and the negative predictive value (NPV) was 92.4% (91.4–93.3). The sensitivity of clinical diagnosis was 28.8% (95% CI 23.9–34.2) and the specificity was 99.4% (99.1–99.7).

Discussion

We used the control arm of a large, phase 3 efficacy dengue vaccine trial to describe the symptomatic and virologically-confirmed dengue burden in five Asian countries. This permitted comparison of clinical vs. laboratory dengue diagnosis for different classifications of symptomatic dengue identified through active surveillance. Importantly, data were consistently collected according to standardized case definitions and with high-quality virological confirmation, allowing IDs to be measured for different clinical outcomes and in different countries, within a single study. Rates observed in study participants were adjusted to match the populations from which they were sampled.

Discrepancies between dengue clinical and laboratory diagnosis typically find case definitions which are sensitive but lack specificity, particularly in episodes of mild disease. [37–39] Our results showed that virological confirmation was the most sensitive means of identifying dengue disease, capturing approximately 3.5 times more episodes than clinical diagnosis alone, even in this acutely febrile patient population. Clinical diagnosis alone captured only 28.8% of

symptomatic cases. Because most passive disease surveillance systems in Asia rely almost entirely on clinical diagnosis, [10,40] it is reasonable to believe a substantial proportion of symptomatic dengue disease is unrecognized and therefore unreported.

The proportion of VCD clinically diagnosed by investigators as dengue varied substantially between countries (range: 10.3%–74.5%), likely resulting from the multifactorial impacts of local clinical guidelines and case definitions that affect diagnostic practices, and variable clinical presentations. Disease severity and clinical manifestation may be affected by factors including circulating viral genotypes; the order and duration between sequential, heterotypic infections; year/season; and subject age [41–43]. Notably, VCD cases appeared to be younger in the Philippines–where dengue was least-frequently diagnosed–than other countries.

Estimates of dengue burden are, in large part, a function of case definition. [9] The active surveillance methods here allowed calculation of IRs according to different case definitions (VCD, cVCD, and CDD) and thus determine EFs for each. The higher rates of VCD captured gave rise to EFs ranging from 5.5 to 31.7, with lower EFs for more specific case definitions of cVCD and CDD. These figures are notable for their variability and emphasize that study and surveillance system methodology and geography are important determinants of under-reporting estimates, as reported elsewhere. [15,19] However, the finding that dengue is under-reported by factors of >30 in some countries and contexts is consistent between these studies. Notable exceptions are the expansion factors <1, observed in Philippines and Vietnam against specific, clinically-diagnosed case definitions (cVCD and CDD): passive surveillance had captured a higher proportion of cases than the active system. There are two likely possibilities: 1) the passive surveillance reported false-positive cases (ie, episodes of febrile, non-dengue disease, thereby increasing the denominator) or, more likely, 2) the active system excluded febrile cases which failed to satisfy case definitions (thereby reducing the numerator). Both scenarios emphasize the heterogeneity of dengue case definitions, surveillance systems and clinical practices, which challenge the generation of consistent burden estimates. Additional complexity has been observed during outbreaks from both over- and under-reporting due to differing levels of disease awareness and/or reporting practices. [44]

A similar analysis was conducted in slightly older Latin American children, focusing on VCD cases and comparing with dengue reported at different levels of the surveillance system (country; state; local). [45] It found lower rates of VCD (from 2,500 cases/100,000 p/y in Mexico to 3,500 in Brazil), and corresponding EFs which varied widely, from 3.5–45.5 (depending on country and comparator), emphasizing that EFs are a complex outcome of local epidemiology, disease awareness, health system characteristics and other factors. [19] Additional analyses of under-reporting according to indicators of socio-demography or dengue awareness, for example, may be illuminating.

Hospitalization was based on local routine practice and rates were shown to be substantial: over 4% of fevers, and over 50% of dengue diagnoses were hospitalized. A clinical diagnosis–rather than virological confirmation–seemed to determine the decision to hospitalize, demonstrated by the successively decreasing incidence and increasing hospitalization rate of episodes of fever; VCD; CDD; and DHF. Interestingly, four cases of clinically diagnosed DHF (13.8% of the total) could not be virologically confirmed, highlighting a possible over-attribution in endemic areas, of consequence for prospective epidemiological or vaccine effectiveness studies using clinical endpoints. Interpretations of the interplay between severity, case definitions and hospitalization are particularly important from a health economics perspective when we consider that a single hospitalization has been reported to cost between USD 289 (Philippines) and USD 863 (Malaysia). [21,46]

The case definition applied in CYD14 (fever $\geq$ 38°C on $\geq$ 2 consecutive days) intentionally captured a broad spectrum of disease, enabling calculation of vaccine efficacy against dengue

of any severity. However, a considerable proportion of VCD episodes (7.8%) were assessed as DHF by the investigators, and while empirically-derived global burden estimates of severe dengue/DHF are not available, [47] our data suggest that in Southeast Asia, the burden is substantial. Extrapolations using appropriate baselines and harmonized case definitions associated with clinical severity could theoretically be used to generate estimates of severe disease, and may be a topic of further research.

Most clinical diagnoses of dengue were virologically confirmed, resulting in a PPV of 85.2%. However the sensitivity was 28.8%, reflecting the significant proportion of VCD which was not clinically diagnosed as dengue. This is likely because local DF or DHF reporting case definitions had not been satisfied, even when investigators suspected dengue as the underlying aetiology, and is a finding which should contribute to the understanding of the clinical and economic burden of mild dengue disease. Using VCD as a denominator, the complement to clinical diagnoses were termed undifferentiated fever VCD in our study, and represent symptomatic, febrile, virologically confirmed cases which were not diagnosed. Policymakers sometimes consider milder manifestations of disease unimportant, but a recent Cambodian study found mild dengue cases are significantly more infectious than those with symptoms. [48] Mild cases may thus contribute significantly to transmission and constitute an important viral reservoir. Additional studies will be required to understand the impacts on population-level immunity and transmission dynamics.

A clinical diagnostic exclusion of dengue following a febrile episode was correct in >90% of instances (NPV: 92.4%) and the specificity of clinical diagnosis was 99.4% in these epidemiological settings where >10% of acute fevers were caused by dengue virus infection. The accuracy of diagnosis was much improved when considering only hospitalized episodes, indicating that surveillance reports and burden estimates of more severe disease are likely more reliable than those of mild cases. However this leaves a considerable burden of mild disease which is unaccounted for. We are not aware of health economic or healthcare utilization studies examining the impact of these mild episodes but their frequency implies a significant source of burden. Additional analyses could consider aggregating costs (including indirect costs), disability-adjusted life years or other measures to quantify impacts.

The study has limitations. The ID of cVCD was low in some settings, with only six episodes in Malaysia and nine in Vietnam. This is an unavoidable consequence of examining infrequent disease outcomes using prospective methods. Our approach annualized incidence, which may have introduced some bias, but our comparison with local surveillance data and overlapping timeframes will limit geographical/temporal distortions. As this was a vaccine trial, sites were chosen for their historically high reported dengue burdens, so results from lower-endemic areas may differ. [38] For this reason, incidence rates and other findings could not be combined between countries. However, the socio-environmental determinants of dengue incidence are poorly understood and in many Asian countries burdens are unpredictable throughout urban endemic areas. Where age-stratified incidence data were unavailable, adjustments were made which introduced slight inaccuracies to the data. More substantial variability was caused by the differences in national surveillance systems, with more sensitive surveillance giving rise to lower EFs. This is an inherent study bias but also an interesting result; the use of a stable denominator in expansion factor calculations provides an insight into surveillance system specificities.

WHO 1997 classifications were applied, as assessed by investigators, because at study initiation 2009 guidelines were not in routine use at all sites. Cases were also classified according to a more inclusive definition of severe dengue, integrating criteria from the WHO 1997 and 2009 and South East Asia Regional Office 2011 guidelines, and applied by the study Independent Data Monitoring Committee (IDMC). [49] Of the 25 VCD cases clinically diagnosed as DHF,

20 met these IDMC criteria indicating, in this clinical trial environment at least, a level of concordance between the two.

This analysis was performed to inform policy making and strengthen evidence for public health decisions, including financing for dengue control efforts such as vaccination. It adds to available evidence indicating that passive surveillance systems greatly underestimate dengue burden and emphasizes that burden estimates are highly sensitive to case definitions. The control arms of vaccine clinical trials can provide valuable data to estimate disease burdens.

Supporting Information

S1 Checklist. STROBE checklist.
(DOC)

S1 File. National dengue surveillance system summaries.
(DOCX)

S1 Table. Characteristics of the control group of the CYD14 study, described by age group, site, and the numbers of dengue and associated clinical episodes occurring during the timeframe of the study.
(DOCX)

S2 Table. Dengue surveillance data from the areas in which the CYD14 clinical trial was conducted, over the period of study and population data.
(DOCX)

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SHORT REPORT
Indonesian dengue burden estimates: review of evidence by an expert panel

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SUMMARY

Routine, passive surveillance systems tend to underestimate the burden of communicable diseases such as dengue. When empirical methods are unavailable, complimentary opinion-based or extrapolative methods have been employed. Here, an expert Delphi panel estimated the proportion of dengue captured by the Indonesian surveillance system, and associated health system parameters. Following presentation of medical and epidemiological data and subsequent discussions, the panel made iterative estimates from which expansion factors (EF), the ratio of total:reported cases, were calculated. Panelists estimated that of all symptomatic Indonesian dengue episodes, 57·8% (95% confidence interval (CI) 46·6–59·8) enter healthcare facilities to seek treatment; 39·3% (95% CI 32·8–42·0) are diagnosed as dengue; and 20·3% (95% CI 16·1–24·3) are subsequently reported in the surveillance system. They estimated most hospitalizations occur in the public sector, while ~55% of ambulatory episodes are seen privately. These estimates gave an overall EF of 5·00; hospitalized EF of 1·66; and ambulatory EF of 34·01 which, when combined with passive surveillance data, equates to an annual average (2006–2015) of 612 005 dengue cases, and 183 297 hospitalizations. These estimates are lower than those published elsewhere, perhaps due to case definitions, local clinical perceptions and treatment-seeking behavior. These findings complement global burden estimates, support health economic analyses, and can be used to inform decision-making.

Key words: Epidemiology, Delphi, dengue, Indonesia, under-reporting.

Dengue is a systemic viral disease, transmitted to humans by the bite of infected *Aedes* spp. mosquitoes throughout the tropical and subtropical world. It results in substantial disease burden, health service disruption and costs [1]. Historically, the World Health Organization (WHO) estimated 50–100 million global infections per year including 500 000 dengue hemorrhagic fever (DHF) cases and 20 000 deaths but more recent modeling studies have found approximately four billion people in over 120 countries at risk, with 50–100 million annual symptomatic cases, mostly occurring in the Asia–Pacific region [2,3].

Indonesia has over 900 permanently inhabited islands extending over 5000 km from east to west. Since the first dengue reports in Jakarta and Surabaya

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in 1968, the disease has been expanding in incidence and geography, and is likely hyperendemic (i.e. multiple co-circulating serotypes) nationwide [4,5]. Notification of DHF is mandatory and Indonesia typically reports the highest number of cases in the WHO Southeast Asia Region [1]. Between 2001 and 2011, there was a reported average of 94 564 cases and between 472 and 1446 deaths per year [6]. The surveillance system uses WHO 1997 case definitions, whether clinically or laboratory diagnosed, but likely captures only a proportion of symptomatic disease due to inconsistent health-seeking behavior, non-specific symptoms, limited use of imperfect diagnostics and health systems issues. Between provinces, significant variation exists in reported incidence rates which may be a function of disease dynamics, surveillance and reporting practices, or both. Under-reporting is thus a complex product of geographical, clinical, epidemiological, laboratory, and health system factors. It may be that the introduction of point-of-care dengue rapid diagnostic tests has increased the reporting rate but data documenting this effect are currently lacking, and the full disease burden is unknown.

Estimating the public health and economic burdens of dengue are elements of the WHO *Global Strategy for Dengue Prevention and Control, 2012–2020*, and are priorities of many ministries of health to support disease control planning, allocation of resources and assessment of the value of novel prevention measures, including vaccination. Accordingly, a range of empirical or extrapolative methods have been employed to make more complete disease burden estimates in Indonesia and other countries [7]. However reliable data to make empirical assessments, particularly robust epidemiological data from active surveillance projects, are often lacking. A Delphi panel is a structured communication process which aims to achieve a convergence of opinion on a specific real-world issue. Experts make iterative estimates to answer a predefined research question, under the assumption that the range of answers will narrow as the process progresses. A group discussion makes each participant aware of the range of opinions and their rationale, information which is used to refine subsequent estimates, typically leading to confluence of opinion based on the expertise of the panel. The process is stopped upon reaching predefined criteria [8]. In combination with statistical methods, this approach has been used to calculate an overall adjustment factor for dengue under-reporting in the Philippines, Malaysia, and India, and it can be used to derive health system parameters which are otherwise unavailable [9,10].

A Delphi panel meeting was convened in Jakarta on 8 December 2015 comprising 14 experts, including infectious disease physicians and pediatricians, national specialists in dengue treatment guidelines and epidemiology, healthcare system managers, surveillance officers, academics, and laboratory workers, from different geographical areas across Indonesia, invited based on the advice of national-level dengue experts (full list of panelists provided in acknowledgments). The panel was expected to estimate the proportion of symptomatic dengue cases captured in the surveillance system and thus enable calculation of national-level dengue burden estimates. The panel also estimated the percentage of hospitalized and ambulatory dengue cases treated in private and public institutions.

A range of epidemiological and clinical data documenting current knowledge and gaps related to dengue in Indonesia was first presented to the panel, to align on recent study results and their methods. With the explanations that: (a) 'dengue case' refers to any patient whose symptoms are the result of infection with a dengue virus, including mild cases (e.g. fever >38°C for $\geqslant$1 day) and those which present atypically; (b) 'dengue diagnosis' refers to a dengue case with a dengue diagnosis from a physician according to local practices (clinical and/or laboratory confirmation); (c) 'healthcare facility' refers to a licensed clinic, hospital, or other health provider (e.g. subdistrict-level primary healthcare center); and (d) 'hospitalized dengue case' is any dengue case spending at least one night in a healthcare facility; panelists were asked five questions, each of which was to be considered from the national perspective:

Q1: What percentage of dengue cases enters a healthcare facility to seek treatment?
Q2: Of all dengue cases entering a healthcare facility, what proportion is diagnosed as dengue?
Q3: Of dengue cases diagnosed in a healthcare facility what proportion is then reported in the routine Indonesian dengue surveillance system statistics?
Q4: Of dengue cases entering an Indonesian healthcare facility, what proportion is hospitalized for any duration?
Q5: Among all dengue cases entering healthcare facilities, what proportion is seen in the public sector if: (a) hospitalized; (b) outpatient (i.e. ambulatory).

Anonymous responses were collected by a moderator who aggregated the data, presented them to the

group and facilitated a discussion. Participants were then invited to re-cast their votes in light of the previous results and discussions. The process was terminated after three rounds of voting (two rounds of discussion). Medians of final round votes were used for analysis, and a bootstrapping resampling method (200 samples; SAS software) employed to provide variability based on the theoretical non-parametric distribution of observed values, enabling estimation of medians and their 95% confidence intervals (CIs) [11]. These medians were used to calculate the total number of symptomatic dengue cases occurring in Indonesia via generation of an overall expansion factor (EF_O):

$$EF_O = \frac{Total\ cases}{Reported\ cases}.$$

By proportionally adjusting a theoretical 100% of symptomatic cases according to the responses to the questions above, this can be logically calculated according to the formula:

$$EF_O = \frac{1}{Q1^*Q2^*Q3}.$$

The total number of cases can be estimated as:

$$Total\ dengue = EF_O{}^*Reported\ dengue\ \ cases.$$

Cases could be further stratified into hospitalized and ambulatory dengue, which are likely under-reported in different magnitudes and incur different public health consequences and costs:

$$Hospitalized\ \ dengue = EF_O{}^*Q1^*Q4$$

and

$$Ambulatory\ \ dengue = EF_O{}^*1 - (Q1^*Q4).$$

Specific EFs for hospitalized/ambulatory dengue EF_H and EF_A are often reported [7,9]. While they do not affect final burden estimates here, they may have value for policy-makers and can be calculated assuming the proportion of hospitalized and ambulatory cases within dengue-reporting systems is known by:

$$EF_H = \frac{Q1^*Q4^*EF_O}{P_h}$$

and

$$EF_A = \frac{1 - (Q1^*Q4)^*EF_O}{P_a}$$

where P_h and P_a are the proportion of reported cases which is hospitalized/ambulatory ($P_h = 100 - P_a$). Based on local experience that most reported cases are hospitalized, we made a base-case assumption of $P_h = 90\%$, with uncertainty assessed by applying rates from 80% to 99% in sensitivity analysis.

Proportions of cases seen in public/private facilities were similarly adjusted using the responses to question 5. National-level estimates were calculated by multiplying these EFs by the number of reported dengue cases in Indonesia, from 2006 to 2015 [12].

One participant departed after the first voting round leaving 13 voting participants at the meeting. At the third vote, four questions were unanswered leaving a total of 74 responses in the analysis. There was significant confluence of opinion by the third round with more than half (45/74) of votes agreeing on the response to each question. Voting summaries from the final round, and median estimates from bootstrapping resampling and their 95% CIs are provided in Table 1. Panelists estimated that, of all symptomatic dengue episodes, 57·8% (95% CI 46·6–59·8) enter healthcare facilities to seek treatment; 39·3% (95% CI 32·8–42·0) is diagnosed as dengue; and 20·3% (95% CI 16·1–24·3) is subsequently reported. In all, 31·5% (95% CI 24·4–35·5) of cases are hospitalized. Of all cases entering the healthcare system, 20·0% (95% CI 14·5–24·2) are hospitalized in the public sector (with a public/private split in hospitalized cases of 64%/36%) and 12·0% (95% CI 9·8–14·1) are outpatients in the public sector (public/private split in ambulatory cases: 45%/55%).

These estimates gave rise to an EF_O of 5·00 (95% CI 4·11–6·21); EF_H of 1·66 (95% CI 1·51–1·86), and EF_A of 34·01 (95% CI 27·85–44·72) and, when combined with passive surveillance data, a 2006–2015 annual average of 612 005 symptomatic cases (Fig. 1). This varied from a low of 328 704 in 2011, to a high of 790 770 in 2007. This equates to a total from 2006 to 2015 of 3 537 238 (95% CI 2 854 797–3 657 332) cases entering health facilities; 2 476 067 (95% CI 1 986 082–2 577 322) dengue diagnoses and 1 832 969 (95% CI 1 665 785–2 052 687) hospitalizations, 1 164 543 of which are seen in the public sector. Varying the hospitalization rate from 80% to 99% led to EF_H ranges of 1·51–1·87 and EF_A from 17·01 to 340·13. As these rates are components of burden calculations they make no difference to final estimates here, but this variability emphasizes the importance of consistent assumptions and accurate methodological reporting.

These findings support previous reports that dengue is significantly under-reported in Indonesia, and provide granularity which was previously lacking, for example the finding that approximately 1/3 of all symptomatic cases is hospitalized for some duration.

Table 1. *Summary final results of the Delphi panel and derived medians and their 95% confidence intervals following boot-strapping resampling*

Question	Number of votes	Mean (%)	Mode (%)	Median response (%)	Bootstrap-derived median (95% CI)
1. What percentage of dengue cases enter a healthcare facility to seek treatment?	13	57	60	60	57·8 (46·6–59·8)
2. Of all dengue cases who enter a healthcare facility, what proportion is diagnosed as dengue?	13	70	70	70	70·0 (69·6–70·5)
3. Of dengue cases diagnosed in a healthcare facility what proportion is then reported in the routine Indonesian dengue surveillance system statistics?	12	54	60	60	56·7 (50·7–59·9)
4. Of dengue cases who enter an Indonesian healthcare facility, what proportion is hospitalized for any duration?	12	56	60	60	59·0 (55·0–60·1)
5 (a). Among all dengue cases who enter healthcare facilities, what proportion is seen in the public sector if hospitalized?	12	68	70	70	68·6 (65·6–70·0)
5 (b). Among all dengue cases who enter healthcare facilities, what proportion is seen in the public sector if outpatient?	12	53	60	52·5	50·9 (50·8–51·0)

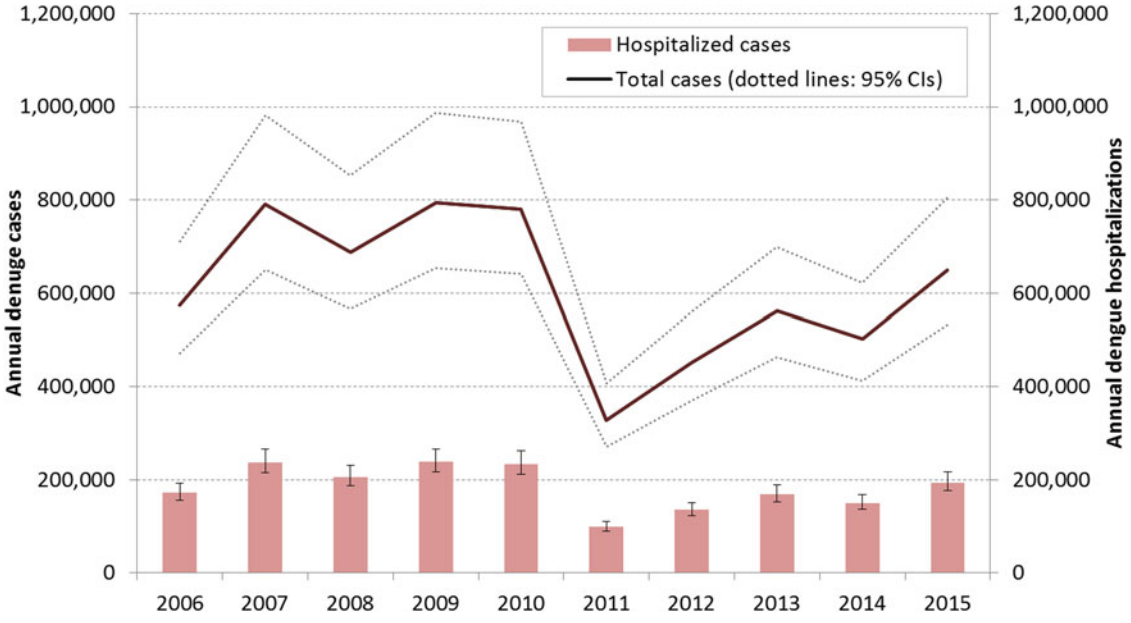

Fig. 1. Estimated annual number of dengue cases and hospitalizations in Indonesia following adjustment of surveillance reports with EFs, and their 95% confidence intervals (CIs), 2006–2015.

However, the magnitude of under-reporting is relatively modest in comparison with other studies: a regional analysis extrapolating from neighboring countries found an overall EF of 7·6 [7] and an analysis of published data found national under-reporting in Indonesia from 36- to 126-fold [13]. More recently, two prospective comparisons between active and passive surveillance systems have been published: a factory-based dengue cohort in West Java identified a dengue incidence rate of 17·3 cases/1000 person-years, 43-fold higher than rates recorded in the passive surveillance system; [14] and a comparative reanalysis of placebo arm data from a dengue vaccine clinical trial in Jakarta, Bandung, and Bali identified an overall EF of 11·5 [15]. Finally, two influential global dengue burden studies using complementary approaches based on dengue occurrence data, incidence rates from published cohorts, or vital registration and verbal autopsy estimated national burdens from which Indonesian EFs of 57 and 106, respectively, can be

derived [2,3]. These recent studies are consistent in finding that dengue is significantly under-reported in Indonesia at magnitudes in significant excess of these Delphi panel estimates.

Dengue causes a spectrum of clinical disease and incidence rates are determined by the surveillance system and case definitions applied to describe symptomatic episodes. The experts participating in this Delphi panel, who are mostly familiar with dengue episodes requiring medical intervention, may be familiar with more severe and less frequent presentations of dengue than considered in other analyses, a possible explanation for these conservative projections [2]. Supporting this hypothesis, our estimates are similar to those from a 2013 paper (which found 792 829 annual cases), conducted before contemporary estimates were available [7]. Additionally, only dengue cases meeting a DHF case definition are notifiable in Indonesia, a probable reason why the nationally reported incidence rates are lower than those from neighboring countries [data not shown]. A recent analysis clearly described a relationship between clinical severity and under-reporting, it therefore remains important for policy-makers to understand methodological study aspects, case definitions, and their implications [15]. Simple comparisons between countries are rarely justified. Some of these observations are limitations of an expert-based approach and are reflective of local expert opinion. However, such a method enables exploration of experimentally challenging research topics in complex countries, understanding of expert views and their rationale, and projection of local experience and data to inform decision-making at the national level.

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DECLARATION OF INTEREST

J.N., S.B., S.N. and A.M. are/were employees of Sanofi Pasteur, a company which produces a vaccine against dengue. A.P. has acted as an investigator for research studies funded by Sanofi Pasteur. T.Y.M. W., H.T. and M.N. declared no conflicts.

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Dengue serotype-specific seroprevalence among 5- to 10-year-old children in India: a community-based cross-sectional study

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SUMMARY

Background: Dengue surveillance data in India are limited and probably substantially underestimate the burden of disease. A community-based study was undertaken to assess the prevalence of dengue-specific immunoglobulin G (IgG) antibodies in children across India and to examine historical dengue exposure rates. Potential associations between socio-economic factors and dengue seroprevalence were also assessed (registered at ctri.nic.in: CTRI/2011/12/002243).

Methods: A convenience sample of 2609 healthy children aged 5–10 years was enrolled; these children were registered at or were living in the vicinity of eight centres located at six geographically distinct sites across India. Blood samples were drawn to test for the presence of dengue IgG antibodies using ELISA. Serotype-specific neutralizing antibody titres were measured in dengue IgG-positive children using dengue plaque reduction neutralization tests. Socio-demographic and household information was collected using a questionnaire.

Results: Overall, 2558/2609 children had viable samples with laboratory results for dengue IgG. Dengue IgG seroprevalence across all sites was 59.6% (95% confidence interval 57.7–61.5%): the lowest (23.2%) was in Kalyani, West Bengal, and the highest (80.1%) was in Mumbai. Seroprevalence increased with age. Multivariate analysis suggested associations with household water storage/supply and type of housing. Half of the subjects with positive IgG results presented a multitypic profile, indicating previous exposure to more than one serotype.

Conclusions: The overall dengue seroprevalence suggests that dengue endemicity in India is comparable to that in highly endemic countries of Southeast Asia. Additional prospective studies are required to fully quantify the disease burden, in order to support evidence-based policies for dengue prevention and control in India.

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1. Introduction

Dengue is caused by a mosquito-borne flavivirus that is endemic in tropical and subtropical countries, including India.[1] Sporadic outbreaks have been reported in India for over 200 years. The scale and severity of two major epidemics in the 1990s prompted the implementation of a number of strategies to aid the control and surveillance of dengue. In particular, a passive surveillance programme and publication of guidelines for dengue prevention and control was launched as an initiative of the National Vector Borne Disease Control Programme,[2] in collaboration with the existing Integrated Disease Surveillance Programme. Due to non-specific and often mild symptoms, dengue is significantly under-reported in nearly all countries. This is exacerbated in India, where dengue surveillance data are collected from only approximately 500 sentinel hospitals.[3] Studies using global or extrapolated data have quantified this under-reporting, and suggest that the dengue disease burden in India is likely to be the highest in the world.[3,4]

Dengue has spread from urban to rural areas of India in recent decades.[2,5] All four virus serotypes – DENV-1 to DENV-4 – have been documented in India, without a clear geographical distribution. Areas where serotypes co-circulate are increasing in number and scale.[2] Specifically, DENV-1, DENV-2, and DENV-4 were isolated during an outbreak of dengue fever in Vellore in 1963 and all four serotypes were isolated during another outbreak in the same city in 1968.[2] DENV-2 was the predominant serotype from the early 1970s to 2000, responsible for large epidemics in 1993 and 1996. DENV-3 was the predominant serotype in a 2003 outbreak and co-circulated with DENV-1 in 2006 in Dehli.[2] Delhi became hyperendemic for dengue, with all four serotypes isolated in 2003 and 2006.[2] No study to date has taken a nationally representative view of serotype distribution.

Cross-sectional, population-based, age-stratified seroprevalence data describe historical disease transmission intensity.[6,7] A seroprevalence study was undertaken to describe the prevalence of dengue-specific immunoglobulin G (IgG) antibodies and the infecting serotype profiles of positive samples, in children from eight sites across India.

2. Methods

2.1. Study design and centres

This was a community-based, descriptive, cross-sectional seroprevalence study and was conducted between January 2011 and October 2012 in healthy children (registered at ctri.nic.in: CTRI/2011/12/002243). A convenience sample of eight private or government medical colleges at six geographically distinct locations was selected (1) to provide a wide geographical distribution across India, (2) to represent rural and peri-urban areas, and (3) based on the recognized ability of the site to conduct epidemiological research. Overall, two sites were selected in New Delhi and Hyderabad, and one site each in Kalyani, Wardha, Mumbai, and Bangalore.

This study was conducted in accordance with the latest revision of the Declaration of Helsinki (Seoul, Korea, October 2008), guidelines for Good Epidemiological Practice,[8] and local regulatory requirements. The study protocol was approved by ethics committees at the study centres and by the Health Ministry Steering Committee (HMSC) of the Government of India.

2.2. Participants

Children, 5–10 years old, who were resident at the study sites, were eligible. This is an age at which blood sample collection is relatively straightforward. Furthermore, seroconversion, and thus the demonstration of age-specific variation in seroprevalence, was considered likely in this age group. Parents or legal guardians were invited to enrol children during routine household visits by community health workers. Written informed consent was obtained from the parents or legal guardians, and children aged 8–10 years also signed an assent form. Enrolment at the two sites in Hyderabad was school-based; parent–teacher meetings were held at randomly selected schools to explain the purpose of the study, and all eligible children at those schools were invited to participate. Permission was obtained from the District Education Officer to perform study visits, complete questionnaires, and collect blood samples from study participants on the premises of each school.

Assuming a dengue seroprevalence of 30%, a sample size of 323 participants at each site was calculated to ensure a precision of 5% for the two-sided 95% confidence interval (CI) around the seroprevalence point estimate.

2.3. Data and sample collection

Socio-demographic data (participant's demographic characteristics, household occupancy, water supply/storage, self-reported history of dengue or Japanese encephalitis (JE) virus infection, and education levels attained by the parents/guardians) were collected using a questionnaire. The questionnaire was administered by the health worker through interviews with the participant's parents or legal guardians during the first visit. Participants were asked to report to the affiliated centre for blood sample collection (5 ml) by a trained laboratory technician. The participant's height and weight were recorded using standard methods. Significant medical history, current or previous medical conditions, concomitant medication, recent vaccinations, and reasons for refusal of blood sampling, where relevant, were recorded.

Blood samples were left at room temperature for 1–2 h before centrifugation. Each serum sample was divided into aliquots and stored in 3-ml cryotubes: 0.5 ml for dengue IgG antibody assessment, 1 ml for dengue plaque reduction neutralization tests (PRNT), and 0.5 ml for JE IgG antibody detection. Serum samples were kept frozen at −20 °C or below until analysis.

2.4. Assays

Samples were sent to the Microbiology Department of the Maulana Azad Medical College (New Delhi) for analysis. Dengue IgG antibody levels were assessed using commercially available ELISA kits. The EL1500G kit (Focus Diagnostics, California, USA) was used for samples from the first two sites (New Delhi); however, due to supply issues, the E-DEN 10G kit (Panbio Diagnostics, Brisbane, Australia) was used for the other sites. A sensitivity analysis of the two dengue IgG-specific ELISA kits performed on 30 samples confirmed 100% concordance; data from all centres were thus pooled. JE IgG antibody testing by indirect ELISA was also performed using commercially available kits (InBios, Washington, USA). Dengue IgG-positive samples were sent to the Centre for Vaccine Development (Mahidol University, Thailand) for measurement of dengue serotype-specific neutralizing antibody titres using PRNT based on 50% or greater reduction in plaque counts ($PRNT_{50}$).[9]

Seropositivity for dengue and JE were defined according to the manufacturer's instructions. Seroprevalence was the percentage of seropositive participants.

For the interpretation of $PRNT_{50}$ titres, participants were classified as follows: 'naïve', if antibody titres were <10 (1/dil) for the four serotypes; 'monotypic', if antibody titres were ≥ 10 (1/dil) for only one serotype or if titres were ≥ 10 (1/dil) for

different serotypes, with a single serotype having a high titre (>80 (1/dil) titre, and ≥5 times higher than other titres); and 'multitypic', if antibody titres were ≥10 (1/dil) for different serotypes without a single predominant titre.

At each site, a designated clinical research associate performed periodic visits to monitor implementation. All serum samples were checked for quantity and storage temperature by a lot quality assurance method.

2.5. Statistical analysis

Descriptive statistics reported baseline characteristics and immunogenicity results. Associations between all demographic–socio-economic factors and dengue serostatus were assessed by univariate analysis using the Chi-square test or *t*-test (for age) and multiple logistic regression with backward selection (significant if the *p*-value is ≤0.05). JE serostatus was not included as a covariate due to possible cross-reaction between flavivirus antibodies. Statistical significance was considered at and below a *p*-value of 0.05. Statistical analyses were performed using SAS software version 9.1 (SAS Institute, Cary, NC, USA) and Stata v. 12.1 (StataCorp LP, College Station, TX, USA).

2.6. Role of the funding source

The sponsor participated in all operational aspects of the study, including data collection, statistical analyses, and the writing of the study report. The sponsor funded medical writing support for the development of this publication. The corresponding author had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

3. Results

3.1. Demographic characteristics of the study population

Overall, 2609 participants from eight health centres were enrolled in the study. A total of 18 participants were excluded, due to age <5 years or >10 years (*n* = 8) and/or assent form not signed (*n* = 13). Thus, 2591 participants were included, all of whom had a blood sample drawn; 1364 (52.6%) were female, and the mean age of all participants was 7.8 (standard deviation (SD) 1.6) years.

3.2. Socio-economic characteristics

The mean number of people living in the participants' households was 5.4 (SD 2.3), including 2.6 (SD 1.2) children under 15 years of age. Most (2170/2591; 83.8%) participants lived in a house, with 381 (14.7%) living in precarious lodgings; 1121 (43.3%) had an indoor piped public water supply, 2343 (90.4%) had water storage in the house, 1675 (64.6%) were connected to the public sewer, and 1339 (51.7%) had regular organized waste collection.

3.3. Medical history

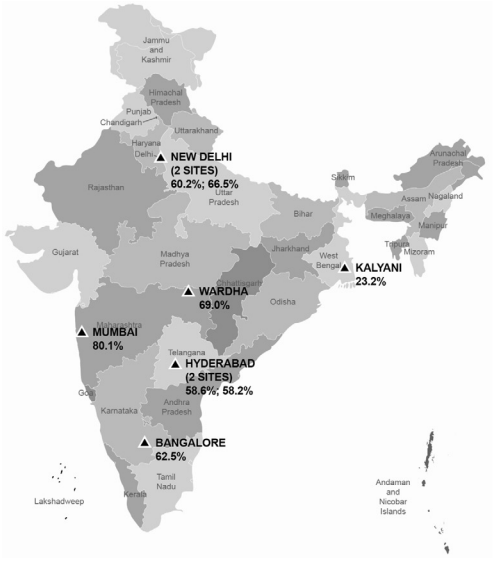
A notable medical history was recorded for 94 (3.6%) children; 61 (2.4%) children were undergoing at least one current treatment at the time of enrolment. A history of dengue or a family history of dengue was reported by 15 (0.6%) and 48 (1.9%) participants, respectively. No participants reported a history or family history of JE infection. Only one participant reported receiving JE vaccination.

3.4. Dengue IgG seroprevalence

Anti-dengue IgG results were available for 2558/2591 (98.7%) participants. Serology data were missing for 33 participants; 22 samples from a single site (Mumbai) could not be analysed due to haemolysed red blood cells. Overall, 1525/2591 (59.6%) participants were dengue seropositive, with similar prevalence in males and females. Six of the eight sites had dengue seropositivity ranging from 58.2% to 69.0%. The sites in Kalyani and Mumbai had the lowest (23.2%) and highest (80.1%) seroprevalence, respectively (Figure 1). Overall, dengue IgG seroprevalence increased with age, from 40.7% (95% CI 36.0–45.5%) in children aged 5 years to 73.4% (95% CI 67.9–78.5%) in 10-year-olds (Figure 2). At the Bangalore site, seroprevalence remained relatively stable across the age strata (varying from 58.8% in 7-year-olds to 70.9% in 8-year-old children).

3.5. Socio-economic characteristics associated with dengue seroprevalence status

In univariate analyses, children seropositive for dengue were found more likely to be from homes with more than two children ($p < 0.0001$), more likely to have water storage ($p < 0.0001$) and



Site	n/N	% IgG positive	95% CI
New Delhi 1	195/324	60·2	54·6–65·6
New Delhi 2	216/325	66·5	61·0–71·6
Kalyani	75/323	23·2	18·7–28·2
Wardha	223/323	69·0	63·7–74·0
Mumbai	241/301	80·1	75·1–84·4
Hyderabad 1	188/321	58·6	53·0–64·0
Hyderabad 2	185/318	58·2	52·5–63·7
Bangalore	202/323	62·5	57·0–67·8
Overall	1525/2558	59·6	57·7–61·5

CI=confidence interval; IgG=Immunoglobulin G; n=number of participants seropositive for dengue; N=total number of participants with blood samples per site

Figure 1. Dengue IgG seroprevalence by site.

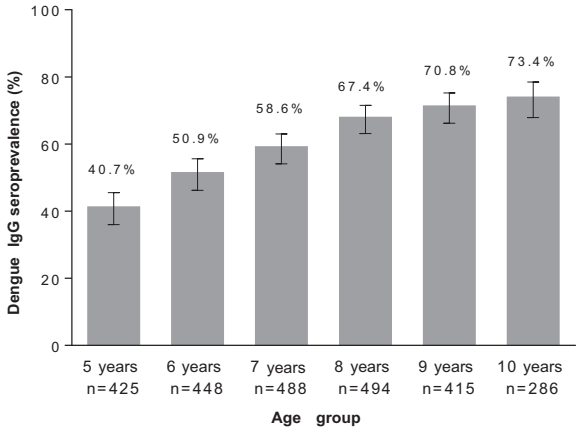


Figure 2. Percentage of participants with antibody titres ≥10 (1/dil) against dengue according to age group (all study sites). Error bars show 95% confidence intervals. n, number of participants with available results per age group.

indoor piped water from the public water supply ($p < 0.0001$), and less likely to be living in precarious housing ($p = 0.0048$) compared with dengue seronegative children (Table 1). Multiple logistic regression confirmed possible positive associations with household water storage (odds ratio (OR) 5.00, 95% CI 3.54–7.06) and indoor piped water from the public water supply (OR 1.49, 95% CI 1.19–1.85). Increasing participant age ($p < 0.0001$) and living in precarious lodgings compared to a house (OR 1.54, 95% CI 1.17–2.03) were also associated with dengue status. In terms of geography, Kalyani was associated with decreased exposure (OR 0.18, 95% CI 0.10–0.31), while Wardha (OR 1.49, 95% CI 1.07–2.08) and Mumbai (OR 2.49, 95% CI 1.70–3.65) had an elevated risk, in comparison with Delhi. The pseudo R^2 of the final model was 0.085.

3.6. Dengue serotype analysis

Of 1525 IgG seropositive participants tested, 1511 had $PRNT_{50}$ data available for all four serotypes. Of these, 1468 (97.2%) had antibody titres ≥10 for at least one serotype and 1205 (79.7%) had antibody titres ≥10 against all four serotypes. Nearly half (736/1511; 48.7%) had a multitypic antibody profile and 732/1511 (48.4%) had a monotypic profile. DENV-1, DENV-2, and DENV-3 were nearly equally represented among dominant serotypes in participants with monotypic profiles overall (Figure 3).

3.7. Japanese encephalitis IgG seroprevalence

Anti-JE IgG results were available for 2544 (98.2%) participants. Of these, 345 (13.6%; 95% CI 12.3–15.0%) participants were seropositive against JE, 327 (94.8%) of whom were also dengue seropositive (Table 2). JE seroprevalence ranged from 4.3% (95% CI 2.4–7.1%) in Kalyani to 20.5% (95% CI 16.2–25.3%) in Wardha.

4. Discussion

These findings demonstrate a high intensity of dengue transmission among children in India; more than 50% of the children had been infected at least once by the age of 6 years, results which are broadly consistent with existing, limited dengue seroprevalence data for adults and children in Chennai and Hyderabad.[10,11] All four serotypes were found to circulate, varying by geographic location. Nearly half of all participants had a multitypic dengue antibody profile. Dengue IgG seroprevalence increased with age at all but one study site, consistent with age-related cumulative exposure to dengue.[10] The exception in Bangalore could be related to epidemiological, behavioural, or environmental factors moderating exposure risk, such as the occurrence of large, infrequent outbreaks.

The observed level of dengue exposure was comparable to that reported in other highly endemic countries of Southeast Asia and Latin America: 56.2% for 4–9-year-olds in Yogyakarta, Indonesia (1995–1996),[12] 65% for 11-year-olds in Rayong, Thailand (2010),[13] 34.4% for under 7-year-olds to 70.5% in 14–16-year-olds from a primary health care facility in Sri Lanka (2013–2014),[14] 53% for under 7-year-olds and 88% by the age of 13 years among primary school children in southern Vietnam,[15] and 35.7% and 52.2% for 5–9 and 10–14 years age groups, respectively, in two localities in Mexico in 2011.[16] A higher seroprevalence was observed in a study in Managua, Nicaragua (2001–2003), where 80% of enrolled children were seropositive by 5 years of age.[17]

Considering these similarities in exposure history, it might be expected that rates of symptomatic, reported dengue are similar in India and other countries. In fact, there are huge disparities: from 2007 to 2011, India reported an approximate average annual incidence of 1.4 cases/100 000 population,[5] whereas case notifications in Cambodia, Malaysia, the Philippines, and Sri Lanka for 2011 were 119, 70.4, 134, and 135 per 100 000, respectively.[18,19] Despite their significant and often multitypic infection history, very few participants in the present study reported a history of dengue. Similarly low reporting was observed in the

Table 1
Demographic and socio-economic characteristics associated with dengue seroprevalence status

	Dengue IgG positive	Dengue IgG negative	*p*-Value (OR[a])
Demographic characteristics			
Overall, *n*	1525	1033	
Age, mean (SD) years	8.13 (1.56)	7.39 (1.56)	<0.0001[b,*]
Socio-economic characteristics			
Number of children living in the household (%)			<0.0001[c]
≤2 children	855 (56.07)	671 (64.96)	
>2 children	670 (43.93)	362 (35.04)	
Type of housing (%)			0.0048[c,*]
House	1290 (84.6)	855 (82.8)	(1.00)
Apartment	29 (1.9)	7 (0.7)	(2.16)
Precarious lodgings	206 (13.5)	171 (16.6)	(1.54)
Water storage in the house (%)	1465 (96.1)	845 (81.9)	<0.0001[c,*] (5.00)
Indoor piped public water supply (%)	721 (47.3)	380 (36.8)	<0.0001[c,*] (1.54)
Connected to public sewer (%)	959 (62.9)	688 (66.6)	0.0541[c]

IgG, immunoglobulin G; SD, standard deviation.
[a] The odds ratio (in parentheses) is provided for significant categorical multivariate results.
[*] $p < 0.05$ in multivariate analysis.
[b] *t*-test.
[c] Chi-square test.

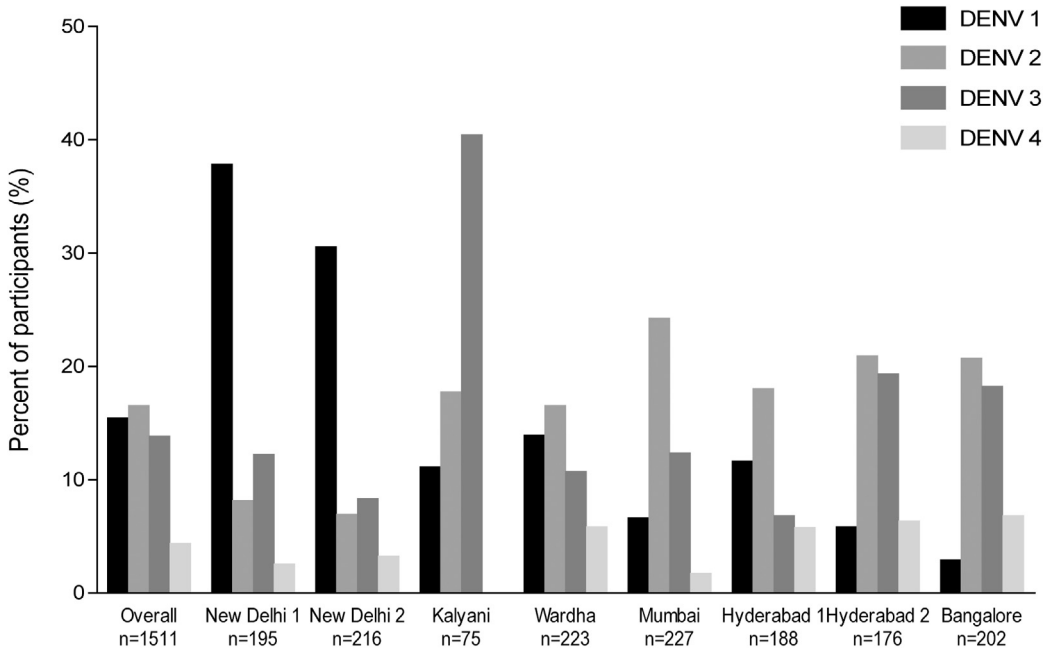


Figure 3. Proportion of participants with $PRNT_{50}$ results showing monotypic profiles, according to dominant serotype, by site and overall.

recent household-based study in Chennai,[10] in which 744/800 (93%) subjects were dengue IgG seropositive, but only 1% of participants reported a history of dengue. The present authors are unaware of virological or genetic factors that might disassociate infection history from the incidence of symptomatic disease; likely explanations include a lack of health-seeking for patients with apparent infection, lack of recognition of the disease, or misdiagnosis of dengue.[1,4] For these reasons, and because dengue surveillance reports are collected from only sentinel sites,[3] it must be assumed that dengue burdens reported in the routine surveillance system represent only a fraction of symptomatic episodes.

In the current study, serotype-specific analyses identified historical circulation of all four dengue virus serotypes at each site, with DENV-1 present in a high proportion of samples in New Delhi and DENV-3 in Kalyani. These serological findings in children are worrying: co-circulation of multiple serotypes is a population risk factor for severe dengue because it allows for sequential infection, and because secondary infection is a risk factor for severe disease.[20,21]

Multivariate risk factor analysis suggested relationships between water availability/storage practices and dengue infection risk, an association with biological plausibility due to the aquatic larval and pupal stages of the vector life-cycle. However, these results should be interpreted with caution because they were mainly driven by data from Kalyani and are thus highly susceptible to confounding with site-specific socio-demographic covariates, or other factors. After excluding Kalyani data from the multivariate analysis, only participant age remained significantly associated with dengue positivity. Furthermore, the determination coefficient (R^2) of the model was lower than 0.1, confirming the limited predictive value of these variables for dengue serostatus, in agreement with inconclusive/variable socio-economic drivers of dengue serostatus identified in other studies. In Chennai, univariate logistic regression showed a negative association with household income and no associations with other household factors.[10] Thai et al. (2005) found associations with littering in and around the home and the types of sanitary facilities in an initial univariate analysis, but these associations were not confirmed on multivariate analysis.[15] A community-based study of potential risk factors for dengue transmission in Venezuela found several household and socio-economic factors, including storing water and used tyres (univariate analysis), and crowding, household size, and living in a shack (multivariate analysis), to be associated with an increased risk of dengue infection.[22]

The seroprevalence of anti JE IgG antibodies was also measured in the participants in the present study. JE is endemic in some regions in India, particularly in the south and north-east;[23] however, during the current study period, none of the study sites were considered to be within a JE endemic area and none were in an area subject to routine JE vaccination. The observed seroprevalence of JE in the current study (13.6%; 95% CI 12.3–15.0%) confirms circulation of the virus, but is lower than that reported in a number of other studies on JE seroprevalence in endemic countries,[14,24,25] perhaps because these sites are located within less-endemic areas of India.

Cross-reaction between anti-flavivirus IgG antibodies has been documented[26] and cannot be excluded here from affecting the observed JEV or dengue IgG rates. The PRNT is a more specific assay and may be used to distinguish between cross-reactive and pathogen-specific responses. Encouragingly, 99% of dengue IgG-positive samples in the current study were also dengue PRNT-positive. However, the lack of JEV PRNT data and dengue PRNT in dengue-negative samples remains a limitation in conclusively addressing this risk.

Table 2
Prevalence of dengue and Japanese encephalitis IgG in participants with blood samples[a]

		Dengue IgG		
		n	Positive	Negative
JE IgG	*n*	2591	**1525**	1033
	Positive	**345**	**327**	18
	Negative	1794	801	990

JE, Japanese encephalitis; *n*, number of participants with results corresponding to the specified category.

[a] 33/2591 total samples had no results available for dengue IgG; 47 were inconclusive and 405 were missing for JE IgG.

Other limitations include the use of different dengue-specific IgG ELISAs at different sites, but the sensitivity test showing 100% concordance was reassuring. In this study, a convenience sample was selected, including some low-income settings, to provide geographical spread across India. However, sites were not randomly selected, subjects were consecutively recruited, and these data cannot be considered nationally or locally representative. As with other epidemiological studies, recall bias (during the questionnaire) and selection bias (e.g., self-selection of healthy subjects) cannot be excluded. Despite these sampling limitations, these data provide a first multi-centre view on dengue seroprevalence in India. The use of a single protocol and consistent methods between the sites strengthens the validity of the data.

In conclusion, high levels of dengue exposure were observed in Indian children, and age-stratified data describe transmission intensity at these locations. This information may inform dengue burden estimates and populate transmission models to assess the potential impact of prevention and control measures, including vaccination programmes.

Author contributions

SG, AC, and GF designed the study. SG, RS, NRRM, RCG, GRJ, EG, NS, MMS, and SO refined the study design, performed the study, and collected demographic and clinical data from study subjects, in the field. AC performed laboratory assays for dengue and JE virus in accordance with good laboratory practice. JN and AM analysed the data. JN provided an initial interpretation and worked to develop a manuscript outline. All authors interpreted the data with refinements and contributed to writing the manuscript. All authors critically reviewed the manuscript while in preparation and approved the final draft.

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Conflict of interest: JN, AM, and SO are employees of Sanofi Pasteur. AC, EG, MMS, RM, and RS have no conflicts of interest to declare.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children

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Abstract

Background

Indonesia reports the second highest dengue disease burden in the world; these data are from passive surveillance reports and are likely to be significant underestimates. Age-stratified seroprevalence data are relatively unbiased indicators of past exposure and allow understanding of transmission dynamics.

Methodology/Principal Findings

To better understand dengue infection history and associated risk factors in Indonesia, a representative population-based cross-sectional dengue seroprevalence study was conducted in 1–18-year-old urban children. From October to November 2014, 3,210 children were enrolled from 30 geographically dispersed clusters. Serum samples were tested for anti-dengue IgG antibodies by indirect ELISA. A questionnaire investigated associations between dengue serologic status and household socio-demographic and behavioural factors. Overall, 3,194 samples were tested, giving an adjusted national seroprevalence in this urban population of 69.4% [95% CI: 64.4–74.3] (33.8% [95% CI: 26.4–41.2] in the 1–4-year-olds, 65.4% [95% CI: 69.1–71.7] in the 5–9-year-olds, 83.1% [95% CI: 77.1–89.0] in the 10–14-year-olds, and 89.0% [95% CI: 83.9–94.1] in the 15–18-year–olds). The median age of seroconversion estimated through a linear model was 4.8 years. Using a catalytic model and considering a constant force of infection we estimated 13.1% of children experience a primary infection per year. Through a hierarchical logistic multivariate model, the subject's age group (1–4 vs 5–9 OR = 4.25; 1–4 vs. 10–14 OR = 12.60; and 1–4 vs 15–18 OR = 21.87; p<0.0001) and the number of cases diagnosed in the household since the subject was born (p = 0.0004) remained associated with dengue serological status.

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Conclusions/Significance

This is the first dengue seroprevalence study in Indonesia that is targeting a representative sample of the urban paediatric population. This study revealed that more than 80% of children aged 10 years or over have experienced dengue infection at least once. Prospective incidence studies would likely reveal dengue burdens far in excess of reported incidence rates.

Author summary

Indonesia reported to the WHO the world's second highest average number of dengue cases and the highest in Asia from 2004 to 2010. These passive surveillance reports vary widely within the country and are likely to be a severe under-estimation of the full disease burden as frequently only dengue haemorrhagic fever is captured. Understanding the intensity of dengue virus transmission and associated risk factors nationwide is necessary to guide and prioritize appropriate prevention and control measures against dengue disease, especially considering the availability of the first dengue vaccine and recent recommendations for its use in areas of high endemicity, as measured by seroprevalence and other indicators. Age-stratified seroprevalence data provide robust estimates of past exposure and can inform on transmission intensity. Therefore, we conducted a seroprevalence study of anti-dengue IgG antibodies in a representative sample of urban-dwelling Indonesian children. We found an overall dengue seroprevalence of 69.4% with half of the children having been infected at least once by the age of 5 years. Age of the subject and the number of dengue cases diagnosed in the household were associated with serostatus. These results confirm the high dengue disease burden in Indonesia and the urgency of implementation of effective prevention and control measures.

Introduction

Dengue is an arbovirus transmitted to humans via the bites of infected *Aedes* mosquitoes. It is the most rapidly spreading mosquito-borne viral disease with a global incidence that has increased 30-fold over the last 50 years [1]. While reliable burden estimates remain elusive, two studies have estimated the global symptomatic disease burden to be 96 million and 58.4 million cases/year, with 70–80% of cases occurring in the Asia-Pacific region [2, 3]. Traditionally an urban disease, dengue disease is increasingly reported in rural areas and its geographic range has expanded to more than 125 tropical countries [1]. There is no specific antiviral treatment; clinical management is focused on careful fluid management and detection of early warning signs of severe disease. Historically, prevention measures have focused on vector control, education and behavioural changes to reduce interactions between humans and vector mosquitoes [4, 5]. Improved clinical management and public awareness have contributed to declining case fatality rates to below 1% in most countries [1]. While this represents important progress, overall dengue incidence continues to rise and fatalities remain unacceptably high, suggesting that traditional control approaches are not sufficient. Vector control measures are important yet operationally challenging, of variable effectiveness and costly to sustain [6]. Routine vaccination is becoming a reality: several dengue vaccines are at different stages of clinical development [7] and a chimeric tetravalent vaccine from Sanofi Pasteur is being licensed in an

increasing number of countries in Latin America and Asia [7, 8]. In this new era of dengue as a vaccine-preventable disease, an accurate understanding of disease burden and transmission patterns will be essential to inform vaccine policy decisions.

Dengue is hyper-endemic with frequent epidemic cycles in Indonesia. The disease is most common in urban areas and in recent years has reportedly spread to smaller, more rural villages. Reported incidence remains highest in children 1–15 years of age, but since the 1980s incidence in persons over 15 years of age has gradually increased [9, 10]. Reporting of dengue haemorrhagic fever (DHF) is mandatory in Indonesia and the country typically reports the highest number of cases in the WHO Southeast Asia Region [1]. Between 2001 and 2011, there was an average of 94,564 reported cases and between 472 and 1,446 reported deaths per year [1, 11]. Dengue disease reporting is acknowledged by Indonesian experts to be incomplete and to vary widely between provinces, with reported incidence rates ranging from 2.2 to 168.5 cases per 100,000 inhabitants in 2013 [12].

An improved understanding of dengue epidemiology, burden and its dynamic characteristics are important for public health planning. Seroprevalence studies in healthy volunteers provide information on infection history in the population, from which inferences about disease burden may be drawn. Since age reflects duration of exposure, age-stratified data provide insights into transmission dynamics [13–17]. There is a lack of dengue seroepidemiological data from Indonesia and no previous study has used a population representative sample of urban Indonesian children [18–20]. This is a particularly important gap as it will provide information on whether the variations in reported incidence from different Indonesian provinces are reflective of underlying transmission dynamics or to the result of the reporting or surveillance practices employed. We conducted a seroprevalence study in urban-dwelling Indonesian children to improve understanding of dengue epidemiology and infection risk factors and inform future dengue vaccine policy decisions.

Methods

The present study is reported according to STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) recommendations (supporting information file).

Ethic statement

The protocol was reviewed and ethical approval was obtained from the Health Research Ethics Committee of Faculty of Medicine of University of Indonesia.

Study area

Indonesia is the largest country in Southeast Asia, with an area of 1.91 million km^2. The country has a population of 252.2 million living on five main islands and four archipelagos (>17,000 islands) administratively divided into 34 provinces [21]. In 2014/2015, approximately 60% of Indonesians were living on the island of Java and 53.3% lived in urban areas [21, 22]. Indonesia is divided into five administrative levels: provinces (n = 34), regencies (n = 416), cities (n = 98), subdistricts (n = 7,024), and villages (n = 81,626). Villages are considered either as rural (*desa*) or urban (*kelurahan*) based on population density, percentage of agricultural household and number of urban facilities such as schools and hospitals [21, 23].

Sampling design

A population-based cross-sectional study design was adapted from the World Health Organization (WHO) Expanded Program on Immunization (EPI) cluster survey method. This

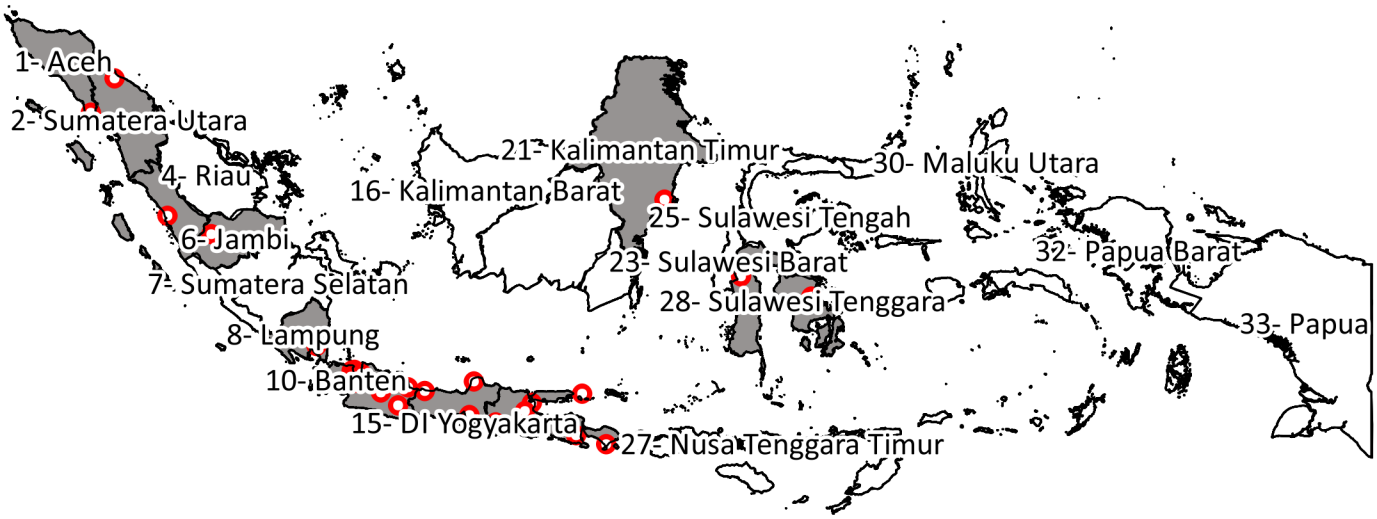

Fig 1. Map of Indonesia showing the ranking of provinces from West to East and study sites' geographic distribution. Provinces with at least one site are coloured in grey. Developed with QGIS 1.8.0 using data from Global Rural-Urban Mapping Project[18].

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approach considers 30 clusters as an adequate number for their means to be normally distributed, thus permitting statistical theory based on the normal distribution to be used to analyse the data [24, 25]. Based on the probability proportional to population size, 30 urban subdistricts were selected using demographic data from 2009 or 2010, provided by the Sub-Directorate of Statistical Services and Promotion, Statistics Indonesia.

The geographical coordinates of Indonesian administrative units were retrieved from the *Global Rural-Urban Mapping Project*, maintained by the *Socioeconomic Data and Applications Center* [26]. Provinces were listed based on their mean geographical coordinates from West to East (Fig 1) and the cumulative urban population of their subdistricts was calculated using 2010 population data. To ensure the population of clusters was sufficient to enrol the desired sample, a minimum population of 1,000 persons per subdistrict was defined and any smaller subdistricts were removed from the list. The first cluster was selected by generating a random number between 1 and $1/30^{th}$ of the total urban population, using Epi Info Version 7, and selecting the first subdistrict for which the cumulative population was superior or equal to this random number. Subsequent clusters were selected by adding $1/30^{th}$ of the urban population to the random number and selecting the first corresponding subdistrict for which cumulative population was higher or equal so that:

$$\text{Cluster}_i \text{ cumulative population} \geq \text{random number} + i * 1/30 \text{ of urban population}$$

The 30 subdistricts selected by this method are listed in Appendix 1. Each subdistrict in Indonesia contains one main health centre (*puskesmas kecamatan*) whose catchment area was the site of the study. Households in the five neighbourhood associations located closest to the health centre (each comprising 30–50 households, giving a total of 150–250 households) were eligible to participate in the study. Household visits were conducted, inviting one child from each household to participate, until the sample size was reached. A table indicating the required number of children from each of four age groups was provided to the health centre study teams. If a household had only one eligible child, the child was invited. When a household had several eligible children, a child in the age group with the fewest children already

participating was selected. Towards the end of the survey, survey teams were allocated a specific number of subjects in each age group to recruit to avoid over-sampling. If the parents refused the participation of the selected child, the household was not included. This process was continued until the desired sample size was achieved in each of the 30 clusters.

Sample size

The sample size was calculated using EpiInfo Version 7 to estimate seroprevalence in each of four age groups (1–4, 5–9, 10–14 and 15–18 years old) with 95% confidence, a margin error of 5% and accounting for clustering with a design effect of 2. The expected national seroprevalence, based on Indonesian expert opinion and published regional data [14, 19, 27, 28], was 25% in the 1–4-year-old group, 45% in the 5–9-year-old group, 55% in the 10–14-year-old group and 65% in the 15–18-year-old group. To account for incomplete data, a 10% contingency was applied. The total sample size was 3,210 children, 660 from the 1–4-year-old group (22 per cluster), 870 from the 5–9-year-old group (29 per cluster), 870 from the 10–14-year-old group (29 per cluster) and 810 from the 15–18-year-old group (27 per cluster). In total, 107 children were enrolled in each cluster.

Enrolment

The study was presented to families during monthly neighbourhood association meetings. After household visits, eligible subjects were invited to the healthcare centre for enrolment and blood sampling if they were healthy, 1–18 years of age on inclusion day, and had lived in the location for at least 1 year. An informed consent form was signed by a parent or legal guardian, and by the subject if aged 13–18 years. Subjects aged 8–12 years provided signed assent.

A questionnaire was administered to collect information on demographics, knowledge of dengue symptoms and transmission, vector control practice, and medical history in the household.

Blood sampling and laboratory analysis

For each subject, 2mL of venous blood was drawn into plain vacutainer tubes. After centrifugation, serum aliquots were frozen at -20˚C before refrigerated transport by courier to a central laboratory for analysis. Each specimen was tested for dengue IgG antibodies by ELISA using the commercial Panbio Dengue IgG Indirect ELISA kit (sensitivity = 96.3%; specificity = 91.4–100% according to manufacturer's instructions; Panbio, Alere, Australia) [29]. Samples were considered positive for previous dengue infection according to the standard protocols of the manufacturer (Panbio units <9 is negative; 9–11 is equivocal; and >11 is positive).

Data analysis and statistics

All analyses were run using SAS 9.4.

Dengue antibody seroprevalence and associations between serologic status and socio-demographic and behavioural factors. The statistical unit was the individual subject.

Seroprevalence and the 95% confidence interval (95% CI) were calculated taking account of the cluster effect. Univariate logistic regression was used to identify variables significantly associated with serologic status. As the data structure was hierarchical with subjects included in clusters, hierarchical logistic regression models were used to consider subject intra-cluster correlation. The clusters account for the random effect and the covariates were taken as fixed effects. As these analyses were considered exploratory, a level of significance (p-value) of <0.15 was applied at univariate level.

The multivariate hierarchical model was reduced by applying a backward descending selection of the non-significant variables at p-value >0.05.

The final model was:

$$P\left(Y=\frac{1}{0}\right)ij=\beta\times Parameter+\mu\times cluster+\varepsilon$$

Where $P\left(Y=\frac{1}{0}\right)ij$ was the probability for a j subject from a i cluster to be seropositive, the βs were the fixed effect describing the subject variables associated with socio-demographic and behavioural factors, μ the cluster random effect and ε the error term.

Median age of conversion. The median age of seroconversion was estimated by fitting a weighted linear regression model to age-specific seroprevalence data. Seroprevalence data were transformed into probits and age values were log transformed to fit the model [30, 31]. However, goodness of fit parameters were not respected. Therefore, a simple linear regression was used.

Force of infection. Catalytic models use seroprevalence data as cumulative markers of past infections that result in life-long immunity from which force of primary infection estimates can be derived. [32, 33], Two force of infection models were developed to describe the rate of infection over the last 18 years and to examine its variability over time. The first model assumed a constant force of infection (model 1) and the second one assumed a force of infection that varied with age (model 2) [13].

The probability of a person living in the area being infected in one year, the force of infection, is estimated by [34]:

$$-p\ =\ 1-e^{-\mu}$$

Where μ is the mean number of infections per year.

The variable force of infection model can be estimated by allowing a separate risk of infection for each age group, were pi is the mean number of infections per year for the i^{th} age group and A is the age midpoint of the i^{th} age group [34]. By fitting a binomial model with a complementary log-log link function and by using X = log(A) as an offset term, α = log(μ) can be estimated as an intercept parameter [34]. The probability of being infected for the ith group at midpoint age A is pi = 1- exp(-μi Ai), so that:

$$\mathrm{Log}(-\log(1-\mathrm{p}i))\ =\ \log(\mu i)\ +\ \log(\mathrm{A}i)$$

Results

Site selection and baseline demographics

From a total of 6,299 Indonesian subdistricts, 2,823 with urban population were identified, 2,756 of which had an urban population >1,000 and were thus used for sampling. A map of the 30 selected clusters is presented in Fig 1. From 30 October 2014 to 27 November 2014, a total of 3,210 subjects were enrolled in the study; 39 subjects (1.2%) were excluded due to at least one criteria of eligibility not being fulfilled and four subjects (0.1%) due to missing or incomplete data (demographic or serologic status result). A total of 3,194 subjects (98.7%) were included in the analyses (Fig 2); there were 107 subjects per site with the exception of four sites with 106 subjects, three sites with 105 subjects and one site with 101 subjects.

There were 672 subjects in the 1–4-year-old age group, 861 subjects in the 5–9-year-old age group, 886 in the 10–14-year-old age group and 775 in the 15–18-year-old age group. Among them, 47.8% were male and the mean age was 9.7 years.

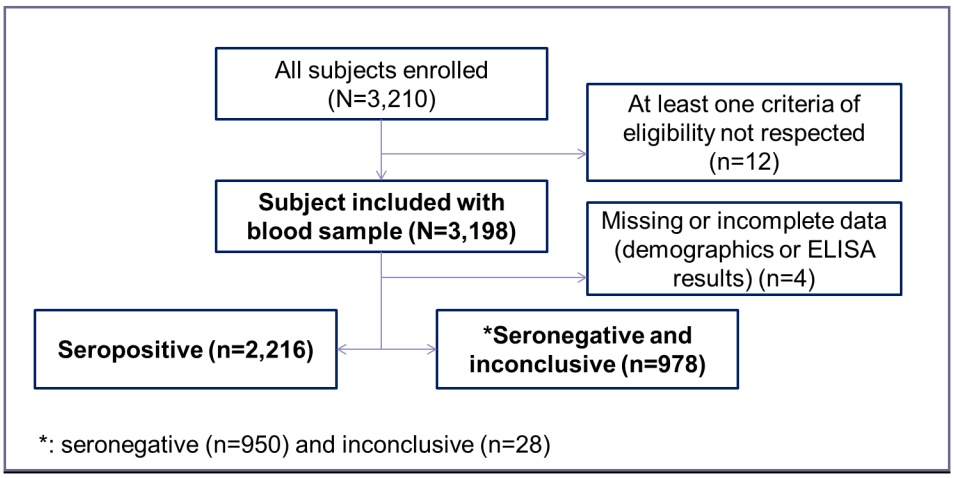

Fig 2. Study flow chart.

https://doi.org/10.1371/journal.pntd.0005621.g002

Dengue antibody seroprevalence and association between serologic status and socio-demographic and behavioural factors

The age-specific seroprevalence ranged from 26.4% (95% CI: 15.8–37.1) in those aged 1-year-old to 95.3% (95% CI: 89.8–100) in the 18-year-old subjects (Fig 3). The median age at seroconversion was 4.8 years. The overall nationwide seroprevalence was 69.4%, with a minimum of 34.6% and a maximum of 87.9% observed per site, and the seroprevalence per age group was 33.8% in the 1–4year-old group, 65.4% in the 5–9-year-old group, 83.1% in the 10–14-year-old group and 89.0% in the 15–18-year-old group (Table 1).

In the final data set, the level of non-response ("no data") varied from 0.4 to 14.0% (Table 1). Subjects were familiar with dengue disease, with 92% having heard about dengue and 91.4% able to cite at least one symptom. Control practices reported included use of repellent cream or mosquito spray (43.8%), elimination of mosquito breeding sites by covering water containers (59.0%) and eliminating stagnant water around the home (85.1%). Most subjects (75.3%) reported they had never been diagnosed with dengue.

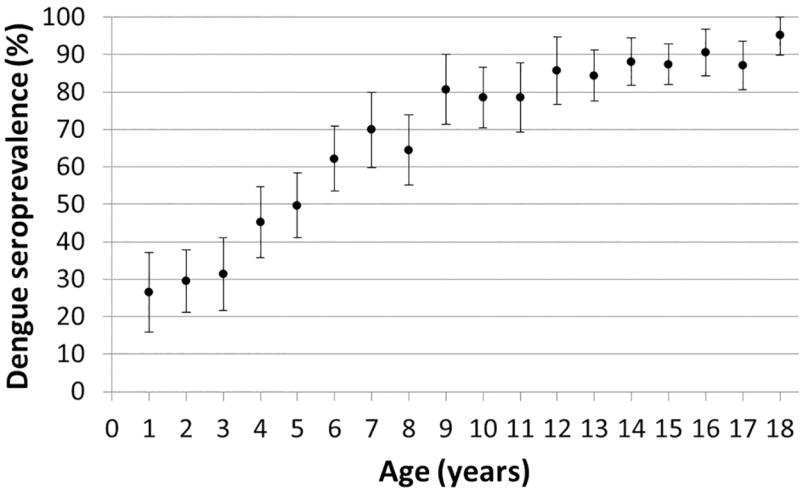

Fig 3. Mean age-specific dengue antibody seroprevalence distribution and 95% confidence interval.

https://doi.org/10.1371/journal.pntd.0005621.g003

Table 1. Description of subject demographics and knowledge of dengue symptoms and transmission, vector control practice, and medical history in the household and results of the univariate hierarchical logistic model.

Variable	Number samples (%)	Response rate	Seroprevalence [95% CI]	Odd ratio [95% CI]	P-value
Overall urban seroprevalence	3,194		69.4 [64.4–74.3]		
Subject demographics					
Gender		100%			
Male	1527 (47.8%)		67.4 [62.4–72.5]	Ref.	0.018
Female	1667 (52.2%)		71.1 [65.9–76.3]	1.21 [1.03;1.41]	
Age		100%			
1–4	672 (21.0%)		33.8 [26.4–41.2]	Ref.	<0.0001
5–9	861 (27.0%)		65.4 [59.1–71.7]	4.40 [3.50;5.52]	
10–14	886 (27.7%)		83.1 [77.1–89.0]	12.95 [10.0;16.79]	
15–18	775 (24.3%)		89.0 [84.0–94.1]	22.24 [16.48;30.01]	
Subject household socio-demographic					
Household		97.6%			0.076
Temporary/unplanned or slum	291 (9.1%)		62.2 [50.1;74.3]	Ref.	
Multi-floored building	277 (8.7%)		68.6 [59.0;78.0]	1.16 [0.79;1.71]	
Single-story attached building	1780 (55.7%)		69.0 [63.3;74.7]	1.25 [0.94;1.67]	
Single-story detached house	769 (24.1%)		72.4 [67.6;77.3]	1.47 [1.07;2.02]	
No data	77 (2.4%)		77.9 [69.5;86.3]	1.93 [1.04;3.59]	
Living in the house since birth		98.3%			0.547
Yes	2790 (87.3%)		69.7 [64.6;74.8]	Ref.	
No	351 (11.0%)		66.1 [58.4;73.7]	1.04 [0.81;1.35]	
No data	53 (1.7%)		75.5 [67.3;83.6]	1.47 [0.74;2.95]	
Average monthly household income		99.0%			0.320
<200000	910 (28.5%)		68.4 [63.0;76.7]	Ref.	
200,000–400,000	241 (7.5%)		68.0 [60.8;75.3]	0.86 [0.71;1.03]	
>400,000	2011 (63.0%)		69.9 [64.0;75.8]	1 [0.74;1.36]	
No data	32 (1%)		75 [65.8;84.2]	1.37 [0.60;3.16]	
Parents/guardian highest education level		99.4%			<0.0001
University	366 (11.5%)		60.4 [53.8;67.0]	Ref.	
Never went to formal school	92 (2.9%)		85.9 [79.0;92.8]	3.62 [1.87;7.00]	
Finished elementary school	628 (19.7%)		72.8 [64.8;80.7]	1.77 [1.30;2.40]	
Finished junior high school	707 (22.1%)		72.0 [65.8;78.2]	1.50 [1.12;2.00]	
Finished senior high school	1383 (43.3%)		67.7 [63.1;72.4]	1.18 [0.91;1.52]	
No data	18 (0.6%)		72.2 [56.8;87.6]	1.55 [0.52;4.60]	
How many persons live in the household		99.6%			<0.0001
1–3	491 (15.4%)		61.1 [54.4;67.8]	Ref.	
4–5	1803 (56.4%)		71.2 [66.4;76.1]	1.66 [1.33;2.07]	
>5	886 (27.7%)		70.2 [63.1;77.3]	1.52 [1.18;1.95]	
No data	14 (0.4%)		71.4 [50.4;72.4]	1.56 [0.47;5.19]	
Dengue knowledge, exposure and control					
Heard about dengue before the study		99.4%			0.168
No	236 (7.4%)		64.8 [52.0;77.6]	Ref.	
Yes	2940 (92.0%)		69.8 [65.0;74.5]	1.34 [0.99;1.82]	
No data	18 (0.6%)		66.7 [47.0;86.3]	1.29 [0.45;3.72]	
Knowledge of dengue illness symptoms*		91.7%			0.145

(*Continued*)

Table 1. (*Continued*)

Variable	Number samples (%)	Response rate	Seroprevalence [95% CI]	Odd ratio [95% CI]	P-value
No symptoms known	9 (0.3%)		55.6 [11.5;99.6]	Ref.	
At least one symptoms	2919 (91.4%)		69.8 [65.1;74.5]	1.94 [0.47;7.97]	
No data	266 (8.3%)		65.4 [53.8;77.1]	1.50 [0.36;6.29]	
How is dengue virus spreading among human		91.1%			0.444
Mosquito bite	2573 (80.6%)		70.0 [65.6;74.4]	Ref.	
Other	336 (10.5%)		66.7 [57.1;76.3]	0.96 [0.72;1.28]	
No data	285 (8.9%)		67.0 [55.8;78.2]	0.83 [0.63;1.10]	
When do mosquito bite		86.0%			0.380
During the day	2603 (81.5%)		69.5 [65.1;73.3]	Ref.	
At night	145 (4.5%)		73.8 [62.1;85.5]	1.14 [0.76;1.69]	
No data	446 (14.0%)		67.3 [56.7;77.8]	0.87 [0.69;1.10]	
Use insecticide spray to kill mosquitoes		98.1%			0.903
No	2037 (63.8%)		68.6 [62.7;74.5]	Ref.	
Yes	110 (3.4%)		75.4 [57.3;93.6]	1.09 [0.67;1.78]	
Yes, all year long	733 (22.9%)		71.5 [67.3;75.6]	1.03 [0.84;1.27]	
Yes, during epidemics	254 (7.9%)		67.3 [60.2;74.4]	0.88 [0.65;1.20]	
No data	60 (1.9%)		68.3 [57.8;78.4]	0.92 [0.52;1.65]	
Use mosquito mat/coil/liquid vaporizer		97.8%			0.905
No	1385 (43.4%)		70.5 [64.8;76.2]	Ref.	
Yes	123 (3.8%)		71.5 [54.4;88.7]	1.04 [0.66;1.62]	
Yes, all year long	1210 (37.9%)		69.1 [64.2;74.0]	1.00 [0.83;1.21]	
Yes, during epidemics	406 (12.7%)		66.0 [58.2;73.8]	1.08 [0.83;1.40]	
No data	70 (2.2%)		67.1 [56.4;77.9]	0.81 [0.47;1.40]	
Sleep under insecticidal bed net		97.2%			0.555
No	2890 (90.5%)		70.1 [65.3;74.8]	Ref.	
Yes	23 (0.7%)		78.3 [60.0;96.6]	1.21 [0.42;3.44]	
Yes, all year long	179 (5.6%)		61.4 [49.1;73.8]	0.88 [0.62;1.24]	
Yes, during epidemics	13 (0.4%)		46.1 [10.5;81.8]	0.57 [1.18;1.81]	
No data	89 (2.8%)		65.2 [55.9;74.4]	0.74 [0.46;1.19]	
Sleep under untreated bed net		97.0%			0.102
No	2563 (80.2%)		70.7 [66.2;75.3]	Ref.	
Yes	43 (1.3%)		72.1 [57.2;86.9]	0.92 [0.46;1.86]	
Yes, all year long	454 (14.2%)		63.4 [52.2;74.7]	0.75 [0.59;0.95]	
Yes, during epidemics	39 (1.2%)		48.7 [32.9;64.5]	0.58 [0.30;1.13]	
No data	95 (3.0%)		69.5 [58.5;80.4]	0.86 [0.55;1.44]	
Use air conditioner at home		97.0%			0.168
No	2854 (89.3%)		69.8 [64.6;75.0]	Ref.	
Yes	20 (0.6%)		65.0 [36.6;93.4]	0.61 [0.23;1.62]	
Yes, all year long	208 (6.5%)		64.9 [55.1;74.7]	0.70 [0.51;0.96]	
Yes, during epidemics	16 (0.5%)		62.5 [38.8.86.2]	0.71 [0.25;2.05]	
No data	96 (3.0%)		68.7 [60.0;77.4]	0.82 [0.31;1.31]	
Use mosquito repellent cream or spray		97.5%			0.259
No	1714 (53.7%)		65.8 [59.7;71.9]	Ref.	
Yes	118 (3.7%)		72.0 [54.7;89.4]	0.90 [0.57;1.44]	
Yes, all year long	940 (29.4%		74.6 [70.2;78.9]	1.20 [0.98;1.47]	
Yes, during epidemics	341 (10.7%)		72.1 [66.2;78.0]	1.25 [0.95;1.66]	

(*Continued*)

Table 1. (*Continued*)

Variable	Number samples (%)	Response rate	Seroprevalence [95% CI]	Odd ratio [95% CI]	P-value
No data	81 (2.5%)		69.1 [58.3;79.9]	1.01 [0.60;1.69]	
Wear long clothing to protect from insect bite		97.2%			0.791
No	2283 (71.5%)		69.3 [64.1;74.4]	Ref.	
Yes	112 (3.5%)		73.2 [55.5;91.0]	0.89 [0.56;1.43]	
Yes, all year long	521 (16.3%)		71.8 [64.2;79.4]	1.05 [0.83;1.33]	
Yes, during epidemics	187 (5.8%)		62.0 [51.9;72.1]	0.84 [0.59;1.19]	
No data	91 (2.8%)		69.2 [60.6;77.8]	0.91 [0.56;1.47]	
Water container in the household		99.3%			0.204
No	605 (18.9%)		72.1 [67.3;76.8]	Ref.	
Yes not covered	682 (21.3%)		67.7 [61.8;73.7]	0.94 [0.72;1.23]	
Yes tightly covered	1884 (59.0%)		69.3 [62.4;76.2]	0.94 [0.75;1.17]	
No data	23 (0.7%)		52.2 [34.9;69.4]	0.38 [0.16;0.92]	
Check and eliminate stagnant water in the property		97.3%			0.306
No	388 (12.1%)		72.4 [67.9;76.9]	Ref.	
Yes	329 (10.3%)		77.2 [65.7;88.7]	1.18 [0.81;1.73]	
Yes, all year long	2204 (69.0%)		68.4 [63.0;73.8]	0.86 [0.66;1.12]	
Yes, during epidemics	185 (5.8%)		64.9 [48.5;81.3]	0.87 [0.58;1.30]	
No data	88 (2.7%)		60.2 [54.2;66.2]	0.96 [0.55;1.67]	
Dengue disease history					
Number of dengue cases in the household since the subject is born		93.7%			<0.0001
No case	1500 (47.0%)		63.5 [56.2;70.7]	Ref.	
One case	288 (9.0%)		83.7 [78.7;88.7]	2.54 [1.80;3.60]	
>1 case	66 (2.1%)		87.9 [78.3;97.5]	3.83 [1.77;8.24]	
Don't know	1140 (35.7%)		71.3 [66.2;76.4]	1.16 [0.93;1.46]	
No data	200 (6.3%)		76 [62.0;90.0]	1.27 [0.80;2.02]	
Has a doctor ever diagnosed the subject with dengue		92.9%			<0.0001
No	2406 (75.3%)		66.7 [61.4;72.1]	Ref.	
Yes	335 (10.5%)		81.5 [74.6;88.4]	2.27 [1.67;3.10]	
Don't know	227 (7.1%)		69.2 [57.7;80.7]	1.05 [0.76;1.44]	
No data	226 (7.1%)		79.6 [70.0;89.3]	1.47 [0.95;2.28]	

*Dengue symptoms: bleeding, fever, headache, muscular pain, nausea and rash

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Age and gender were associated with dengue serological status, with seroprevalences increasing with age ($p<0.0001$) and values of 71.1% (95% CI: 65.9–76.3) in females versus 67.4% (95% CI: 62.4–72.5) in males ($p = 0.018$) (Table 1). After univariate analysis, the type of household ($p = 0.08$), the level of education of the parents/guardians ($p<0.0001$), the number of persons living in the household ($p<0.0001$), knowledge about dengue symptoms ($p = 0.14$), sleeping under an untreated bed net ($p = 0.10$), the number of dengue cases identified since the subject was born ($p<0.0001$), and a previous clinical diagnosis of dengue for the subject ($p<0.0001$) were also associated with dengue serological status. In the multivariate model (Table 2), two variables remained associated with the dengue serologic status, the subject age group (1–4 vs 5–9 OR = 4.25; 1–4 vs. 10–14 OR = 12.60; and 1–4 vs 15–18 OR = 21.87; $p<0.0001$) and the number of cases diagnosed in the household since the subject was born ($p = 0.0004$).

Table 2. Result of the multivariate hierarchical logistic model of variables associated with dengue seropositive status.

Variable	N	Odd ratio [95% CI]	P-value
Age group			<0.0001
1–4	672 (21.0%)	Ref.	
5–9	861 (27.0%)	4.25 [3.39;5.37]	
10–14	886 (27.7%)	12.60 [9.72;16.35]	
15–18	775 (24.3%)	21.87 [16.16;29.59]	
Number of dengue cases in the household since the subject is born			0.0004
No	1500 (47.0%)	Ref.	
One	288 (9.0%)	2.05 [1.39;3.01]	
>1	66 (2.1%)	2.96 [1.29;6.79]	
Don't know	1140 (35.7%)	1.06 [0.82;1.38]	
No data	200 (6.3%)	0.81 [0.48;1.36]	

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Force of infection

The constant force of infection model was valid and estimated a force of primary infection of 13.1% per year in dengue-naïve children. As a result of the goodness of fit statistic being close to 0.05, a model of varying force of infection (age groups of one year) was run to examine the homogeneity of the force of primary infection estimates per age group. As suggested by the first model, there was no clear trend in changes in force of infection with age; the estimates were overlapping, ranging from 10.2% to 18.5% per year. The highest force of primary infection was observed in the 1-year-old age group (Table 3).

Table 3. Dengue virus force of infection time varying and constant risk model.

Model		Estimated force of infection (%)	[95% CI]	P- value	Goodness of fit statistics
Model 1	1–18	13.1	12.5–13.6	<0.0001	>0.05**
Model 2	1	18.5	13.2–24.8	<0.0001	1.00***
	2	13.1	9.9–16.7	<0.0001	
	3	10.2	8.0–12.7	<0.0001	
	4	12.5	10.1–15.3	<0.0001	
	5	11.7	9.5–14.3	<0.0001	
	6	13.9	11.6–16.5	<0.0001	
	7	14.8	12.4–17.5	<0.0001	
	8	11.5	9.5–13.7	<0.0001	
	9	15.9	13.3–18.8	<0.0001	
	10	13.6	11.5–16.0	<0.0001	
	11	12.6	10.5–14.9	<0.0001	
	12	14.4	12.0–17.2	<0.0001	
	13	12.9	10.7–15.3	<0.0001	
	14	13.7	11.5–16.1	<0.0001	
	15	12.5	10.8–14.5	<0.0001	
	16	13.4	11.4–15.5	<0.0001	
	17	11.1	9.4–13.0	<0.0001	
	18	15.2	10.9–21.3	<0.0001	

** Pearson (0.063) and Deviance tests (0.068)

*** Hosmer and Lemeshow test

https://doi.org/10.1371/journal.pntd.0005621.t003

Discussion

This is the first dengue antibody seroprevalence study conducted in a representative population of urban dwelling Indonesian children. The findings benefit from a cluster sampling design with probability proportional to size method, and sensitive and specific dengue diagnostic assays performed in the same laboratory.

This study found that 69.4% of children had been previously infected with dengue virus, more than 80% of children aged 10 years or over, indicating that the disease burden is extremely high. A seroprevalence study conducted in 1995 in healthy children in Yogyakarta, Indonesia, using the plaque reduction neutralization test to determine previous exposure, reported the presence of neutralizing antibodies in 56.2% of 4–9-year-old children, ranging from 37.2% in 4-year-old subjects to 69.7% in those 9 years of age. These are slightly lower than the rates observed in our study (Fig 3 and Table 1) and may be reflective of increasing dengue endemicity in the intervening decades, or geographic variability [19]. Our results also show higher levels of dengue virus exposure than those reported in other dengue endemic countries such as Sri Lanka (Colombo, 2008, 52.0% in those <12 years of age, and median age of seroconversion of 4.7 years) [13, 35], and Vietnam (Binh Thuan, 2003, 65.7% in 7–13 year olds) [14]. This elevated dengue exposure risk was also observed during a 2011 dengue vaccine trial in 5 Asian countries, where baseline dengue seroprevalence was highest in Indonesian children [36].

Our constant force of infection model estimated a 13.1% annual rate of primary infection among 1–18-year-old children, while the variable model estimated a force of infection that varied from 10.2% to 18.5%. These estimates are similar to those reported in Sri Lanka in 2008 (14.1% in those aged <12 years) and Southern Vietnam in 2003 (11.7% in 7–13-year-old children) [13, 14]. Despite these similarities between Vietnam and Indonesia in terms of transmission dynamics, the reported incidence of disease in Vietnam is more than twice that in Indonesia. [37]. A number of hypotheses could explain this difference in findings: most likely, it is reflective of Indonesia's specific case definition for reported dengue disease (only DHF is reported), but underlying virological, genetic or epidemiological differences could play a role. From the constant force of primary infection model, it can be assumed that the average rate of primary infection was not highly variable over the past 18 years. Additional analysis may be needed to better understand infection risk over time. The recently observed increase in age distribution of reported cases may have been driven by more variable virologic, demographic, reporting or other determinants of disease [10]. A similar phenomenon was illustrated by a study conducted in Thailand showing that the upward shift in dengue case age was associated with demographic changes [38].

It can be assumed that dengue awareness, through social mobilization and education campaigns, begun in the 1970s, and the increasing public health importance associated with high media coverage, has steadily increased [39]. Knowledge of dengue transmission and symptoms was high within the study subjects; 92% of households had heard about dengue before our study and were able to cite at least one of the disease symptoms, and more than 80% knew that dengue virus is transmitted by diurnal mosquito bites. In term of exposure, household practices were focused on destroying mosquito breeding sites rather than personal protection. The level of exposure to the virus, however, is strong evidence that these reported behaviours are inadequate to protect against infection and additional prevention and control measures are urgently required.

In the multivariate model, only subject age group and the number of dengue cases that occurred in the household were associated with seropositive status. Some of the parameters significantly associated with dengue seropositivity in univariate models were also implicated in

other dengue studies conducted in Latin America and Asia. For example, parental level of education and dengue illness history in the household have been associated with dengue seropositivity [17]. Other parameters, such as household size, exhibit an association inverse to that previously reported in the literature [40]. This is most likely explained by confounding effects from known risk factors such as age or unknown, socio-demographic drivers of exposure risk. The lack of significant associations between socio-demographic and behavioural factors with serological status provides evidence that essentially everyone is at risk of infection; that knowledge of prevention and control at the individual/household level is not protective against infection; and that additional measures to prevent transmission are required. The retrospective nature of our questionnaire limits the robustness of our results; recall bias may have been an issue.

A recent expansion in dengue virus transmission from urban to peri-urban and rural areas has been described [15] and the identification of provinces or areas of high transmission risk is a focus of prevention and control planning. This study showed a high level of exposure across urban Indonesia and, while we excluded rural areas from this study for operational reasons, it is likely that nearby peri-urban populations may have experienced similar high levels of exposure [40]. Another possible limitation is that cross-reaction between flaviviruses has been documented and the risk of false positives cannot be excluded. We consider this risk as low, because reports of other viruses such as Japanese encephalitis and Zika, in Indonesia, are rare. This study was not designed to make national-level infection or disease burden estimates but the observation that 13.1% of children suffer a primary infection per year translates into many millions of infections per year. Adults are presumably infected with a similar frequency. A proportion of these infections will be secondary, predisposing to symptomatic and severe disease. While a modelling approach would be required to quantify this burden, these data are strongly suggestive that dengue infections result in a significant burden of symptomatic and severe disease in urban Indonesia.

Supporting information

S1 Checklist. STROBE Checklist.
(DOC)

S1 Appendix. List of the 30 clusters selected in Indonesia.
(DOCX)

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Dengue virus serotype distribution based on serological evidence in pediatric urban population in Indonesia

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Abstract

Background

Dengue is a febrile illness transmitted by mosquitoes, causing disease across the tropical and sub-tropical world. Antibody prevalence data and serotype distributions describe population-level risk and inform public health decision-making.

Methodology/Principal findings

In this cross-sectional study we used data from a pediatric dengue seroprevalence study to describe historical dengue serotype circulation, according to age and geographic location. A sub-sample of 780 dengue IgG-positive sera, collected from 30 sites across urban Indonesia in 2014, were tested by the plaque reduction neutralization test (PRNT) to measure the prevalence and concentration of serotype-specific neutralizing antibodies according to subject age and geography. PRNT results were obtained from 776 subjects with mean age of 9.6 years. 765 (98.6%) neutralized one or more dengue serotype at a threshold of >10 (1/dil). Multitypic profiles were observed in 50.9% of the samples; a proportion which increased to 63.1% in subjects aged 15–18 years. Amongst monotypic samples, the highest proportion was reactive against DENV-2, followed by DENV-1, and DENV-3, with some variation across the country. DENV-4 was the least common serotype. The highest anti-dengue antibody titers were recorded against DENV-2, and increased with age to a geometric mean of 516.5 [1/dil] in the oldest age group.

Conclusions/Significance

We found that all four dengue serotypes have been widely circulating in most of urban Indonesia, and more than half of children had already been exposed to >1 dengue serotype, demonstrating intense transmission often associated with more severe clinical episodes. These data will help inform policymakers and highlight the importance of dengue surveillance, prevention and control.

manuscript for important intellectual content and approved the final version published.

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: Anne-Frieda Taurel, Alain Bouckenooghe, Hermin Sitompul and Joshua Nealon are employees of Sanofi Pasteur, a company engaged in the production of vaccines including against dengue. R. Tedjo Sasmono, Ari Prayitno and Sri Rezeki Hadinegoro have been investigators for clinical or epidemiological studies sponsored by Sanofi Pasteur, and have been remunerated accordingly.

Author summary

Dengue is a febrile illness transmitted by mosquitoes, causing disease across the tropical and sub-tropical world. Antibody prevalence data and serotype distribution describe population-level risk and inform public health decision-making. We present data from a dengue seroprevalence study in children in Indonesia; circulation of the four dengue serotypes (DENV-1, -2, -3, -4) was assessed, by age and location. Samples collected from 30 urban Indonesian sites were tested using the plaque reduction neutralization test (PRNT), which enabled us to measure prevalence and concentration of antibodies specific to dengue virus serotypes. Results were obtained from 776 subjects (mean age: 9.6 years). 765 (98.6%) neutralized $\geq$1 dengue serotype; the highest proportion was reactive against DENV-2, followed by DENV-1, and DENV-3, with some variation across the country. Reaction to multiple serotypes was observed in 50.9% of samples. The highest anti-dengue antibody titers were recorded against DENV-2, and increased with age. The fact that all four dengue serotypes have been widely circulating in urban Indonesia, and more than half of children had been exposed to >1 dengue serotype, shows intense transmission, often associated with more severe clinical episodes. These data will help inform policy-makers and highlight the importance of dengue surveillance, prevention and control.

Introduction

Dengue is a febrile illness caused by dengue virus (DENV) infection. The clinical manifestations of dengue occur on a spectrum, ranging from asymptomatic or a mild flu-like syndrome known as classic dengue fever (DF), to a more severe form known as dengue hemorrhagic fever (DHF) and the potentially fatal dengue shock syndrome (DSS) [1]. DENV, which belongs to the family *Flaviviridae*, is transmitted by mosquitoes of the genus *Aedes*; predominantly *Aedes aegypti*. There are four evolutionarily distinct, antigenically related DENV serotypes; DENV-1, -2, -3, and -4 causing disease across the tropical and sub-tropical world [2, 3].

Neutralizing antibodies (NAbs) against the four serotypes are considered a critical component of the protective immune response which is achieved when adequate, specific antibody titers circulate [4]. Accordingly, plaque reduction neutralization tests (PRNT), which quantify serum concentrations required to neutralize live viruses, are the most specific assays for detecting flavivirus exposure history [5]. The dengue PRNT is able to target individual viral serotypes, and therefore can infer serotype-exposure history, however, interpretation of heterotypic responses is complicated for reasons including original antigenic sin [6, 7].

Indonesia is the largest archipelago country in the world with over 17,000 islands, inhabited by around 240 million people. Dengue was first reported in 1968, and has been expanding ever since, in both incidence and geography, with an annual burden of >750,000 cases [8]. The disease is likely hyperendemic across most islands [9, 10]. Reporting of DHF in Indonesia is mandatory within 72 hours of diagnosis, health centers and public/private hospitals use the World Health Organization's (WHO) 1997 case definitions [11] and only DHF/DSS cases are reported. Laboratory confirmation of dengue is rare, especially in health services with limited facilities although dengue IgG/IgM and NS1 rapid tests are increasingly used in hospitals and health clinics. Indonesia does not conduct nationally-representative dengue serotype surveillance. Genotypic and serological surveillance has been undertaken by some Indonesian institutions, on a project basis which confirmed the dengue serotypes in symptomatic individuals [12–14]. Those studies include in Makassar, South Sulawesi from 2007–2010, where dengue infection was confirmed in >100 patients, many of whom were aged 11–20 years old.

Serotyping revealed that DENV-1 was the most common form (41%) followed by DENV-2 (31%), DENV-3 (20%), and DENV-4 (7%) [15]. In Surabaya, East Java, in 2012, dengue RNA was isolated from 79 of 148 suspected dengue patients (53%), with DENV-1 as the predominant serotype (73%), followed by DENV-2 (8%), DENV-4 (8%), and DENV-3 (6%), while 5% were found to have mixed serotypes [16]. In Semarang, Central Java in 2012, 66 of 120 suspected cases (55%) were serologically confirmed and viral RNA was detected in 31 samples [12]. DENV-1 was the predominant serotype, followed by DENV-2, DENV-3, and DENV-4. DENV-1 predominance has also been reported from other studies and cities in Indonesia, including Surabaya [17] and Makassar [15]. Finally, from urban and rural areas of Bali (Denpasar and Gianyar), in 2015, 205 adult patients with suspected dengue were recruited in a prospective cross-sectional study. Of these, 161 patients had virologically-confirmed dengue; DENV-3 was predominant (48%), followed by DENV-1 (28%), DENV-2 (17%), and DENV-4 (4%). Five samples (3%) were detected which contained two different serotypes, and it was noted that the proportions varied in urban and rural areas [18].

Understanding antibody prevalence is an important consideration in the interpretation of epidemiological data, especially when reviewing interactions with other flaviviruses or considering vaccine introduction. The co-circulation of multiple dengue serotypes is a population-level risk factor for severe dengue disease because of the increased likelihood of a second or subsequent infection, and also due to the fact that sequential infections are associated with increased severity [19]. Serotype distribution may be predictive of future epidemiology and is important information for dynamic transmission models. The objective of this study was to use data from a dengue seroprevalence survey to describe the historical serotype (DENV-1, 2, 3, 4) circulation based on the prevalence of serotype-specific anti-DENV antibodies, according to age and geographic location, in a pediatric population in Indonesia.

Materials and methods

Study design

In this cross-sectional study, serum samples and data from a national-level pediatric dengue seroprevalence study were used to describe historical dengue serotype circulation, according to age and geographic location. Dengue IgG-positive sera, collected from 30 sites across urban Indonesia, were tested by the PRNT to measure the prevalence and concentration of serotype-specific dengue neutralizing antibodies according to subject age and geography.

Sample collection and selection

Surveillance and sample collection methods were previously described [20]. Ethical approval was obtained from the Health Research Ethics Committee of Faculty of Medicine of University of Indonesia (No. 462/H2.F1/ETIK/2014). Briefly, between 30 October 2014–27 November 2014, blood samples were collected from 3,210 children aged 1–18 years in 30 urban Indonesian subdistricts, randomly selected from west to east based on the probability proportional to population size. The blood samples were to be tested for dengue IgG by enzyme-linked immunosorbent assay (ELISA). A sub-sample of 780 dengue IgG positive sera was used to estimate the prevalence of serotype-specific neutralizing antibodies by PRNT. The sample size was estimated to provide 95% confidence and a margin error of 5%; this is accounting for the 30 clusters with a design effect of two and assuming the "worst case" of 50% exposure to any one serotype. The sample was not strictly representative of the dengue IgG positive population as the samples were selected equally from each of the four age groups, *i.e.* 195 samples per age group, and, to provide geographical representativeness, from clusters in proportion to dengue IgG seroprevalence rates. This method over-sampled from younger subjects to; 1) increase the

number of samples tested from children recently infected with dengue, to provide a record of recent dengue circulation; 2) reduce the number of PRNTs performed on samples from older children, likely to have been infected with many serotypes, which may therefore be impossible to meaningfully interpret.

Dengue plaque reduction neutralization test (PRNT)

The PRNT method was performed based on optimized and validated $PRNT_{50}$ assay for the detection of neutralizing antibodies to four serotypes of DENV [21]. Each serum sample was heat inactivated at 56˚C and assayed in four separate PRNT runs, which corresponded to four different DENV serotype challenge viruses. Vero cells (CCL-81) were obtained from American Type Culture Collection (ATCC). Cells were grown and maintained in Minimum Essential Medium (MEM) (Gibco-Thermo Fisher Scientific, CA, USA), supplemented with 5% heat-inactivated Fetal Bovine Serum (FBS), 2 mM of L-glutamine, and 1% of antibiotic/antimycotic (Gibco-Thermo Fisher Scientific, CA, USA) at 37˚C in an atmosphere of 5% CO_2. Working banks of Vero cells were prepared in-house, qualified, and confirmed to be free of any microbial, mycoplasma, and viral contaminants. Purified mouse monoclonal antibodies (MAbs) specific to the DENV serotype envelope protein were used as the primary antibodies for virus detection according to the corresponding serotype: anti-DENV-1 (D2-1F1-3), anti-DENV-2 (3H5-1-12), anti-DENV-3 (8A1-2F12), and anti-DENV-4 (1H10-6-7) (Biotem, Le Rivier d'Apprieu, France). Alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used as the secondary antibody. The parental DENVs of the recombinant CYD vaccine viruses, i.e., DENV-1 strain PUO-359, DENV-2 strain PUO-218, DENV-3 strain PaH881/88, and DENV-4 strain 1228, were used as challenge viruses in the PRNT. The initial source, and the suitability of these four DENV serotypes to be used in dengue neutralization assay have been described elsewhere [21–23]. Dengue-antibody positive and negative human serum sample controls were obtained from healthy adult donors from Indonesia. The serum controls were used in each assay run, and served to monitor its performance and validity.

The neutralization titer ($PRNT_{50}$) of the test serum sample was defined as the reciprocal of the highest test serum dilution for which the virus infectivity was reduced by 50% when compared with the average plaque count of the challenge virus control, calculated using a four-point linear regression method. Since the lowest starting dilution of serum in the assay was 1:5, the theoretical lower limit of quantitation of the assay was a titer of 10 (reciprocal dilution).

Statistical analysis

This is a descriptive analysis, no hypotheses were tested. The study population mean age was calculated and geographic distribution described. Dengue serotype specific PRNT profiles were defined according to the following algorithm; categorizing samples as naïve (no previous dengue infection), monotypic (infection with one serotype), or multitypic (>1 serotype)[24]:

- Naïve: antibody titers <10 for the four serotypes
- Monotypic: antibody titers >10 (1/dil) to only one serotype **or** titers ≥ 10 for different serotypes with a high titer (>80 (1/dil)) and for a single predominant serotype (> 5 times higher than other titers)
- Multitypic: antibody titers ≥10 (1/dil) for different serotypes without a single predominant titer.

PRNT profile distribution by age and geography were described. PRNT profile prevalence and their 95% confidence intervals (95% CI) were calculated, the clusters results were aggregated at province level and a map was generated using QGIS 2.16.2 "Nødebo".

The mean PRNT titer, GMT (Geometric Mean Titer), and the 95% CI for each age group and dengue serotype was calculated for all samples based on their DENV PRNT results. To calculate the GMT, samples with an antibody titer T <10 (1/dil) were given the value 5 and the mean titer was calculated using the equation:

$$\widehat{GMT}_{jh} = 10^{\wedge} \frac{\sum_{1}^{n} \log_{10} T_i}{n}$$

Where $\widehat{GMT}_{jh}$ is the mean titer for the dengue serotype h of the age group j, T_i is the PRNT titer of the subjects i and n the number of subjects with a PRNT titer in the age group j for the serotype h.

All statistical analyses were performed using Excel 2013.

Results

Description of sample set

Blood samples were collected from 3,210 children aged 1–18 years in 30 urban Indonesian subdistricts, randomly selected from west to east. From a sub-sample of 780 dengue IgG positive sera, $PRNT_{50}$ results were obtained from 776 participants, equally sampled from each age group (1–4, 5–9, 10–14 and 15–18 years old). In the youngest, 1–4 years old group, four serum samples were of insufficient quantity to be tested. The mean age was 9.6 years old (95% CI [9.3–10.0]. The 30 clusters were represented with 14–39 samples per cluster. Of these, 765 (98.6%) neutralized one or more dengue serotypes at a threshold of >10 (1/dil), a proportion which varied by age: 95.3% in the 1–4 years old, 99.5% in the 5–9 years old, 99.5% in the 10–14 years old and 100% in the 15–18 years old.

PRNT profile distribution stratified by age and geographic level

Samples were categorized according to $PRNT_{50}$ profile. Multitypic profiles were observed in 50.9% of the subjects, with 28.3% in those aged 1–4 years old, 48.2% in the 5–9 years old, 63.6% in the 10–14 years old and 63.1% in those aged 15–18 (Fig 1). The proportion of monotypic profiles decreased with increasing age, representing 67.0% of those aged 1–4 years, 51.3% of the 5–9 year old group, 35.9% of the 10–14 years old group, 36.9% of the 15–18 years old and 47.7% of the overall sample. There were no naïve subjects in the 15–18 years old group whereas 4.7% of the 1–4 years old group; 0.5% of the 1–9 and 10–14 years old groups, and 1.4% of the overall sample had no detectable neutralizing dengue antibodies at the 10 (1/dil) threshold. Amongst monotypic samples, the highest proportion of samples were reactive against DENV-2, followed by DENV-1, and DENV-3, a trend which was also observed in the two youngest age groups, while the three serotypes were more evenly distributed amongst the 10–14 and 15–18 years old age groups (Fig 1).

The clusters were aggregated within 14 provinces, resulting in samples per province ranging from 15 to 183 serum samples. In seven provinces multitypic profiles were the most common (from 52.2% to 69.4% of samples). In seven provinces the monotypic profile was more prevalent (from 49.7% to 68.8%). DENV-4 was dominant in one province, in the 13 other provinces DENV-1, DENV-2, DENV-3 or a combination of these serotypes were dominant, with DENV-2 dominance being more common (Fig 2). The four monotypic serotypes were

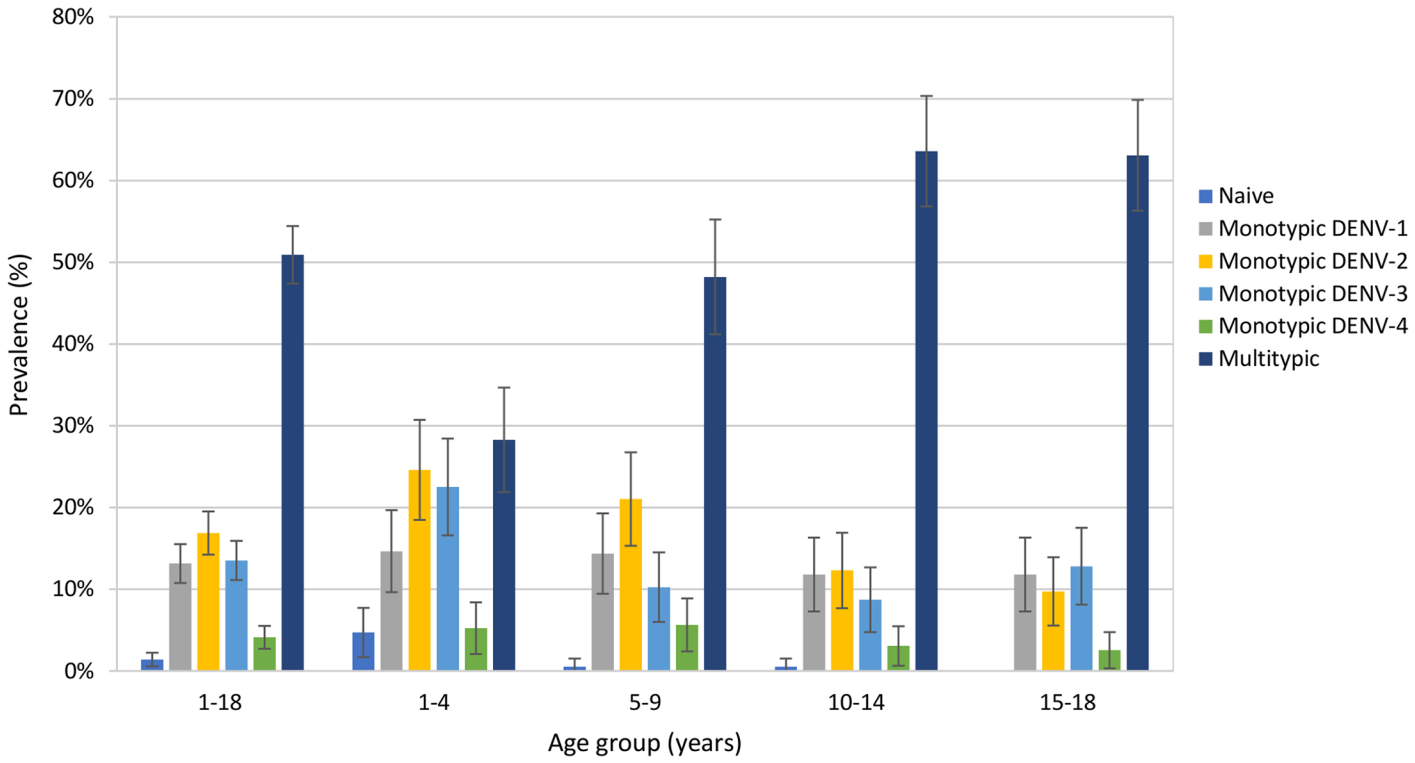

Fig 1. Proportion of individuals with naïve, monotypic (for each dengue serotype), or multitypic PRNT profiles, by age group.

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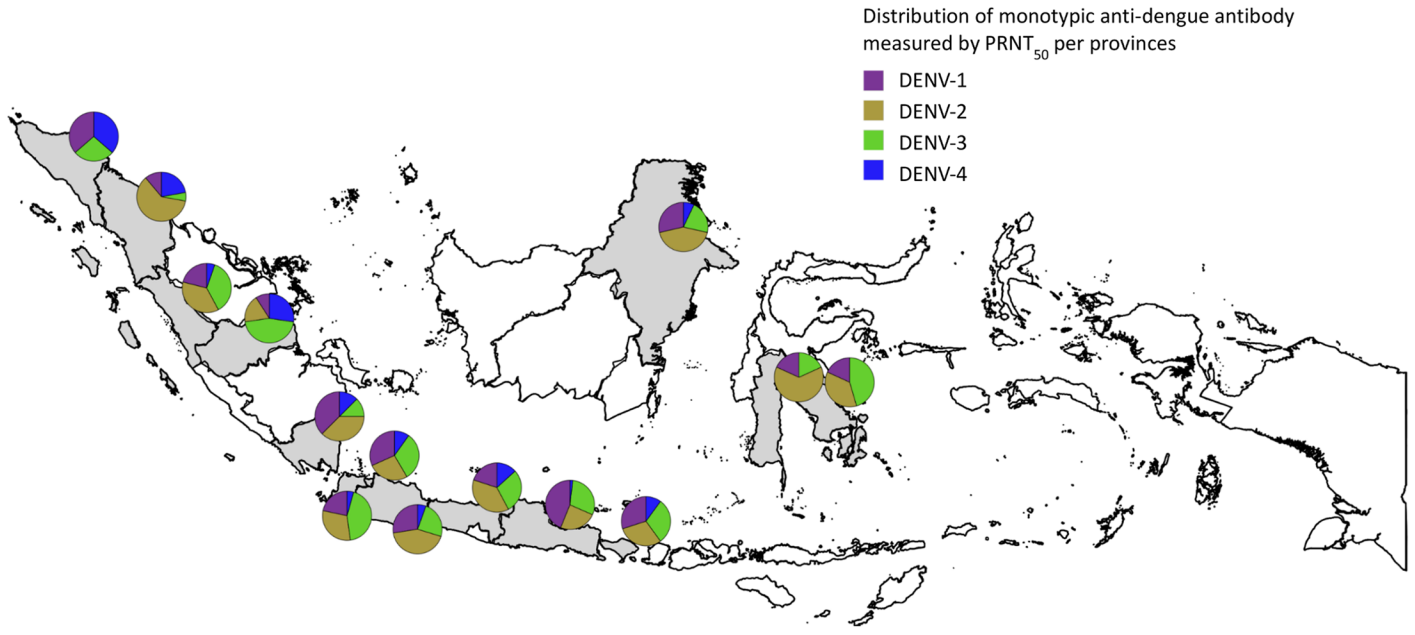

Fig 2. Map showing the proportion of monotypic dengue antibody profiles against each dengue serotype, by Indonesian province containing at least one study site (shown in grey).

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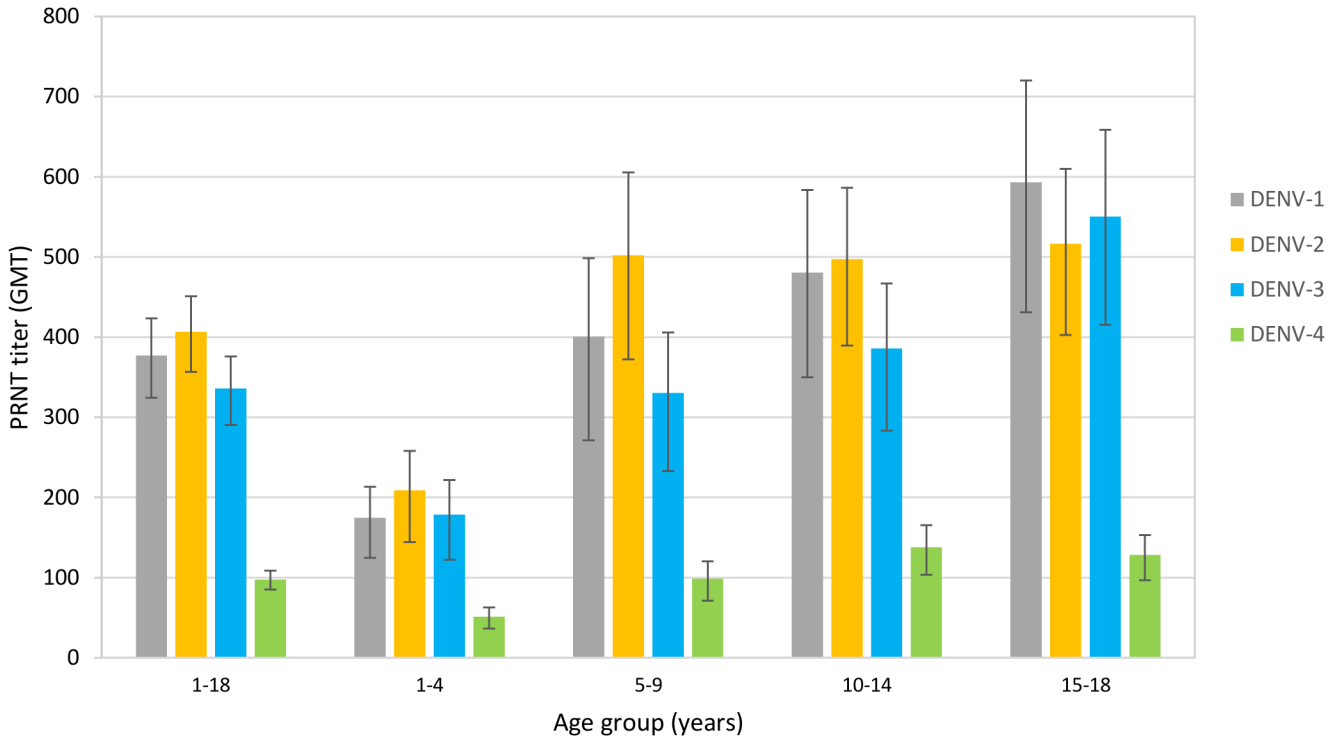

Fig 3. Dengue serotype specific GMT by age-group.

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identified in every province, with the exception of DENV-2 in Nanggroe Aceh Darussalam and DENV-4 in Sulawesi Tenggara and Sulawesi Selatan.

GMT stratified by age

GMTs increased with age. DENV-2 had the highest GMT overall (406.5 [1/dil]) and for three of the four age groups with titers of 208.8, 502.2, 497.4 and 516.5 [1/dil], respectively (Fig 3). DENV-4 had the lowest GMT for each age group (51.2, 98.9, 138.1 and 128.2 [1/dil]) and overall (97.6 [1/dil]). In the oldest subjects, titers against DENV-1 were highest (593.08 [1/dil]) followed by DENV-3 (550.2 [1/dil]) and DENV-2.

Discussion

We conducted a dengue seroprevalence study which identified serological evidence for the circulation of all four dengue serotypes across urban areas of Indonesia, in children who were exposed to infection from 1996 to 2013. The proportion of children with exposure to >1 serotype increased with age, and children were more likely to have been infected with DENV-2, DENV-1 and DENV-3 than DENV-4. Nonetheless, these results show that all four serotypes have been widely circulating in most of Indonesia, as is common in hyper-endemic countries. This study generated data on serotype-specific prevalence in areas where little or no data were previously available, with the exception of historical data from Yogyakarta, Java island [32].

Available dengue serotype data collated from 1994 to 2012 (n = 596) [25] and recent publications from all over Indonesia confirm the concomitant presence of the four DENV serotypes [10, 12, 15–18, 26–28]. Samples were collected from suspected cases and therefore suffer a potential selection bias towards serotypes associated with more symptomatic/severe cases. The

serological data we report here indicate a consistent pattern of distribution of serotypes, a finding which may indicate that the cases captured within these surveillance studies is broadly reflective of the DENV serotype circulation in the country.

PRNT enables the interrogation of samples according to their exposure history. In this study, it was remarkable to observe that in this pediatric population more than half (50.9%) had already been exposed to >1 dengue serotype, a proportion which increased with age. This rate is important because it demonstrates early and intense transmission in Indonesia; and we know that second infections have been described as more likely to be symptomatic, severe and hemorrhagic [29]. Individuals of an age likely to have received one natural exposure, but before their second, may represent an attractive target for dengue vaccination programs [30]. The observed GMT increase with age is most likely explained by continuous re-exposure to DENVs over time, further boosting antibody levels. These profiles imply that existing vector control activities in urban areas are largely insufficient at preventing infection; and that investments in novel methods may be warranted. The prevalence of multitypic profiles further reinforces the requirement for development of a safe and effective, quadrivalent dengue vaccine which could be used in children at highest risk of developing symptomatic and severe disease episodes. Additionally, these data can be useful for the calibration of dengue transmission models which may help to understand disease dynamics and the likely effects of dengue control interventions.

There are several limitations to our study. Sera collected during the convalescent phase represent infection history in the population, but are limited by the sensitivity and specificity of the serological methods used to quantify antibodies. We had the benefit of analyzing samples in this study by PRNT; however interpretation of data can be confused by heterotypic cross-neutralization between serotypes. For this reason, we did not interpret the serotype distributions of multitypic infections. Only samples positive for dengue IgG in ELISA screening assay were selected to undergo PRNT, therefore these may not be fully representative of dengue positive sera. We also observed discrepancies between IgG ELISA and PRNT data in which some samples that were positive by IgG ELISA were negative in PRNT (1.4%). This may be a consequence of the well-documented serological cross-reactivity across the flavivirus group [31] Our sample collection was also limited to urban areas and subjects consenting to the study which may have introduced additional bias.

In conclusion, this study confirmed the distribution of multiple dengue serotypes across urban Indonesia. Many children were infected with multiple serotypes, and the accompanying risk of severe disease, from an early age. DENV-1, DENV-2 and DENV-3 may play a more significant epidemiological role than DENV-4. It is hoped that these data influence policymakers to afford increased attention to dengue surveillance, prevention and control.

Supporting information

S1 Checklist. STROBE checklist.
(DOC)

S1 Table. Description of mean age, sample size and dengue serotype specific prevalence (naïve, monotypic, or multitypic) per province.
(DOCX)

S2 Table. IgG seroprevalence distribution per cluster and age group and derived PRNT sample size.
(DOCX)

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MAJOR ARTICLE

Serological Evidence of Japanese Encephalitis Virus Circulation in Asian Children From Dengue-Endemic Countries

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Background. Japanese encephalitis virus (JEV) is a zoonotic, mosquito-borne flavivirus, distributed across Asia. Infections are mostly mild or asymptomatic, but symptoms include neurological disorders, sequelae, and fatalities. Data to inform control strategies are limited due to incomplete case reporting.

Methods. We used JEV serological data from a multicountry Asian dengue vaccine study in children aged 2–14 years to describe JEV endemicity, measuring antibodies by plaque reduction neutralization test ($PRNT_{50}$).

Results. A total 1479 unvaccinated subjects were included. A minimal estimate of pediatric JEV seroprevalence in dengue-naive individuals was 8.1% in Indonesia, 5.8% in Malaysia, 10.8% in the Philippines, and 30.7% in Vietnam, translating to annual infection risks varying from 0.8% (in Malaysia) to 5.2% (in Vietnam). JEV seroprevalence and annual infection estimates were much higher in children with history of dengue infection, indicating cross-neutralization within the JEV $PRNT_{50}$ assay.

Conclusions. These data confirm JEV transmission across predominantly urban areas and support a greater emphasis on JEV case finding, diagnosis, and prevention.

Keywords. epidemiology; flavivirus; encephalitis; Japanese; seroepidemiologic studies.

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus, distributed across endemic or epidemic-prone countries in East, Southeast, and South Asia. Extending from North Korea, southeastern Russia, Japan, and Northern China to Western Pacific islands including the Philippines, Papua New Guinea, and the far north of Australia, and west to India and southern Pakistan: >3 billion people are at risk of infection [1]. A variety of vertebrate hosts sustain transmission in zoonotic cycles with mosquitoes, predominantly *Culex tritaeniorhynchus* [2]. Humans are infected as incidental, dead-end hosts and may be at particular risk when in proximity to pigs and ardeid birds, which experience durations and levels of viremia capable of infecting vector mosquitoes [2, 3].

After humans are bitten by an infected mosquito, the virus is thought to amplify in the cells of the peripheral lymphatic system causing a transient and mostly low-grade viremia for ~1 week. Although infections are common in endemic areas, most are either asymptomatic or resolve after acute undifferentiated fever and are unlikely to be diagnosed as Japanese encephalitis [2, 4]. Estimates of the proportion of infections that lead to symptomatic disease vary widely from 1:25 to 1:1000 [5]. Estimates of the proportion of symptomatic disease are higher in studies from non-indigenous US servicemen in Asia than indigenous populations, perhaps a consequence of (1) viral or human genetics, (2) level of health, (3) immune status, (4) more sensitive surveillance, and (5) increased laboratory confirmation [6–8].

A small proportion of infections proceed to more severe disease after invasion of the central nervous system, leading to an encephalitis; this results in a broad range of neurological disorders including convulsions, prolonged seizures, respiratory abnormalities, and spasms [9]. In hospitalized individuals, approximately 30% will die, and approximately 50% of survivors will suffer severe residual neurological disease [9, 10].

Although considered rare, Japanese encephalitis cases cause significant morbidity and mortality with an estimated 67 900 incident symptomatic cases per year across affected countries [11]. Even severe cases may be unreported to public health authorities due to a combination of low level of clinical suspicion, infrequent use of laboratory confirmation, and a wide spectrum of symptoms [9, 12]. Several licensed vaccines are available, and vaccination is recommended both for those living in and traveling to endemic areas. Underrecognition of disease contributes to undervaccination [1, 13].

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In the absence of reliable incidence data, seroepidemiological methods can be used to measure exposure and make inferences around endemicity of diseases [2, 14]. Age reflects duration of exposure, and because JEV antibodies persist for life, age-stratified data describe the proportion of individuals historically infected, from which the infection rate can be calculated [15]. A challenge in this approach stems from the specificity of diagnostics that have well documented cross-reactivity between members of the flavivirus family [16]. Assays detecting immunoglobulin G antibodies, raised after recent vaccination or recent wild-type infection, are particularly cross-reactive, and a positive result cannot be considered specific in areas where multiple flaviviruses cocirculate [17, 18]. Cross-reactivity typically decreases as the immune response evolves from an initial more heterotypic to a homotypic response. Neutralizing assays including the plaque reduction neutralization test (PRNT), in which the dilution of serum required to neutralize live viruses is quantified, are more specific and are considered the gold standard in detecting historical flavivirus exposure [19]. For JEV, a PRNT titer ≥1:10 dilution by $PRNT_{50}$ is considered protective from infection; a more stringent threshold, $PRNT_{90}$, may be preferred for epidemiological studies of historical exposure, reducing the risk of background serum cross-reactivity [20, 21].

CYD14 (ClinicalTrials.gov number NCT01373281) was an observer-blinded dengue vaccine study conducted in 2011–2017 in 10 275 children aged 2–14 years in Indonesia, Malaysia, Thailand, the Philippines, and Vietnam [22]. From an immunological study subset, JEV seroprevalence was ascertained by PRNT at the study start before any vaccines were administered. Sites were urban, selected based on their high dengue incidence rates, and most were not considered areas of high JEV endemicity, although some (eg, Bali, Indonesia) have recorded JEV cases and outbreaks in the past [11]. At the time of the study, Japanese encephalitis vaccine was not in routine use at most study sites [23].

In this study, we used the age-stratified serological data to describe JEV endemicity, and we estimated the force of infection (FOI) at the sites where this clinical trial was conducted. To account for cross-reactive, anti-flavivirus neutralizing antibodies, we stratified by dengue virus (DENV) serological status thereby providing minimal estimates of JEV infection in individuals who had never experienced a dengue infection; and higher estimates in those who have been infected with dengue in the past.

METHODS

Ethics Statement

This was a secondary analysis using records from a vaccine clinical trial, CYD14. The original clinical trial that generated the data (ClinicalTrials.gov number NCT01373281) underwent ethics committee approval of the protocol, amendments, consent, and assent forms and was funded by Sanofi Pasteur [22].

Study Sample Set and Data

CYD14 was an observer-masked, randomized, controlled, multicenter, phase 3 dengue vaccine trial conducted in children aged 2–14 years old in 5 countries in the Asia-Pacific (3 sites in Indonesia [Bandung, Jakarta, and Bali]; 2 sites in Peninsular Malaysia [Kuala Lumpur and Penang]; 2 sites in the Philippines [San Pablo City and Cebu]; 2 sites in Thailand [Ratchaburi and Kamphaeng Phet]; and 2 sites in Southern Vietnam [My Tho and Long Xuyen]) and has been described previously [22]. Parents or legal guardians provided informed consent before participation, and written assent was obtained from older children, in compliance with the regulations of each country. Subjects received either 3 doses of a recombinant, live, attenuated, tetravalent dengue vaccine or placebo at months 0, 6, and 12.

In an immunological subset of approximately 20% of participants, serum was collected at baseline (before injection at month 0). Baseline concentrations of neutralizing antibody against JEV and DENV were measured by PRNT at the Centre for Vaccine Development (Mahidol Univeristy, Thailand) (for JEV) and at Sanofi Pasteur's Global Clinical Immunology laboratory (Swiftwater, PA) (for DENV) using the method described by Timiryasova [24]. For DENV, challenge viruses were for DENV-1 strain PUO-359, DENV-2 strain PUO-218, DENV-3 strain PaH881/88, and DENV-4 strain 1228. Neutralizing antibody titers were expressed as the reciprocal serum dilution (1/dil) achieving 50% reduction in plaque count and a lower limit of quantification of ≥10, as calculated by probit analysis [25]. After an observation that JEV seroprevalence varied according to DENV serostatus, neutralizing JEV titers achieving 90% plaque reduction ($PRNT_{90}$) were subsequently calculated from the same laboratory data to explore the impact of increasing specificity of the assay by decreasing the background serum cross-reactivity from other flaviviruses [21].

Individuals with a history of JEV or another flavivirus vaccination before blood sampling were removed from the analysis to ensure that serological status was a consequence of natural infection. Subjects from Thailand were excluded because Japanese encephalitis vaccination had been practiced nationwide for several years before the study and >95% of children were vaccinated, leaving a sample too small for meaningful analysis (n = 15).

Japanese Encephalitis Virus Seroprevalence

Japanese encephalitis virus seroprevalence, defined as the proportion of subjects with a JEV-neutralizing antibody concentration of ≥10 (1/dil), was calculated according to $PRNT_{50}$ and $PRNT_{90}$, overall, and by age for each country. To control for the influence of cross-reactive DENV antibodies and generate a minimal estimate of true JEV-positive samples, JEV seroprevalence by $PRNT_{50}$ was calculated separately for DENV seropositive and seronegative populations.

Force of Infection

Catalytic models use seroprevalence data as cumulative markers of past infections that result in lifelong antibodies, from which force of primary infection estimates can be derived [26, 27]. An FOI model was developed to describe the rate of JEV infection over the period of time covered by the subjects' age. The model assumed a constant FOI that does not vary with age whereby the probability of an individual being infected in 1 year is estimated by the following [28, 29]:

$$p_i = 1 - e^{-\mu Ai}$$

Where p_i is the probability for the i^{th} group of Ai years old of being positive and μ is the proportion of individuals infected per year, FOI. Using a maximum likelihood regression method, fitting a binomial model with a complementary log-log link function and using X = log(A) as an offset term, the intercept parameter α = log (μ) was estimated as follows:

$$\text{Log}\,(-\log\,(1-p_i)) = \log\,(\mu) + \log\,(Ai)$$

The exponential of α provided an estimate of the FOI, μ. Model fit was assessed using the Pearson and deviance test for goodness-of-fit statistics with a significance level of $P < .05$.

The proportion of individuals seropositive per age group, p_i was subsequently estimated with the following:

$$pi = 1 - e^{-\mu Ai} = 1 - e^{-(e^{\alpha} Ai)}$$

We considered that JEV serostatus in the DENV-naive population could not have been affected by cross-reactive flavivirus antibodies and therefore treated the resulting FOI estimate as a minimal estimate of annual infection risk.

All data were analyzed anonymously. Analyses were conducted using SAS 9.4, and figures were developed using Microsoft Excel 2013 and STATA, version 15.

RESULTS

Sample Set Description

We conducted an epidemiological reanalysis of clinical trial data to document historical JEV exposure in 1479 children from 4 Asian countries, as shown in the study flow chart (Figure 1). The database included 239 subjects from Vietnam, 295 subjects from Malaysia, 345 subjects from Indonesia, and 600 subjects from the Philippines (Table 1). The mean age in each country was 8.24 in Indonesia, 8.25 in Malaysia, 8.18 in Philippines, and 7.55 in Vietnam.

Japanese Encephalitis Virus Seroprevalence and Rate of Infection

By $PRNT_{50}$, overall JEV seroprevalence was 46.1% in Indonesia, 22.4% in Malaysia, 45.7% in the Philippines, and 47.5% in Vietnam. Seroprevalence increased with age, reaching >70% in the 13- to 14-year-old children in Indonesia, the Philippines, and Vietnam and 40% in Malaysia (Table 1). When stratified by DENV serostatus, JEV seroprevalence was 54.4% in Indonesia, 41.0% in Malaysia, 55.3% in the Philippines, and 59.4% in Vietnam in DENV seropositive individuals and 8.1% in Indonesia, 5.8% in Malaysia, 10.8% in the Philippines, and 30.7% in Vietnam in DENV seronegative individuals. By JEV $PRNT_{90}$, seroprevalence was considerably lower: 1.7% in Indonesia, 2.4% in Malaysia, 3.7% in the Philippines, and 11.3% in Vietnam. FOI estimates revealed an annual infection rate within DENV-positive subjects of 9.1% (95% confidence interval [CI], 7.7–10.7) in Indonesia, 5.4% (95% CI, 4.1–6.9) in Malaysia, 9.3% (95% CI, 8.2–10.6) in the Philippines, and 11.1% (95% CI, 8.8–13.8) in Vietnam. In DENV seronegative subjects, FOI was considerably lower:

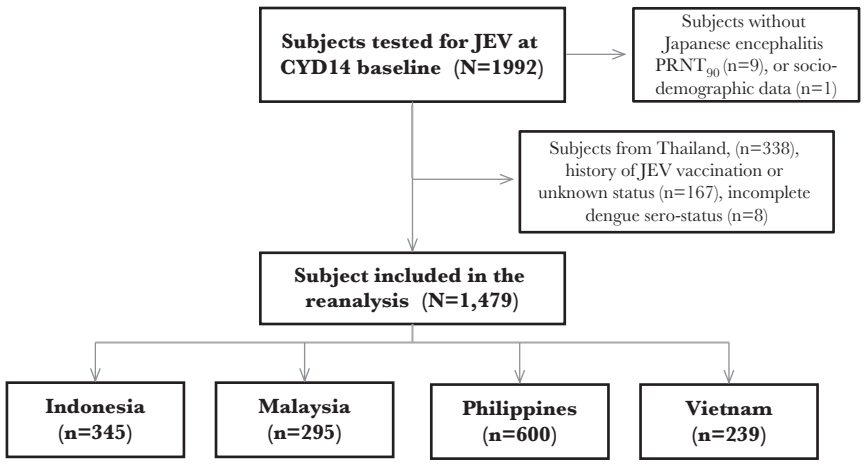

Figure 1. Study flow chart.

Table 1. Number of Subjects Included by Age (N) and Japanese Encephalitis Seroprevalence by $PRNT_{50}$ (%) According to DENV Serostatus

	Indonesia				Malaysia				Philippines				Vietnam			
Dengue Status	Positive		Negative		Positive		Negative		Positive		Negative		Positive		Negative	
Age	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
2	13	23%	11	9%	1	0%	21	0%	21	19%	18	22%	3	100%	7	14%
3	19	37%	10	0%	9	11%	12	0%	33	30%	24	8%	9	56%	11	46%
4	18	56%	13	8%	12	42%	12	0%	33	30%	20	10%	14	43%	8	38%
5	27	33%	6	0%	6	50%	25	8%	35	40%	17	6%	13	15%	22	9%
6	14	21%	2	0%	8	75%	10	0%	41	51%	14	7%	12	33%	10	30%
7	21	52%	2	50%	4	0%	9	11%	29	52%	6	0%	8	75%	4	25%
8	14	43%	3	0%	9	22%	10	10%	20	55%	5	20%	11	72%	8	38%
9	14	36%	3	0%	9	44%	9	11%	31	61%	4	25%	9	67%	6	50%
10	23	52%	3	0%	8	25%	4	0%	21	67%	1	0%	13	54%	7	43%
11	13	69%	2	50%	11	36%	8	0%	29	45%	-	-	12	75%	4	25%
12	51	75%	6	17%	30	57%	20	5%	58	64%	12	0%	28	71%	13	39%
13	34	74%	1	0%	24	42%	14	14%	68	75%	8	25%	6	100%	1	100%
14	22	73%	-	-	8	38%	2	50%	51	80%	1	0%	-	-	-	-
Total	283	54%	62	8%	139	41%	156	6%	470	55%	130	11%	138	59%	101	31%

Abbreviations: DENV, dengue virus; $PRNT_{50}$, 50% plaque reduction neutralization test.

1.4% (95% CI, 0.5–3.0) in Indonesia, 0.8% (95% CI, 0.4–1.4) in Malaysia, 1.8% (95% CI, 1.0–2.9) in the Philippines, and 5.2% (95% CI, 3.6–2.3) in Vietnam. The goodness-of-fit statistics were respected for all models (Pearson test, $P > .05$; deviance test, $P > .05$), except for the DENV-positive population in Vietnam (Figure 2).

DISCUSSION

This study documented serological evidence of JEV circulation in urban and periurban areas of Indonesia, Malaysia, the Philippines, and Vietnam, countries with differing epidemiology and JEV risk. Our study assessed historical exposure to viruses but collected no data on symptomatic episodes.

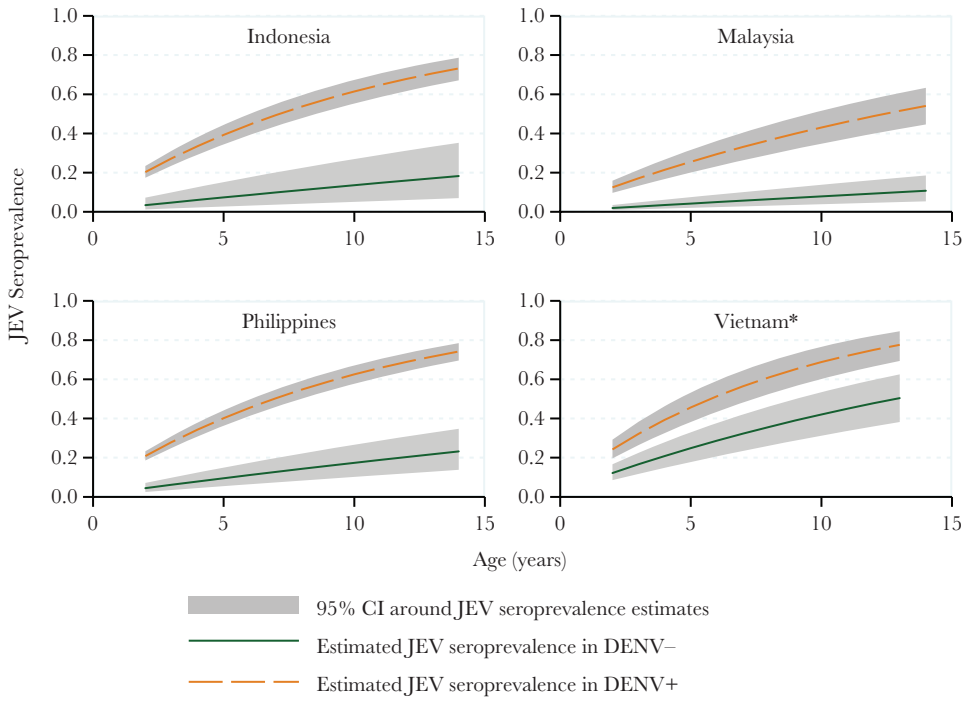

Figure 2. Force of infection-derived Japanese encephalitis age-specific seroprevalence estimates by country in dengue virus (DENV)-positive ($DENV^+$) and DENV-negative ($DENV^-$) subjects. *, Pearson and deviance test $P < .05$ for the $DENV^+$ population in Vietnam. Abbreviations: CI, confidence interval; JEV, Japanese encephalitis virus.

However, the World Health Organization's vaccine-preventable disease monitoring system reports an annual average for these countries over recent years varying from 35.2 cases (Malaysia) to 310.6 cases (Vietnam) [30]. Even after correcting for a low proportion of symptomatic infections, the levels of pediatric infection documented here imply a significant level of underreporting of symptomatic cases. Measures to improve disease awareness and increase use of confirmatory diagnostics and surveillance enhancements may be justified in response.

Seroprevalence, a function of exposure, increased with age. Therefore, these age-stratified data allowed estimation of FOI, and, to our knowledge, this is the first time this has been done in a multicountry JEV study. JEV seroprevalence varied according to DENV status, which is likely a consequence of cross-reactive antibodies raised after DENV infections. Indeed, these sites were selected for their high levels of dengue endemicity, with annual attack rates of symptomatic dengue of 2%–11% per year [31]. Therefore, we estimated JEV FOI for individuals with no previous DENV exposure, resulting in minimal JEV exposure estimates, which provide strong evidence for JEV circulation within these study populations. By this measure, between 0.8% and 5.2% of children were estimated to be naturally infected by JEV annually, findings that may be considered high in areas that do not include JEV vaccination in their national immunization programs. The estimated JEV FOI in DENV-exposed individuals was considerably higher, and the true infection rate is likely somewhere in between. Although direct comparisons of JEV FOI are unavailable, historically, Japanese encephalitis has been a pediatric disease in endemic areas with seroprevalence increasing to 100% in adults [2].

For Vietnam, the goodness-of-fit statistics for the constant FOI risk model was statistically significant ($P < .05$), indicating that the assumption of constant risk of infection was not correct. This may be due to differential exposure at different ages or epidemic prone rather than endemic epidemiology. Vietnam is the country with highest infection risk, a finding aligned with the current knowledge of risk and epidemiology [32].

It is well known that flavivirus genera share epitopes that induce cross-reactive antibodies, which leads to difficulty in differentially diagnosing flaviviral infections [16, 17]. More recent or secondary infections generate broader, heterotypic, cross-reactive responses, and—because these sites were chosen due to their high level of DENV endemicity—we considered that anti-DENV antibodies would be more likely to cross-react with JEV virus in the PRNT than the reverse [16]. However, it is important to remember that cross-reactivity or neutralization does not mean cross-protection, and interactions between flavivirus antibodies are complex and poorly understood [17, 33]. Our additional observation that JEV seroprevalence by $PRNT_{50}$ in DENV-naive children was higher than corresponding rates derived from JEV $PRNT_{90}$ implies that $PRNT_{90}$ is overly specific, excluding true-positive samples, for epidemiological studies such as this.

We assumed that the association between JEV and DENV serostatus was a product of cross-reactive antibodies, but this could also be caused by confounding by similar exposure risk to JEV and DENV. Japanese encephalitis virus and DENV are transmitted by different vector mosquitos, but behavioral or ecological risk factors such as increased outdoor exposure time or use of mosquito protective tools may predispose to risk of both [6, 34]. Well defined behavioral or ecological risk factors for these infections are poorly understood or lacking, and results of studies conducted across different geographies and time periods are seldom in agreement. In addition, although JEV infection in DENV-naive individuals provides confirmation of JEV circulation in a population with a low risk of flavivirus cross-reactivity, this may represent a specific population with less exposure to mosquito vectors, a selection bias that would underestimate true JEV transmission risk.

Limitations of this study include that subjects were not selected using a randomized or representative method and that, in the absence of virological confirmation of historical infections, it remains impossible to quantify the relative contribution of cross-reactivity in the PRNT assays. Interpretations cannot be generalized nationwide, and local experts and policymakers will need to decide the broader relevance of these findings for their countries.

CONCLUSIONS

We report a clear demonstration of JEV infection risk and human transmission in regions of 4 countries. These regions were previously considered of low JEV risk and have no JEV vaccination programs in place.

Notes

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Potential conflicts of interest. J. N., A.-F. T., A. M., M. B., D. C., and A. B. are employees of Sanofi Pasteur, a company engaged in the production of vaccines including against Japanese encephalitis and dengue viruses. S. Y., L. C. Q., M. R. C., S. R. H. and A. P. have been investigators for clinical or epidemiological studies sponsored by Sanofi Pasteur and have been remunerated accordingly. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Economic burden of dengue in Indonesia

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Data Availability Statement: The raw data were collected from hospital and health centers and are not publicly available since they are from confidential hospital medical records and billing statements. However, all data underlying the findings in the manuscript, including unit cost and denominator population sizes used to make national estimates, are included in the manuscript Tables 1,2,3

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Abstract

Background

Dengue is associated with significant economic expenditure and it is estimated that the Asia Pacific region accounts for >50% of the global cost. Indonesia has one of the world's highest dengue burdens; *Aedes aegypti* and *Aedes albopictus* are the primary and secondary vectors. In the absence of local data on disease cost, this study estimated the annual economic burden during 2015 of both hospitalized and ambulatory dengue cases in Indonesia.

Methods

Total 2015 dengue costs were calculated using both prospective and retrospective methods using data from public and private hospitals and health centres in three provinces: Yogyakarta, Bali and Jakarta. Direct costs were extracted from billing systems and claims; a patient survey captured indirect and out-of-pocket costs at discharge and 2 weeks later. Adjustments across sites based on similar clinical practices and healthcare landscapes were performed to fill gaps in cost estimates. The national burden of dengue was extrapolated from provincial data using data from the three sites and applying an empirically-derived epidemiological expansion factor.

Results

Total direct and indirect costs per dengue case assessed at Yogyakarta, Bali and Jakarta were US$791, US$1,241 and US$1,250, respectively. Total 2015 economic burden of dengue in Indonesia was estimated at US$381.15 million which comprised US$355.2 million for hospitalized and US$26.2 million for ambulatory care cases.

Conclusion

Dengue imposes a substantial economic burden for Indonesian public payers and society. Complemented with an appropriate weighting method and by accounting for local

Pasteur; authors/researchers declare full independence of the contract and were solely responsible for concept development, methods, analysis and results of the study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: the study was funded by Sanofi Pasteur; two authors are Representatives of the funding body and provided input in the discussion process on study design and data analysis. However, the final decision on analytical methods, data analysis, data interpretation and content of the manuscript were made by the corresponding and lead author. We did not involve any intervention associated with the funding authority. MN received payment from University of Indonesia under contract from Sanofi Pasteur for work related to this study. ES received payment from the University of Indonesia under contract from Sanofi Pasteur for work related to this study. SP received consultation fees from the University of Indonesia for work related to this study. JN is an employee of Sanofi Pasteur. SB is an employee of Sanofi Pasteur SRH received consultation fees from the University of Indonesia for work related to this study. VYP received consultation fees from the University of Indonesia for work related to this study. KS received consultation fees from the University of Indonesia for work related to this study. TMW received consultation fees from the University of Indonesia for work related to this study. EK received consultation fees from the University of Indonesia for work related to this study. DNW received consultation fees from the University of Indonesia for work related to this study. HT received consultation fees from the University of Indonesia for work related to this study.

specificities and practices, these data may support national level public health decision making for prevention/control of dengue in public health priority lists.

Author summary

Dengue, an infection transmitted by mosquitos, is a public health concern particularly in tropical/subtropical areas and the Asia Pacific region where it is associated with a significant cost to society. Indonesia has one of the world's highest dengue burdens but Indonesia-specific data on cost are lacking. To estimate the annual economic burden of dengue in Indonesia, this study collected data from public/private hospitals and health centres in three provinces (Yogyakarta, Bali and Jakarta) during 2015. We estimated cost of illness using the societal perspective: calculations of costs included those that were directly paid by the healthcare system, as well as costs incurred by the patients (or their family/care givers) and their lost productivity. The costs from the three provinces were then used as the basis for extrapolating cost of illness in Indonesia. The authors confirmed that dengue imposed a substantial economic burden for Indonesian public payers and society. Based on 2015 data, the authors estimated total economic burden of dengue in Indonesia at US $381.15 million. Of this, US$355.2 million related to patients treated in hospitals and US $26.2 million was for patients treated in health centres. Establishing a better understanding of the burden of dengue in Indonesia will help to guide public health decision-making at a national level and support prevention and control initiatives for this disease.

Introduction

Dengue is an arboviral infection transmitted between humans by *Aedes* mosquitoes. Globally, dengue is a major public health concern that has rapidly spread across the tropics and subtropics.[1, 2] Between 1990 and 2013 the estimated number of global dengue cases doubled every decade,[3] and up to 3.9 billion people remain at risk in endemic countries.[4] Recent global modelling studies estimate between 55–100 million dengue cases occur annually; and estimate an increasing dengue mortality reaching over 38,000 deaths in 2016.[3, 5, 6] Of the global population at risk, more than 70%–or about 1.8 billion people–live in the Asia-Pacific region and as such, Asians contribute the most to the overall burden of dengue.[1] In addition, the incidence of the severe forms of disease is higher in Asia-Pacific compared with other endemic regions perhaps for reasons of genetic susceptibility, but more likely because secondary infection is more common, due to the higher levels of endemicity and that all four dengue virus serotypes continually co-circulate.[7–10]

In Indonesia, *Ae. aegypti* and *Ae. albopictus* are the primary and secondary vectors for transmission, respectively. The average number of annual dengue cases reported to health authorities in Indonesia was more than 129,000 for the period between 2004 and 2010, the second highest incidence rate in the world after Brazil.[1] Reporting of dengue in Indonesia is acknowledged to be incomplete and reporting procedures vary widely among the provinces. [11] A 2013 cartographical modelling study estimated that approximately 7.6 million dengue infections may have occurred in in Indonesia in 2010, the majority of which went unreported. [5] The disease typically is most common in urban areas, however, rural areas are increasingly affected.[7] Furthermore, the traditionally cyclical epidemic outbreaks of dengue appear to have become more erratic in recent decades.[9]

The costs associated with dengue illness are substantial, in 2012 the WHO ranked dengue as the most important mosquito-borne viral disease across the globe, noting that outbreaks "exert a huge burden on populations, health systems and economies in most tropical countries of the world".[1] Recognizing the substantial impacts in endemic regions, several economic burden studies have been conducted in various regions of the Americas,[12–18] and several countries in Asia and South Asia including Thailand,[19] Malaysia,[20, 21] India,[22] Singapore,[23] Cambodia [24] and the Philippines.[25] These studies confirmed the considerable direct and indirect impact of dengue on individuals, families and communities.

In Indonesia, some initial insights could be derived from the study by Shepard and colleagues, who estimated the annual economic burden of dengue in 12 countries of Southeast Asia at US$950 million; for Indonesia the annual cost over the period 2001–2010 was US$323 million.[26] A subsequent estimate based on revised global dengue incidence estimates and extrapolations of costs from scientific literature estimated the costs in Indonesia in 2016 to have been US$2 billion.[27] Another study by Stahl and colleagues estimated the cost of dengue outbreaks by conducting a literature review and case studies in four countries including Indonesia.[28] The estimated costs of an Indonesia dengue outbreak in 2011 were US$6.75 million (adjusted to 2012 US$). However, these studies did not collect local empirical cost data and instead relied on estimates derived from a literature review on unit costs for inpatient and outpatient care and used extrapolations of proportionality of costs from other nearby countries.[26–28] One study conducted in Surabaya, Indonesia in 2007 examined treatment costs at the hospital level and estimated inpatient costs per episode related to dengue were in the range of 1–2 million Indonesian Rupiah (IDR) or approximately US$106–212. However, the scope of this study was limited to that single area and did not provide country-wide estimates for total healthcare costs.[29]

We are not aware of a study which has collected comprehensive primary data on the economic burden of dengue in Indonesia. Such studies are needed to inform policy making, provide information to support healthcare resource allocation including prioritizing research and disease prevention and control measures, as well as promote public awareness.[30] Due to the country's economic, geographic and sociological heterogeneity, the best way to represent national level burden and expenditure would be to use data from multiple sites and treatment facilities, taking a broad economic perspective. The aim of this study was to estimate the economic burden–including direct and indirect costs–associated with hospitalized and ambulatory dengue cases in Indonesia, first by determining costs at the facility level across three provinces, then extrapolating these using local epidemiological data to make the first, empirically-derived national economic burden of dengue estimates for Indonesia.

Methods

This study used a combination of retrospective and prospective methods and multiple data sources to estimate the direct and indirect costs of dengue in Indonesia as of 2015.

Ethical considerations

The ethics Committee of the Faculty of Public Health at Universitas Indonesia approved this study. Ethical approval for data collection at public hospitals and health centres was received from the local authorities (Dinas Kesehatan or District Health Office). Interview participants or their parents/guardians signed informed consent (signed assent forms were also required for those aged 8–18 years) before study entry.

Study sites

In Indonesia, tertiary healthcare facilities are divided into type A facilities, which provide the full spectrum of specialist medical services and type B facilities, where specialist services are limited. Both types provide basic and supportive care to both in- and out-patients. Of the 34 provinces in Indonesia, three were selected to represent low- (Yogyakarta), medium- (Bali) and high-income (Jakarta); from these three a total of nine facilities were selected for inclusion in the study. Public and private healthcare facilities were selected according to their research experience; and to provide a range of dengue and cost perspectives, including those treating inpatients and outpatients. Four facilities were selected in Jakarta: RSUPN Cipto Mangunkusumo (public type A hospital), RSUD Pasar Rebo (public type B hospital), RS Pelni (private hospital) and Tambora (Puskesmas [a sub-district level public health centre]); three facilities in Yogyakarta: RSUD Wirosaban (public type B hospital), RS Bethesda (private hospital), Puskesmas kota Yogyakarta (Puskesmas); two facilities in Bali: Sanglah Hospital (public hospital) and Puskesmas VI Denpasar (Puskesmas).

Sampling strategy

Patient records were randomly selected from a list of all age-stratified dengue diagnoses, maintained in facility diagnosis log books, in the 12 months preceding the beginning of the study. We planned to assess 50 inpatient and 50 outpatient records from each hospital (total sample from six hospitals = 600); and 50 outpatient records from each Puskesmas (total sample from three sites = 150). It was expected that the sample would comprise an equal number of children ($\leq$18 years old) and adults ($\geq$ 19 years old) due to the approximately equal distribution of dengue cases occurring in these categories. Additionally, we intended to interview 30 inpatients and 30 outpatients or their respective parents/guardians at each hospital (total sample from six hospitals = 360) and 30 outpatients or their parents/guardians from each Puskesmas (total sample from three sites = 90). Sample sizes were chosen to be operationally feasible and sufficiently large that analysis methods based on the normal distribution may be used for the analysis.

Sources of data–direct medical costs from patient records

Direct medical costs were retrospectively assessed through review of medical records and billing/charges made to patients who received treatment at selected hospitals or Puskesmas (sub-district level health centres) in the 12 months prior to the beginning of the study (April 1st 2014 until March 31st 2015) with a diagnosis of dengue or dengue haemorrhagic fever (with ICD 10 code A90 and A91).

Sources of data–direct non-medical and indirect medical costs from interviews

Direct non-medical costs and indirect costs were assessed from data collected during face-to-face interviews with patients or their parents/guardians at selected hospitals or Puskesmas. Patients with clinically diagnosed/laboratory confirmed dengue or those with evidence of fever $>$38˚C for $>$1 day, plus symptoms compatible with dengue fever were recruited to participate in two interviews. The first was a face-to-face interview with patients or their parents/guardians using a questionnaire and conducted at the health facility at discharge/during an ambulatory visit. The second interview was conducted by telephone two weeks later to determine subsequent costs of treatment and any absenteeism from work/school. Direct non-medical costs were defined to include all expenses incurred due to the treatment, such as meals,

transport, accommodation for care givers, etc. The interviews documented: the use of medical services; missed schooling; lost work productivity; out-of-pocket spending (e.g. transportation, meals, hotel/house rental, etc) and income lost due to the episode of illness. In the event that participants chose not to disclose their income and in the absence of reliable data on average wages including in the informal economy, we applied the standard minimum wages as a proxy, which are regulated in Indonesia and differ by province. Lost productivity was not calculated for children; rather, for each affected school child lost productivity was calculated for the caregiver (as a result of leaving work to care for the child).

Cost of dengue cases

Costs were expressed in US dollars (as of 2016 with a conversion rate: US$1 = IDR13,000). For those regions where a particular type of facility was not included in the study, gaps in the data were filled via weighted adjustment from neighbouring sites. For example, private outpatient costs were captured by recording treatment bills paid by the patient in Jakarta. Because private outpatient facilities were absent in Yogyakarta, these costs were estimated by adjusting Jakarta values weighted according to outpatient public costs for Jakarta and Yogyakarta. In Bali, private outpatient and inpatient costs were estimated based on the ratio observed in Yogyakarta.

Extrapolation of the dengue cost burden to the national level

Passive reporting of dengue in Indonesia is mandatory within 72 hours of diagnosis according to SEARO-WHO dengue diagnosis guidelines 2011.[31] Notification follows diagnosis by clinical and/or laboratory confirmation (by detection of NS1 antigen and/or IgM/IgG). Cases are reported to provincial health offices and pooled at the provincial and national levels by the Directorate General of CDC.[32]

Costs at the national level were estimated by multiplying cost per case (outpatient/inpatient) by an estimate of the number of cases occurring in Indonesia in 2015. National burden estimates were generated using a) provincial-level surveillance data from each of the 34 provinces; b) estimates of hospitalization rate derived from an expert consensus technique in Indonesia;[11] and c) a study which observed a magnitude of dengue under-reporting of 11.5-fold in the placebo group of a dengue vaccine clinical trial in Jakarta, Bandung and Denpasar, Bali.[33]

The expert panel that gave rise to the estimates of hospitalization rate has been described previously;[11] briefly, it comprised a group of Indonesian dengue experts (clinicians, hospital managers, epidemiologists and Ministry of Health officials) who reviewed existing data sources and made iterative estimates of epidemiological parameters by which full burden estimates could be made. These were balanced against published analyses.[3, 5, 33–37] The panel concluded that 60% of dengue cases in Indonesia were hospitalized; a figure which, when combined with an estimated reported hospitalization rate and under-reporting factor of 11.5, generated the final expansion factor for hospitalized patients (EFH; 7.65) and expansion factor for ambulatory patients (EFA; 45.90) used for calculation of the cost-of-illness. The numbers of ambulatory and hospitalized dengue cases for each province in Indonesia during 2015 were estimated by multiplying these expansion factors by the numbers of reported cases in each province.

To calculate the economic burden of dengue nationally the three sites in our study: Jakarta, Yogyakarta and Bali, were used as references for other provinces arranged into three groups according to their fiscal capacity index (FCI). Yogyakarta was the reference for low FCI province (FCI <0.5), Bali for middle FCI (0.5–2.0) and Jakarta for high FCI (>2.0) provinces. Unit outpatient and inpatient costs of each province were proportionally weighted by the consumer

price index (CPI) or Indeks Harga Konsumen (IHK) using Jakarta, Bali and Yogyakarta dengue unit costs as the baseline. By multiplying the number of ambulatory/hospitalized cases by the unit cost estimates for each province, the total economic burden in each province was calculated.[11]

Sensitivity analyses

To assess the uncertainty surrounding estimated overall dengue burden,[38] deterministic sensitivity analyses were performed to examine the effect of parameters' variations i.e. costs in each setting (inpatient, outpatient, by region) and expansion factor. Each parameter was manually varied by an arbitrary value of ±10% to examine the impact on the total economic burden. Calculations were performed using Microsoft Office Excel 2010.

Results

Data collection, recruitment and timelines

A total of 615 patient records were reviewed for the retrospective, direct medical cost calculation (262 in Jakarta, 251 in Yogyakarta and 102 in Bali) during the period from the 15th of June to the 31st of July 2015. The regional distribution of patients included, by province, inpatient/outpatient and dengue classification is shown in Table 1. A total of 199 patients were involved in the prospective phase of the study (94, 43 and 62 from each site); data were collected from interviews during the period from the 3rd of August to the 15th of September 2015. Combining both retrospective and prospective elements, the study sample was 68% of the enrolment target.

Direct and indirect medical cost for inpatient and outpatient care of dengue cases in Jakarta, Yogyakarta and Bali

The total costs (combined direct and indirect costs) per patient episode for outpatient cases were, US$103, US$252 and US$179 for Yogyakarta, Bali and Jakarta, respectively. For inpatients these costs were US$689, US$989 and US$1071 respectively. With the exception of inpatient costs in Jakarta, direct medical costs were higher from private hospitals compared with public facilities. Table 2 describes cost of illness results per episode for each site. Direct medical cost were the largest proportion of costs for inpatient care, while indirect costs were the largest proportion of costs for outpatient care. Outpatient costs in Jakarta were slightly lower than in Bali. Overall, the mean length of hospital stay was 3.9 days. By region, it was 4.4 days in Yogyakarta, 3.5 for Bali and 3.8 days in Jakarta.

Table 1. Distribution of patient records used in the retrospective analyses, by province, type of service and severity of dengue during the period from 15th of June to 31st of July 2015.

Province	Type of services	Dengue fever	Dengue haemorrhagic fever	Dengue shock syndrome	Total
Yogyakarta	Outpatient	33	15		48
	Inpatient	3	48	3	54
Bali	Outpatient	109	8		117
	Inpatient	50	82	2	134
Jakarta	Outpatient	62	56		118
	Inpatient	43	94	7	144
Total		300	303	12	615

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Table 2. Cost of illness per patient per episode (in US$) by component and site in Yogyakarta, Bali and Jakarta.

Province	Type of services	Health facility	Direct costs: medical (US$)	Direct cost: non-medical (US$)	Indirect cost (US$)	Total cost (US$)
Yogyakarta	Outpatient	Puskesmas	3.32	21.15	8.02	32.49
		Public hospital	7.33	10.00	8.02	25.35
		Private hospital	26.66	10.00	8.02	44.68
	Inpatient	Public hospital	222.89	18.31	46.16	287.35
		Private hospital	334.30	24.19	43.06	401.55
Bali	Outpatient	Puskesmas	16.73	15.14	30.64	62.51
		Public hospital	20.08	21.31	49.54	90.92
		Private hospital	28.13	21.31	49.54	98.98
	Inpatient	Public hospital	229.55	76.89	122.31	428.75
		Private hospital	344.30	101.61	114.11	560.01
Jakarta	Outpatient	Puskesmas	14.53	6.64	13.77	34.94
		Public hospital	23.41	10.47	30.32	64.19
		Private hospital	32.80	17.64	29.08	79.52
	Inpatient	Public hospital	407.07	84.33	153.79	645.19
		Private hospital	248.61	73.24	104.12	425.97

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Total economic burden due to dengue in the provinces of Indonesia

The results of the extrapolated regional costs (by CPI and FCI) are shown in Table 3. Jakarta was the province with the highest dengue-related cost, followed by Yogyakarta, West Java and West Kalimantan.

Total economic burden due to dengue in Indonesia

The annual total cost of dengue-related illness in Indonesia was estimated at US$381.5 million, with US$354,802,570 for hospitalised and US$26,249,519 for ambulatory cases (Table 3). Considering the total number of inpatient cases and costs, the average cost per dengue patient was lowest in region 1 ($346.38) and highest in region 3 (US$535.91). Similarly, average cost per outpatient was lowest in region 1 (US$34.38) and highest in region 2 (US$84.48).

Sensitivity analysis

Results from the sensitivity analyses are presented in the Tornado diagram (Fig 1), which represents baseline value (per US$ million). The parameters included in sensitivity analysis were the costs of outpatient and inpatient treatment by province; and EFA and EFH. Variation in any of these parameters resulted in overall economic burden varying from US$166–557 million. The greatest variation in the final estimate followed variation in outpatient costs in Jakarta; followed by costs in outpatient facilities in type A clinics, and in Bali.

Discussion

We estimated the average annual economic burden of dengue-related illness in Indonesia in 2015 to be US$381.5 million with more than 90% of this cost associated with hospitalized care. Jakarta was the province associated with the greatest cost, which is a function of the greater population and the higher average costs of treating hospitalized dengue episodes. In Jakarta, inpatient, direct medical costs were higher from public facilities than in private hospitals. This was thought to result from the fact that the public study sites included Ciptomangunkusumo Hospital which is a type A public hospital, a top referral hospital in Indonesia and therefore

Table 3. Extrapolated total economic burden (in US$) of dengue in Indonesian provinces by income-category (Region 1: Low income context, Region 2: Medium income context, Region 3: High-income context). Number of cases estimated by extrapolation from cases reported from passive surveillance in 2015 using expansion factors.

	Actual inpatient cases	Total inpatient cost (US$)	Actual outpatient cases	Total outpatient cost (US$)	Total economic burden (US$)
REGION 1					
Yogyakarta	139,556	48,070,196	92,635	3,165,523	51,235,719
Bengkulu	4,317	1,535,502	2,866	101,116	1,636,618
Banten	34,331	12,152,270	22,788	800,252	12,952,522
West Sumatra	26,376	9,233,246	17,508	608,029	9,841,274
North Sumatra	36,549	12,738,624	24,260	838,865	13,577,489
Jambi	23,534	8,146,013	15,622	536,432	8,682,445
West Java	113,638	39,173,980	75,431	2,579,688	41,753,669
Special Region of Aceh	10,457	3,521,684	6,941	231,910	3,753,594
South Sumatra	6,951	2,340,043	4,614	154,097	2,494,140
North Kalimantan	4,484	1,628,835	2,976	107,262	1,736,097
West Kalimantan	74,179	25,967,594	49,238	1,710,020	27,677,614
East Java	20,866	7,209,095	13,851	474,734	7,683,829
Central Java	23,701	8,186,530	15,732	539,100	8,725,631
West Nusa Tenggara	10,395	3,637,047	6,900	239,507	3,876,554
Maluku	3,368	1,177,939	2,236	77,570	1,255,509
East Nusa Tenggara	1,601	551,161	1,063	36,295	587,456
Southeast Sulawesi	10,887	3,732,207	7,227	245,774	3,977,980
North Sulawesi	10,409	3,546,774	6,909	233,562	3,780,337
West Sulawesi	4,983	1,696,603	3,307	111,725	1,808,328
South Sulawesi	5,059	1,721,320	3,358	113,353	1,834,673
Gorontalo	25,419	8,546,321	16,873	562,793	9,109,114
Central Sulawesi	44,754	15,659,385	29,707	1,031,203	16,690,588
Special Region of Papua	457	60,387	304	10,562	170,949
Lampung	12,439	4,467,725	8,257	294,209	4,761,934
Total region 1	648,710	224,700,481	430,603	14,803,581	239,604,063
REGION 2					
Bali	27,540	13,615,216	18,280	1,538,048	15,153,264
Special Region of West Papua	825	396,267	547	44,764	441,031
Riau	22,599	11,207,903	15,001	1,266,105	12,474,008
Bangka–Belitung Islands	21,795	11,038,270	14,467	1,246,942	12,285,212
Riau Islands	9,383	4,609,852	6,228	520,754	5,130,605
Central Kalimantan	8,676	4,246,122	5,759	479,665	4,725,787
South Kalimantan	3,507	1,704,769	2,328	192,580	1,897,349
North Maluku	541	272,137	359	30,742	302,879
Total region 2	94,866	47,090,536	62,969	5,319,600	52,410,135
REGION 3					
Jakarta	147,172	78,822,769	97,690	5,817,201	84,639,971
East Kalimantan	7,727	4,188,784	5,129	309,137	4,497,920
Total region 3	154,899	83,011,553	102,819	6,126,338	89,137,891
NATIONAL TOTAL	**898,475**	**354,802,570**	**596,391**	**26,249,519**	**381,152,089**

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responsible for treating the most severe cases requiring intensive, expensive, specialist care. Sensitivity analyses identified uncertainty around outpatient cost in Jakarta as the variable with the largest impact on the overall economic burden, due to the relatively higher cost of episodes in Jakarta, and their frequency. Notably, the overall estimates are directly influenced by the expansion factors used to estimate the number of cases. These numbers were derived from high-quality epidemiological studies in tandem with local expert opinion. But studies have

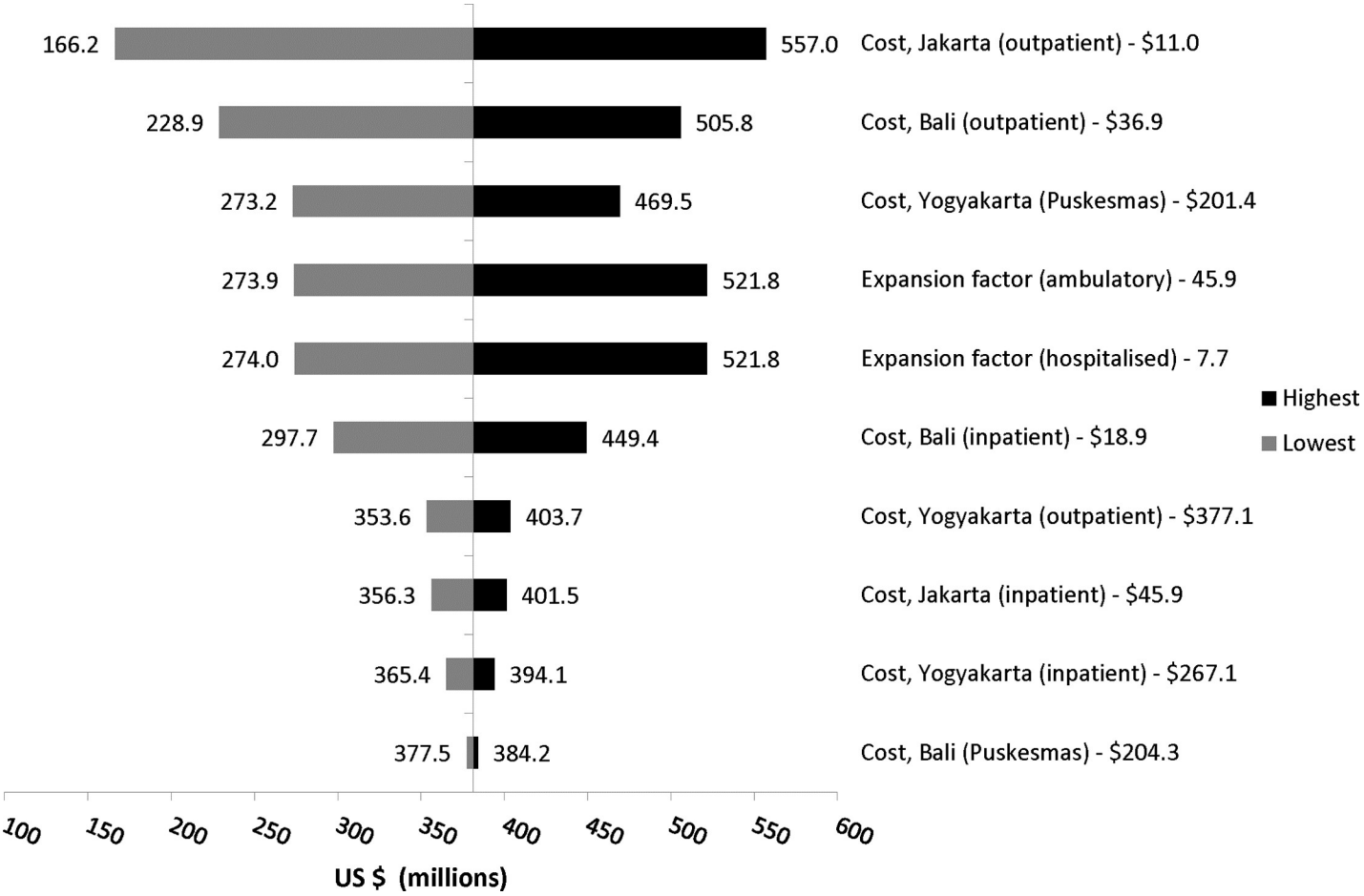

Fig 1. Tornado diagram for the deterministic sensitivity analysis of variability of the Indonesian national-level, annual cost of dengue illness in US$ million. Black represents the lowest value, grey represents the largest value. Parameters were varied by ±10% as a subjective scenario and the base case was US$381.15 million. The point estimate for each parameter is included in the label for each bar.

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shown reporting completeness can be affected by changes in disease severity, level of epidemic activity and other external factors, which could limit the generalizability of these numbers at different time points. 2015 was a fairly "typical" year in Indonesia, with the number of cases being close to the average from 2010–2016.[39, 40] Future analyses will hopefully allow for a more refined understanding of the level of dengue reporting in Indonesia.

Our estimate of the cost per episode in type B hospital was~US$150 (~IDR 2 million), which is consistent with a previous Indonesian estimate from East Java of IDR1–2 million published in 2008.[29] Our unit cost estimates are also similar to those reported in the regional analyses of Shepard in 2013 and 2016.[26, 27] Our study found that dengue is associated with considerable economic burden, which is in agreement with other studies conducted in Asian countries, especially those in Thailand and the Philippines. In Thailand, Philippines and Malaysia, total economic burdens were estimated at US$486 million (in 2005 costs),[19] US $345 million (in 2012 costs),[25] US$102.25 million (in 2009 costs),[20, 21] respectively. However, estimates in the much smaller (Singapore) and larger (India) countries were considerably higher than our estimate at more than US$1 billion in each country.[23, 41]

With regard to existing national level burden estimates for Indonesia, our results are similar to those published by Shepard and colleagues in 2013 who concluded that the annual

economic burden of dengue for Indonesia was US$323 million.[26] This was slightly lower than our 2015 estimate, caused by an increasing disease burden; and slightly higher outpatient unit costs. However, this group refined their estimates in a 2016 [27] publication using a different method of epidemiological burden estimation and concluded that the dengue burden in Indonesia was US$2 billion.[27] Costs in our study were calculated from primary data sources and clinically diagnosed dengue, including medical record review and patient interview. Unit costs were broadly similar to those estimated by Shepard and colleagues and the variation is predominantly driven by different epidemiological estimates: Shepard and colleagues' estimated >11 million annual dengue cases, while we assumed ~640,000. Such variation in dengue burden estimates are difficult to reconcile; the paper by Shepard and colleagues applied regression methods from the Global Disease Burden group; in contrast we used local surveillance data combined with expert opinion and empirical under-reporting calculation. Much of this variation likely stems from case definitions, particularly those around mild cases of dengue whose clinical and economic significance is very difficult to calculate with confidence, and whose full economic impacts are very difficult to measure. In addition, the Shepard 2016 study included estimates for non-medical cases (i.e. patients that did not seek professional medical advice but may have had laboratory testing or purchased therapeutic products outside the professional healthcare system), which we did not include in this analysis.

The strength of this study is that it is based on empirical, patient-specific data for medical care and out-patient costs in Indonesia. Furthermore, it considered both public and private hospitals and included costs derived from different treatment settings and economic backgrounds. To address limitations in the available passive surveillance data, expansion factors were used to fully describe the number of dengue cases and expert opinion employed to desegregate data into outpatient and inpatient cases. We consider this approach, underpinned by gold-standard epidemiological clinical trial data with local expert opinion to stratify cases by severity, is likely a realistic representation of the health-seeking dengue case population in Indonesia. The costs captured from the three reference provinces (Jakarta, Bali and Yogyakarta) were extrapolated to other regions based on weighted average costs linked to the consumer price index to ensure relevant estimates from other regions. Other variables such as type of hospitals (private/public, type A or B) were also taken into account in the extrapolation to get a mixed representation of healthcare setting throughout the country.

We acknowledge several limitations to our study, mostly due to the patients' clinical pathway i.e. most patients generally received outpatient services at type B hospitals, hence had an impact on type A sample size; also the number of ambulatory patients was generally lower than expected (potentially due to the local regulation at Jakarta and Yogyakarta whereby laboratory-confirmed dengue patients were referred directly to hospital). Furthermore, we did not enroll as many patients as planned and were only able to achieve 68% of the target enrolment. The primary reason for lower-than-expected enrolment was the relatively small number of dengue cases occurring in 2015, especially in Yogyakarta in which enrolment was especially challenging. Outpatient recruitment was additionally complicated by local clinical practice guidelines which advise that all dengue cases should be hospitalized. There is uncertainty in income loss calculations due to illness because most patients or their parents/guardians did not disclose their actual income during the interviews; so the national minimum wage was used as proxy. Some studies also included 'outside hospital costs', such as vector control activities, in the overall cost estimates, but this was beyond the scope/focus of our study. Lastly, our estimates are based on data from one year (2015), corresponding to the period over which primary data were collected. As a result, the estimates are subject to vary with epidemic activity.

Conclusion

The total direct costs of dengue illness in Indonesia were estimated at US$381.15 million. Our analysis provides results that are relevant to public health policymakers in Indonesia, helping to strengthen local knowledge and informing decision-making regarding the prevention and control of dengue in public health priority lists. These results can also be used in health economic studies of novel dengue prevention and control technologies or vaccine programs.

Supporting information

S1 Table. STROBE statement—Checklist of items that should be included in reports of cross-sectional studies.
(DOC)

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Feasibility of case-control and test-negative designs to evaluate dengue vaccine effectiveness in Malaysia

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ABSTRACT

Background: The world's first dengue vaccine [Dengvaxia; Sanofi Pasteur] was licensed in 2015 and others are in development. Real-world evaluations of dengue vaccines will therefore soon be needed. We assessed feasibility of case control (CC) and test-negative (TN) design studies for dengue vaccine effectiveness by measuring associations between socio-demographic risk factors, and hospitalized dengue outcomes, in Malaysia.

Methods: Following ethical approval, we conducted hospital-based dengue surveillance for one year in three referral hospitals. Suspected cases aged 9–25 years underwent dengue virological confirmation by RT-PCR and/or NS1 Ag ELISA at a central laboratory. Two age- and geography-matched hospitalized non-dengue case-controls were recruited for a traditional CC study. Suspected cases testing negative were test-negative controls. Socio-demographic, risk factor and routine laboratory data were collected. Logistic regression models were used to estimate associations between confirmed dengue and risk factors.

Results: We recruited 327 subjects; 155 were suspected of dengue. The planned sample size was not met. 124 (80%) of suspected cases were dengue-confirmed; seven were assessed as severe. Three had missing RT-PCR results; the study recruited 28 test-negative controls. Only 172 matched controls could be recruited; 90 cases were matched with ≥1 controls. Characteristics of cases and controls were mostly similar. By CC design, two variables were significant risk factors for hospitalized dengue: recent household dengue contact (OR: 54, 95% CI: 7.3–397) and recent neighbourhood insecticidal fogging (OR: 2.1; 95% CI: 1.3–3.6). In the TN design, no risk factors were identified. In comparison with gold-standard diagnostics, routine tests performed poorly.

Conclusions: The CC design may be more appropriate than the TN design for hospitalized dengue vaccine effectiveness studies. Selection bias in case control selection could be minimized by protocol changes more easily than increasing TN design control numbers, because early-stage dengue diagnosis in endemic countries is highly specific. MREC study approval: (39)KKM/NIHSEC/P16-1334.

1. Background

Dengue, a mosquito-borne flavivirus, causes around 100 million clinical episodes, and likely results in 10.5 million hospitalizations annually, mostly in Asia [1–3]. The disease has a wide and unpredictable range of clinical presentations, from mild/asymptomatic flu-like illness, progressing to acute, febrile, and severe/haemorrhagic disease and rarely, death [4,5]. Risk factors for severe outcomes may include the presence of heterologous antibodies from a previous infection, viral characteristics, and the age and genetic background of the infected human host [6]. Population-level risk

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factors include urbanization, high population density, and presence of *Aedes* mosquito vector breeding sites [7].

The world's first dengue vaccine [Dengvaxia; Sanofi Pasteur] was licensed in 2015 and has since been introduced in Asia and Latin America [8]. A number of other dengue vaccines are in clinical development and evaluations of the real-world performance of dengue vaccines will therefore soon be needed [8–10]. A workshop of international experts took place in 2014 to discuss the underlying principles; participants agreed that case-control (CC) and test-negative (TN) designs should be considered for this purpose [11].

CC studies are established methodologies for assessing associations between vaccine exposure and infectious disease outcomes including for influenza [12]; Japanese encephalitis [13]; whooping cough [14], and pneumococcal pneumonia [15]. For dengue, CC studies have been used to evaluate individual- and population-level risks factors [16,17]. The TN design is a variant of the CC study whereby suspected cases with negative laboratory results – and who are therefore considered absent of the outcome of interest – are used as controls, and has been used extensively for evaluating the effectiveness of influenza vaccines [18–21] and other vaccines [22–24]. The TN design has advantages in reducing bias in control recruitment, and has been used to understand dengue risk factors [25].

Post-licensure dengue vaccine effectiveness studies have not been published and, given the clinical and epidemiological specificities of dengue, challenges may be expected which warrant preparatory study [26]. These include the ability to recruit dengue patients satisfying relevant case definitions; laboratory capacity to confirm infection with adequate specificity and sensitivity; and the practical infrastructure to identify and recruit suitable control subjects.

In Malaysia, dengue outbreaks occur nationwide with increased risk in urban and *peri*-urban areas. Peaks in transmission often coincide with rainfall but cases occur year-round and reported cases have doubled since 2010 [27,28]. Although cases showed some reduction in 2016, the disease was highlighted in the Eleventh Malaysia plan of 2016–2020 to expand health promotion programmes for communicable diseases, which aims primarily to mitigate dengue risk [29]. Dengvaxia was granted a two-year conditional registration in October 2016 by the Drug Control Authority of Malaysia for post-registration study, with conditions to monitor long term risks, safety and efficacy over a wider population [30].

To prepare for vaccine introduction, we assessed the feasibility of conducting traditional CC and TN studies for dengue vaccine effectiveness evaluation, in Malaysia, by measuring associations between socio-demographic and environmental risk factors and dengue outcomes. We considered hospitalized/severe dengue as policy-relevant and specific endpoints, so we conducted hospital-based dengue surveillance for a period of one year, matching cases to control subjects who were hospitalized for a non-dengue condition. We assessed feasibility of recruitment, logistics, and laboratory confirmation as well as likely biases and potential remedies to minimize them to improve the design of future dengue vaccine effectiveness studies.

2. Methods

2.1. Ethical approval for study

This study was conducted in accordance with the Declaration of Helsinki [31], the Guidelines for Good Epidemiology Practices [32] and local regulatory requirements. Before subjects were enrolled the protocol and study documents were approved by the Medical Research and Ethics Committee of the Ministry of Health, Malaysia (study approval: (39)KKM/NIHSEC/P16-1334). Institutional approval was obtained from each Hospital Director and relevant Head of Department before data collection commenced.

2.2. Study design

Prospective, hospital-based enhanced surveillance. Suspected dengue cases who were laboratory-confirmed were enrolled as cases and matched to two hospitalized non-dengue, age- and geography-matched controls, to conduct the traditional CC study. Laboratory-confirmed dengue cases were considered test-positive cases for the TN study; suspected cases testing negative were considered as the TN controls.

2.3. Study sites and population

Surveillance for suspected dengue cases starting from October 2016, over a period of 12 months, among hospitalized patients at three Malaysian study sites: (1) Raja Permaisuri Bainun Hospital, Ipoh, Perak; (2) Selayang Hospital, Selangor; and (3) Sungai Buloh Hospital, Selangor. Study sites are large, tertiary care hospitals operating within the Malaysian Ministry of Health system. Two (Selayang and Sungai Buloh Hospitals) are located within large urban areas and one (Ipoh Hospital) is located in a smaller, more *peri*-urban city. The hospitals accept referrals from health districts within their catchment areas, ranging from 16 km to 76 km. Nonetheless, patients living outside of the catchment areas (in regional and rural areas) may be referred for tertiary care services. An estimated 1000–2000 febrile cases are seen in these hospitals each month. All hospitals are centres of excellence for dengue, treating several thousand hospitalized dengue cases, annually.

Study subjects were classified according to the following case definitions:

- Suspected dengue: patients on whom the attending clinician makes a diagnosis of probable dengue according to clinical history, physical examination and results of routine diagnostic tests which may have been used.
- Virologically-confirmed dengue (VCD): suspected dengue cases that are virologically confirmed by the central laboratory by dengue RT-PCR and/or NS1 antigen (Ag) ELISA.
- Severe dengue: a patient presenting with fever of 2–7 days plus any of the following: severe plasma leakage, severe haemorrhage or severe organ impairment, as derived from raw clinical data, based on WHO 2009 definitions [4].
- Case-controls: non-dengue patients, age- and geographically-matched to VCD cases.
- TN controls: suspected dengue cases who tested negative for dengue by both RT-PCR and NS1 Ag.

2.4. Inclusion criteria for cases and controls

Inclusion criteria for suspected dengue were: age 9–25 years; acutely ill and suspected of dengue infection; admitted to the study hospital within 5 days of fever onset; resident of the hospital catchment area. Due to low case enrolment, a protocol amendment was approved on 31st July 2017, extending the recruitment window to within 7 days of fever onset.

For each laboratory-confirmed dengue case, study teams attempted to identify two hospitalized, matched case-controls. Inclusion criteria were: hospitalized in the same hospital as cases; with no suspicion of dengue infection; with a final diagnosis other than dengue; admission within one month (before or after) of the laboratory confirmation of the case. The last control subject was enrolled on 3rd December 2017. Controls were age-matched to cases in three age groups: 9–12 years; 13–17 years; and 18–25 years; and geographically-matched based on the catchment areas of district health offices (*Pejabat Kesihatan Daerah*).

2.5. Subject screening and enrolment

Screening from medical and paediatric wards was performed during weekdays and within working hours by the study coordinators. Eligibility was assessed based on clinical history, physical examination and following discussions with attending physicians. Typically in Malaysia, individuals are suspected of dengue based on clinical signs and symptoms and, at these referral centres, it is likely that most subjects already received either IgM/IgG and/or NS1 Ag rapid diagnostic tests (RDT) and/or a previous clinical diagnosis of dengue at primary care clinics or hospital emergency departments. Children suspected of dengue are typically admitted, whereas adults will be hospitalized following a poor or worsening clinical condition.

Suspected dengue cases were screened by study coordinators and principal investigators for other inclusion criteria before being invited to join the study. Informed consent and assent forms, available in English, Malay, Tamil, and Chinese languages, were reviewed and signed by subjects and parents of subjects aged <18 years. Subjects' identification cards (18 years and above) and birth certificates (below 18 years old) were collected to verify legal relationships, as required by the Medical Research and Ethics Committee.

2.6. Data collection and laboratory analysis

Following enrolment, a standardized questionnaire was administered by study staff which collected socio-demographic information, reported dengue histories of subjects and household contacts, other risk factor data (e.g., household and neighbourhood vector control practices; time spent outdoors) and flavivirus vaccination history. Final discharge diagnoses, made by attending physicians based on routine clinical practice, were retrieved from electronic medical records upon discharge, verified by the investigator and recorded.

For suspected cases, during routine blood sampling in the wards, an additional aliquot of 5 mL venous blood was collected. Blood was kept at room temperature for 30–60 min (or refrigerated at 2–8 °C for $\leq$24 h) before centrifugation. Serum was transferred into two 650 μL aliquots, frozen at −20 °C and shipped in dry ice to the central laboratory, the Department of Medical Microbiology, University of Malaya Medical Center in Kuala Lumpur. Virological confirmation of dengue was by RT-PCR and NS1 Ag ELISA. RNA extraction was performed using Roche High Pure viral RNA extraction kit; RNA purity and concentration were assessed by spectrophotometry. One step real-time Sybr Green RT-PCR was performed using Bio-rad iTaq universal one step Sybr Green premix and in-house designed primers [33]. The SD Dengue NS1 ELISA kit was used according to the manufacturer's instruction.

The results of routinely-performed dengue diagnostic testing, which could include RDTs and ELISAs detecting IgM, IgG and NS1 Ag, before or during hospitalization, were recorded.

All data were entered into an electronic database by study teams and verified through computerized logic and consistency checks to detect errors or omissions.

2.7. Sample size

In the context of vaccine effectiveness study preparation the sample size was based on a hypothetical effectiveness objective comparing the odds ratio (OR) of having a virologically confirmed, hospitalized dengue episode between vaccinees and non-vaccinees, assuming a power of 80%, a two-sided alpha of 5%, vaccine coverage of 50%, and an expected vaccine effectiveness of 50%. Assuming 70% of suspected cases test positive, the TN design would require 223 cases and 96 TN controls. A CC design would require 88 cases and 352 controls (with a case:control ratio of 1:4) or 110 cases and 220 controls (with a 1:2 ratio). Expecting a minimum of 20% non-evaluable cases, a target of 300 confirmed cases (meaning 400 suspected cases) and 600 controls was planned. Targets were provided for each site to enrol equal numbers, stratified into age categories, resulting in a total of 100 suspected cases aged 9–12 years, 100 aged 13–17 years, and 200 aged 18–25 years.

2.8. Statistical analysis

We compared socio-demographic characteristics of VCD cases and controls enrolled for both designs. Univariate logistic regression models were used to estimate associations between confirmed dengue and risk factors using the CC (in which only subjects with at least 1 matched control was included, by conditional logistic regression) and TN study designs. Variables with a P-value <0.2 on univariate analysis were included in a final multivariable model and were backward-selected to retain in the model at a P-value of <0.05.

Dengue discharge diagnoses were compared with WHO 2009 case definitions, including severity assessment, as derived from subjects' clinical data [4]. The sensitivity and specificity of each diagnostic test used in routine practice were calculated using RT-PCR and/or NS1 Ag ELISA positive test results as the reference standard, with confidence intervals computed using the normal approximation method.

All statistical analyses were performed with SAS 9.4 using Enterprise Guide 5.1 software or later.

3. Results

3.1. Characteristics of study subjects

Fig. 1 is a study flow chart. The study recruited 327 subjects; the mean age was 18 (SD 4.2) years for VCD cases, 18 (SD 3.7) for TN controls, and 19 (SD 4.3) for case-controls. There were 155 subjects suspected of dengue within 5 days of fever of whom 18 were aged 9–12 years; 48 were aged 13–17 years, and 89 were aged 18–25 years. The planned sample size was therefore not met in any age group. Many suspected cases were ineligible to participate because they were not aged 9–25 years old; had experienced onset of fever >5 days previously; parents were unavailable to provide informed consent and/or birth certificates. Following protocol amendment, ten suspected dengue cases were enrolled, admitted between 5 and 7 days of fever, two of whom were VCD. Due to the low impact on overall results, these subjects were not considered in further analyses. Table 1 summarises the socio-demographic characteristics of study subjects. Of the 155 suspected dengue cases, 124 (80%) were VCD. Three subjects had missing RT-PCR results and the study therefore recruited 28 TN controls. To match 124 confirmed dengue cases in a 1:2 ratio, 248 controls were required. A total of 172 matched controls were recruited and some cases therefore lacked controls: 90 cases were matched with 1 or 2 controls. Time between case and matched control recruitment was on average 74 days.

3.2. Dengue risk factors – univariate analysis

The characteristics of cases and controls were similar in terms of most baseline clinical characteristics, individual dengue history and educational and socio-demographic dengue risk factors (Table 1; complete table of risk-factors in supplementary Table S1). Differences were observed in the sex distribution: of VCD cases, 43 (34.7%) were female in comparison with 98

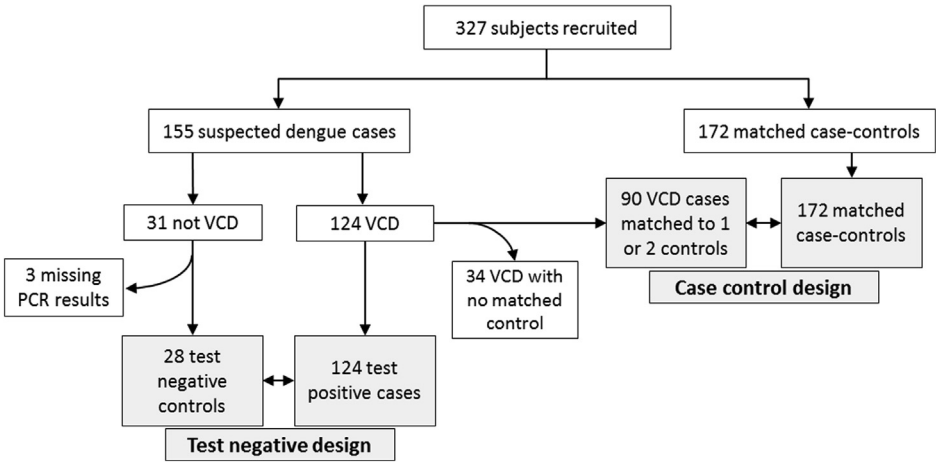

Fig. 1. Study flow chart. VCD = virologically confirmed dengue.

Table 1
Numbers (%; SD for Mean age) of subjects with different socio-demographic characteristics and risk factors recruited as VCD cases and case- or test-negative controls. P-values are in bold font with ORs below, vs the reference category.

	Case-control design			**Test-negative design**		
	VCD cases[^]	**Case-controls**	**P-value** OR (95% CI)	**VCD cases**	**Test-negative controls**	**P-value** OR (95% CI)
N	90	172		124	28	
Mean age, years (SD)	18 (4.3)	19 (4.3)		18 (4.2)	18 (3.7)	
Sex			**0.003**			**0.181**
M	57 (63)	74 (43)	Ref	81 (65)	22 (79)	Ref
F	33 (37)	98 (57)	0.4 (0.3; 0.8)	43 (35)	6 (21)	1.9 (0.7; 5.2)
Site[#]			–			**0.1697**[*]
Ipoh, Perak	31 (34)	59 (34)	–	45 (36)	5 (18)	Ref
Selayang, Selangor	32 (36)	62 (36)	–	44 (36)	14 (50)	0.3 (0.1; 1.1)
Sungai Buloh, Selangor	27 (30)	51 (30)	–	35 (28)	9 (32)	0.4 (0.1; 1.4)
Education level			**0.270**			**0.110**[*]
No formal or primary	5 (5.6)	14 (8.1)	Ref	10 (8)	2 (7.1)	Ref
Secondary	58 (64.4)	114 (66)	4.4 (0.5; 37)	76 (61)	23 (82)	0.7 (0.1; 3.2)
Tertiary	27 (30.0)	44 (26)	5.6 (0.6; 50)	38 (31)	3 (101)	2.5 (0.4; 17)
Type of dwelling			**0.135**[*]			**0.589**
Individual house	58 (64)	127 (74)	Ref	82 (66)	17 (61)	Ref
Apartment/flat/others	32 (36)	45 (26)	1.5 (0.9; 2.6)	42 (34)	11 (39)	0.8 (0.3; 1.8)
Number of family members in household			**0.596**			**0.224**
≤3	11 (12)	31 (18)	Ref	14 (11)	7 (25.0)	Ref
4–5	32 (36)	54 (31)	1.8 (0.8; 4.2)	44 (36)	11 (39)	2 (0.7; 6.1)
6–7	31 (34)	59 (34)	1.6 (0.7; 3.7)	45 (36)	7 (25)	3.2 (1; 10.7)
≥8	16 (18)	28 (16)	1.7 (0.7; 4.3)	21 (17)	3 (10.7)	3.5 (0.8; 16)
Household member diagnosed with dengue within the past month?			**<0.001**[*,z]			**0.630**
No	62 (69)	171 (99)	Ref	92 (74)	22 (79)	Ref
Yes	28 (31)	1 (0.6)	54 (7.3397)	32 (26)	6 (21)	1.3 (0.5; 3.4)
Subject previously diagnosed with dengue?			**0.589**			**0.202**
Yes	12 (13)	19 (11)	Ref	15 (12)	6 (21)	Ref
No	78 (87)	153 (89)	0.8 (0.4; 1.8)	109 (88)	22 (79)	2 (0.7; 5.7)
Average time spent outdoors, daily (hours)			**0.213**			**0.280**
<4 h	17 (19)	19 (11)	Ref	23 (19)	3 (11)	Ref
4 h ≤ time < 8 h	57 (63)	122 (71)	0.5 (0.2; 1.1)	77 (62)	16 (57)	0.6 (0.2–2.3)
≥8 h	16 (18)	31 (18)	0.6 (0.2–1.4)	24 (19)	9 (32)	0.3 (0.1–1.4)
Insecticidal fogging in neighbourhood in the past month?			**0.0049**[*,z]			**0.174**[*]
No	39 (43)	108 (63)	Ref	55 (45)	16 (59)	Ref
Yes	51 (57)	64 (37)	2.1 (1.3–3.6)	68 (55)	11 (41)	1.8 (0.8–4.2)

[#] Study site not included in CC design because controls were matched to cases based on site.
[^] Includes only cases with ≥1 matched control.
[*] Variables included in multivariate model.
[z] Variables retained in final multivariate model.
Ref = reference category.
Columns totals may vary due to lack of responses; or not equal 100% due to rounding.

(57.0%) case-controls and 6 (21.4%) TN controls. There were also differences in reported recent dengue history in the household (32 [25.8%] cases; 6 [21.4%] TN controls and one [0.6%] case control) and reports of recent neighbourhood fogging (68 [55.3%] VCD; 11 [40.7%] TN controls and 64 [37.2%] case-controls). Previous flavivirus vaccination was rare: only one subject reported having received a yellow fever vaccine.

3.3. Utility of case-control and test-negative design for risk factor identification

In the CC study, only the 90 VCD cases with at least one matched control were included in the analysis. Two variables remained significant in the final model: respondents who reported a recent household dengue contact (OR: 54; 95% CI: 7.3–397; P < 0.001) and those reporting neighbourhood insecticidal fogging in the last month (OR: 2.1; 95% CI: 1.3–3.6; P = 0.005) were associated with an increased risk of hospitalized dengue as compared to subjects without household dengue contacts or neighbouring fogging. In the TN analysis, no risk factors were identified. This might be partially a result of the number of controls (n = 28), resulting in imprecise estimates. No risk factors associated with severe VCD could be calculated as the number of severe dengue cases was too small (n = 7).

3.4. Dengue severity

Of the 124 hospitalized VCD cases, according to discharge diagnoses 69 (55.6%) were dengue fever (clinically/serologically diagnosed); one (0.8%) was dengue fever (virologically confirmed); 53 (42.7%) were dengue with warning signs and one (0.8%) was severe dengue. According to WHO 2009 criteria, classified from clinical data, seven (5.6%) were severe, and 117 were non-severe. The seven cases classified as severe presented with severe bleeding, mainly epistaxis and gum bleeding, either at admission (4 subjects) and/or during the hospitalization (3 subjects). The one case who was additionally diagnosed as severe also presented with severe plasma leakage. No severe organ impairment was observed.

3.5. Routine laboratory diagnosis of dengue

The most commonly-used dengue confirmatory test in routine practice was the NS1 Ag RDT, in 148 (95.5%) of 155 suspected cases, followed by the IgM RDT (110; 71.0%) and the IgG RDT (109; 70.3%). The IgM ELISA, IgG ELISA and NS1 ELISA were used in 45 (29.0%), 31 (20.0%) and 2 (1.3%) subjects, respectively. The NS1 Ag RDT correctly identified 108 of the 118 VCD cases on which the test was used, a sensitivity of 91.5% (95% CI 86.5–96.6%). However, 14 of 27 negative samples were incorrectly classified as positive, giving a specificity of 48.1% (29.3–67.0%). IgM rapid tests correctly identified 8 out of 87 VCD cases, a sensitivity of 9.2% (3.1–15.3%) and specificity of 81.0% (64.2–97.7%; correctly identifying 17 of 21 negative cases). The IgM ELISA had a sensitivity of 47.2% (30.9–63.2%; 17/36 VCD cases positive) and specificity of 25.0% (0–55.0%; 2/8 negative cases correctly identified). The NS1 Ag ELISA misclassified both VCD cases on which it was used as dengue negative (Table 2).

4. Discussion

We aimed to assess feasibility in recruitment, logistics and laboratory confirmation of a traditional CC or TN design to evaluate dengue vaccine effectiveness in Malaysia. The study also aimed to assess biases, stemming primarily from the methods of control recruitment and misclassification of disease and vaccine status [34]. We considered that such an assessment was needed because many of these aspects depend on the characteristics of specific pathogens and healthcare systems and will therefore be different for dengue than for other vaccine-preventable disease studies in the past [26]. Primarily due to low levels of TN design control recruitment and selection bias resulting in unbalanced case and control populations in the CC study, it is likely that protocol changes would be required before embarking on a hospitalized dengue effectiveness evaluation. Selection bias in case control selection could potentially be minimized whereas low recruitment of TN design controls will likely persist in current healthcare settings where dengue diagnoses prior to hospitalization are specific. Key challenges and possible solutions are provided in Table 3.

4.1. Identified dengue risk factors

The exposures under assessment were a selection of sociodemographic and behavioural risk factors which were generally well-matched between cases and controls, and were therefore not identified as risk factors in multivariable models. Two risk factors were identified with the CC method: living with household members recently diagnosed with dengue (OR: 54), and neighbourhood insecticidal fogging conducted in the last month (OR: 2.1). Biologically plausible explanations could explain these findings: case-contacts may be more likely than other individuals to become infected with dengue due to geographical clustering of cases; [35] and it may be reasonable to suggest that insecticidal fogging is directed towards outbreak-prone areas. Alternatively, recall or reporting bias may be responsible: perhaps hospitalized

Table 2
Results (number of subjects) of diagnostic tests used in routine practice and confirmed VCD using the gold standard of PCR and/or NS1 ELISA; and resulting sensitivity and specificities. RDT = rapid diagnostic test. ND = not done.

Test	Result	VCD Positive	Negative	Sensitivity, %	Specificity, %
NS1 Ag, RDT	Positive	108	14	91.5 (86.5; 96.6)	48.1 (29.3; 67.0)
	Negative	10	13		
	ND	6	1		
IgM, RDT	Positive	8	4	9.2 (3.1; 15.3)	81 (64.2; 97.7)
	Negative	79	17		
	ND	37	7		
NS1 Ag, ELISA	Positive	0	0	–	–
	Negative	2	0		
	ND	122	28		
IgM, ELISA	Positive	17	6	47.2 (30.9; 63.5)	25 (0; 55)
	Negative	19	2		
	ND	88	20		

Table 3
Requirements for a dengue vaccine effectiveness study; challenges encountered and potential remedies.

Study requirement	Challenge encountered	Potential remedies
Sufficient sample size and characteristics of cases	Few hospitalized suspected dengue cases	- Increase number and/or range of study sites (e.g., include emergency department) - Assess and improve enrolment mechanisms - Assess local ethics administrative requirements and incorporate mechanisms to ease enrolment
	Few severe dengue cases	- Recruit retrospectively using stored serum samples and/or medical records - Assess and improve enrolment mechanisms
Sufficient number of case-controls and test-negative controls	Few case-controls recruited	- Consider community-based control recruitment (family members; neighbours; etc.) - Assess logistics of hospital-based recruitment during site selection - Relax matching criteria based on expected exposure status
	Few test-negative controls recruited due to high confirmation rates in suspected cases	- Enrol suspected cases prior to use of rapid tests - Recruit from primary health centres or otherwise earlier in the patient pathway - Recruit TN controls separately from routine clinical practice with a follow-up to assess severity/hospitalization
Exposure history (e.g., exposure to risk factors under study) of controls representative of source population of cases	Duration between case and control recruitment may introduce bias in exposure (during a vaccination campaign; or if vaccination increases during an outbreak)	- Enrol controls immediately after identification of suspected cases - Consider community-based control recruitment (family members; neighbours; etc.) - Improve laboratory test turnaround time
	Females over-represented as controls in CC design which could bias results if vaccination rates are unequal	- Match controls on sex - Recruit from alternative hospital wards
Controls have similar outcome risk (e.g., reporting to study site with hospitalized dengue) as cases	Severity of conditions suffered by case-controls may have differed from hospitalized dengue	- Assess impact of using different control populations - Make changes to study enabling test-negative design after assessing misclassification bias arising from imperfect confirmatory diagnostics

dengue cases preferentially recall dengue episodes in household contacts; are more likely to report fevers and thus become hospitalized because of recent dengue cases at home; and recall vector control activities having been conducted in their communities more readily than non-dengue controls. Reported rates of household dengue/recent fogging were higher in TN than case-controls, providing evidence for reporting bias.

4.2. Recruitment challenges

We recruited a lower-than-expected number of suspected dengue cases and controls. This is partially associated with epidemiology: Malaysia reported ~20,000 fewer dengue cases in 2017 than in preceding years [36]. But even those hospitalized dengue cases were often ineligible for study inclusion, for a number of interrelated reasons associated with local care-seeking and hospitalization practices. Some suspected cases were monitored as outpatients within the emergency department but were never admitted; others were admitted after >5 days of fever due to late care-seeking or hospital referral; and a proportion of subjects and/or their parents declined to participate in the study. Local ethical committee regulations stating that parents must provide birth certificates at study enrolment were particularly challenging to satisfy. Scheduled laboratory operating hours resulted in loss of potential cases, particularly on Friday afternoons or weekends when clinical samples could not be processed. To remedy this, we relaxed inclusion criteria, enrolling subjects with onset of fever $\leq$7 days. This change is not aligned with WHO guidance on dengue confirmation; [4] it was included for exploratory purposes only, and yielded few additional cases during the short period in which it was implemented.

For each VCD case we also failed to recruit two matched case-controls. Our study enrolled adolescents, teenagers and young adults, a healthy demographic unlikely to be hospitalized in Malaysia. Additionally, the logistics of identifying suitable controls within large, complex hospitals was challenging, resulting in over-sampling from some wards in which eligible controls were likely to be found (e.g., gynaecology/orthopaedic surgery). Perhaps the age- and geographical matching used here should be relaxed in the future; or alternative methods of control selection, including recruiting community-based controls, could be considered. Such an approach may facilitate age-matching but would be labour-intensive for study teams. Because virological confirmation rates were high and also to reduce potential bias, it may be beneficial to recruit controls immediately following suspected case enrolment to better-match on exposure risk which may vary over time.

For the TN study, recruitment of controls was low because a higher-than expected (80% vs. 70%) proportion of suspected cases was VCD. This may be due to clinical expertise and familiarity with dengue in Malaysia and/or frequent use of RDTs in Malaysian clinics and emergency departments, and subsequent decisions to admit based on their results. Indeed, 95.2% of VCD cases had received an NS1 Ag RDT as part of their routine care; and 87% of VCD cases had a positive NS1 Ag RDT result. The frequency of pre-admission testing and subsequent hospitalization are likely influenced by epidemic activity, availability of RDTs at health facilities and hospital congestion, effects which have been shown to introduce bias to TN studies of influenza vaccines.[37] The proportion of suspected cases testing negative is also likely to vary across time and study setting, requiring conservative sample size estimates in future studies. Probably, a TN study would only be efficient if a higher proportion of suspected cases tested negative, perhaps by using a less specific case definition, and/or enrolment at an earlier stage of the treatment pathway and before full clinical assessment, for example in the clinic before RDTs are used, with a follow-up to assess severity and hospitalization at a later timepoint. This approach would be less specific and require a larger sample to capture the same number of outcomes.

4.3. Impact of disease severity and routine clinical practice

The efficacy of dengue vaccination varies according to disease severity, and vaccination has been shown to increase the risk of hospitalized dengue in seronegative vaccine recipients [8,38]. It is therefore likely that effectiveness studies should capture severe disease outcomes and we considered this an indicator of study

feasibility. Here, only seven cases had symptoms of severe dengue. This may be associated with changing dengue epidemiology in Malaysia, the cyclical nature of outbreaks or, perhaps more likely, due to challenges in recruiting subjects from intensive care units or who are otherwise clinically severe. This represents an important study bias, confining analysis to milder cases and prohibiting effectiveness estimation against severe outcomes which may be of particular relevance for policymakers and in whom vaccine performance may differ. A study design should consider this bias – perhaps by retrospective testing of stored biological specimens after recovery or death of severe cases, for example, or by designing streamlined methods of enrolment of severe patients.

Rates of confirmatory diagnostics used in routine clinical practice were variable and of inadequate sensitivity/specificity to conclude on infection status. This was most concerning for the NS1 Ag RDT which is most-commonly used in Malaysia and displayed specificity much lower than reported elsewhere (many false-positive results) [39]. This low specificity could be caused by false-negative results in the reference assays but we have no evidence of operational failings in sampling, specimen collection and shipment. RT-PCR is considered the gold-standard. This study was not designed specifically to assess diagnostic test performance and subjects are not representative of the full spectrum of suspected dengue cases in Malaysia. Nonetheless, the observation deserves additional investigation, for example via clinical assessment of discordant cases; or programmatic evaluation of RDTs in the field.

4.4. Potential biases identified

CC studies are vulnerable to a number of biases, most notably due to challenges in control selection [34,40]. Our approach was to use hospitalized controls, matched to cases and recruited within a similar time window. Hospitalized dengue is a rare outcome and in this scenario, resulting ORs approximate the rate/risk ratio [40]. To minimize bias, controls should represent the population at risk; and should be selected independent of the exposure of interest [41]. Important biases may therefore arise if family dengue history or community fogging – rates of which were elevated in cases over controls – led to the decision to vaccinate. In such a scenario the case-control population would have lower vaccination exposure rates than cases, under-estimating the protective effects of vaccination. Consideration of this and other related biases deserves further assessment when patterns of dengue vaccine distribution after launch are better-understood, including by verifying the accuracy of patient-reported data with family members or public health authorities to limit recall bias. We similarly observed gender differences between cases and controls, perhaps caused by the wards used for control identification. This may constitute a bias because the sex-distribution of dengue in Malaysia is not equal [27]. Matching controls to cases based on sex may be advisable in the future.

We considered virological, rather than serological confirmation essential to avoid misclassifying vaccinated controls as cases due to false-positive serological test results [42]. However we cannot exclude misclassification of cases as non-cases due to lack of sensitivity of PCR/NS1 Ag ELISA, which we considered the gold-standard assays. We also only enrolled subjects reporting $\leq$5 days' fever in whom viremia and NS1 Ag circulation is most likely [4]. Modelling experiments indicate that misclassification in outcome can constitute a significant source of bias, particularly in TN studies and under relatively extreme diagnostic test sensitivity/specificity scenarios, depending on vaccination coverage rate and other parameters. It may therefore be prudent in future, if practical, to minimize this bias by restricting TN control enrolment to those with confirmed alternative discharge diagnoses. We have no data on exposure misclassification because dengue vaccination is not practiced in Malaysia, but we expect recollection of dengue vaccination history to be good and the potential bias to be modest.

4.5. Limitations

This study was conducted in three sites in Malaysia over only one year. Results should be generalized only in the context of local epidemiology and treatment practices. Lower-than expected recruitment led to frozen samples being stored for $\leq$6 weeks for shipment but we do not anticipate an adverse impact on results. Our difficulty in recruiting matched hospitalised controls resulted in low statistical power which may have prevented identification of risk factors, an effect difficult to describe because strong socio-demographic risk factors for hospitalized dengue are unknown. Practical limitations also led to lower-than-possible recruitment.

5. Conclusions

It is likely the TN design would not be efficient for a dengue vaccine effectiveness study in Malaysia unless a less-specific endpoint were used to recruit subjects, enabling recruitment of higher numbers of TN controls. The CC method, with adjustments to methods of control recruitment, may be feasible: we recruited 124 confirmed dengue cases, an approximate minimum sample size. However, there is a risk of significant bias and a full bias assessment after vaccination patterns are better-understood would be needed. Case-based methods with retrospective ascertainment of vaccination status have limitations. The feasibility of population-based/community evaluation methods should be explored to assess VE according to serostatus prior to vaccination; and measure herd immunity.

Declaration of Competing Interest

- JN, CR, RLO, AM are employed by and own stocks in Sanofi Pasteur, a company producing a dengue vaccine.
- BJC has received honoraria from Sanofi Pasteur and Roche.
- JPN has been involved with an Industry Sponsored Research with Sanofi Pasteur.
- SJ, WYL, SLL, SK, JPN, AR and Amar-Singh declare no conflict of interest.

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Contributions

RLO led development of the study protocol with inputs from SJ, WYL, SLL, AM, JN and AS.

SK, JPN and AR were responsible for data collection, site management and study management.

CR, SJ, WYL were responsible for study management.
SDS was responsible for laboratory analyses and interpretation.
AM was responsible for the statistical analysis.
JN, SK, JPN and AR coordinated writing of the research report.
JN drafted the manuscript outline with support from SK.
All authors revised the article and approved the final publication and are accountable for the final paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.083.

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RESEARCH ARTICLE Open Access

Estimated dengue force of infection and burden of primary infections among Indian children

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Abstract

Background: Comprehensive, age-stratified dengue surveillance data are unavailable from India and many more dengue cases occur than are reported. Additional information on dengue transmission dynamics can inform understanding of disease endemicity and infection risk.

Methods: Using age-stratified dengue IgG seroprevalence data from 2556 Indian children aged 5–10 years, we estimated annual force of infection (FOI) at each of 6 sites using a binomial regression model. We estimated the ages by which 50 and 70% of children were first infected; and predicted seroprevalence in children aged 1–10 years assuming constant force-of-infection. Applying these infection rates to national census data, we then calculated the number of primary dengue infections occurring, annually, in Indian children.

Results: Annual force-of-infection at all sites combined was 11.9% (95% CI 8.8–16.2), varying across sites from 3.5% (95% CI 2.8–4.4) to 21.2% (95% CI 18.4–24.5). Overall, 50 and 70% of children were infected by 5.8 (95% CI 4.3–7.9) and 10.1 (95% CI 7.4–13.7) years respectively. In all sites except Kalyani, > 70% of children had been infected before their 11th birthday, and goodness-of-fit statistics indicated a relatively constant force-of-infection over time except at two sites (Wardha and Hyderabad). Nationwide, we estimated 17,013,527 children (95% CI: 14,518,438- 19,218, 733), equivalent to 6.5% of children aged < 11 years, experience their first infection annually.

Conclusions: Dengue force-of-infection in India is comparable to other highly endemic countries. Significant variation across sites exists, likely reflecting local epidemiological variation. The number of annual primary infections is indicative of a significant, under-reported burden of secondary infections and symptomatic episodes.

Trial registration: Registered retrospectively with clinicaltrials.gov (NCT01477671; 18/11/2011) and clinical trials registry of India (ctri.nic.in; CTRI/2011/12/002243; 15/12/2011). Date of enrollment of 1st subject: 22/9/2011.

Keywords: Dengue, Endemic diseases, Flavivirus, India, Infection, Seroepidemiologic studies

Background

Dengue has become hyperendemic in many parts of India [1, 2]. The disease is being reported from an increasing number of states, and the number of cases reported to the National Vector Borne Disease Control Program (NVBDCP) has been increasing over recent years. In 2010, the incidence of reported dengue was 2.3 cases per 100,000 individuals, increasing to 11.7 per 100,000 in 2017 [3]. In 2016, for the first time, more than 100,000 cases were reported (total: 129, 166 with 245 deaths). However, reported cases represent only the tip of the iceberg, and the true disease burden is likely significantly higher [4]. Mild cases are particularly susceptible to under-reporting [5]. Notably, a global cartographic modeling study by Bhatt et al. provided comprehensive global dengue burden estimates, and projected > 32 million cases in India in 2010 [6]. A complementary study by Stanaway and colleagues from the Institute for Health Metrics and Evaluation, using verbal autopsy, vital registration and surveillance data estimated 18.6 million cases in 2013 [7]. A local estimate

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focusing on the city of Chennai (population of 4.7 million) used seroprevalence data to estimate 89,700 new infections and 138,100 secondary infections every year [8]. This distinction is important because dengue has four serotypes; and second infections are more commonly severe [2].

In the absence of incidence data including cases which were not recognized as dengue and those who did not access healthcare, seroprevalence data provide an alternative indicator of transmission intensity. [9] Seroprevalence describes historical infection and, when derived with standardized diagnostics, is a relatively unbiased indicator of viral exposure when compared with surveillance data. Age-stratified surveys provide data from which one can derive force of infection (FOI) estimates and therefore understand the infection rate [10, 11]. Understanding endemicity is important for a wide range of public health decision-making and, given that the world's first dengue vaccine's efficacy is associated with baseline serostatus, population level seroprevalence is an important predictor of population-level vaccine impact [12].

In India, as elsewhere, few studies have documented the seroprevalence of dengue in healthy subjects. In the earliest, Padbidri et al. measured exposure to various arboviruses, including dengue serotype-2, in the Andaman and Nicobar Islands. This 1988–89 study found 25.4% of subjects with neutralizing antibodies against dengue type-2 [13]. More recently, Oruganti et al. examined the presence of antibodies in healthy individuals attending routine health check-ups in Hyderabad, Andhra Pradesh by indirect IgG ELISA [14]. They found 89.5% of subjects aged 19 to 70 years of age were seropositive for dengue: 100% of those 40 years of age or older had seroconverted. In another community-based study Rodriguez et al. estimated seroprevalence in 5–40 year old healthy subjects in 2011 in Chennai [8] They demonstrated that 93% of subjects in this age group had been exposed to dengue at least once in their lifetime, a level of exposure which was consistent with long-term endemic circulation.

We previously published results of a community-based multi-centric, cross-sectional study (DNG10) on dengue seroprevalence in Indian children aged 5–10 years (CTRI/2011/12/002243 and NCT01477671) [15]. The study was conducted at 8 sites in 6 distinct urban and rural areas in 2011–12. Overall seroprevalence was 59.6% and increased with age. We also described monotypic serological profiles demonstrating that all four dengue serotypes circulate in India.

No previous analysis has assessed dengue FOI and its variability across multiple Indian sites. Here, we conducted a secondary analysis to estimate dengue FOI in healthy children in different geographic regions of India. In combination with census data, this enabled estimation of the number of primary dengue infections occurring annually. We also predicted seroprevalence in children aged 1–10 years of age and the ages at which 50 and 70% of children have experienced at least 1 dengue infection, to inform vaccination policy.

Methods

Ethics statement

As this was secondary analysis, no additional ethical approvals were needed. Details of ethical approvals for the original study are provided in Garg et al. [15].

Source of dengue seroprevalence data

DNG10 was a dengue seroprevalence study which collected blood samples from children aged 5–10 years old between January 2011 and October 2012. There were 8 sites across 6 districts spread over India (two nearby sites each from Delhi and Hyderabad; and one site each from Kalyani, Wardha, Mumbai and Bangalore), which have been described before [15]. Briefly, a convenience sample of children was drawn from the community by household visits (6 sites) or school visits (2 sites). Community health workers obtained informed consent and drew blood samples. The presence of anti-dengue IgG antibodies was measured using one of two commercial ELISA (Focus Diagnostics, California, USA and Panbio Diagnostics, Brisbane, Australia) whose performances were shown to be concordant [15]. We performed a re-analysis of data from this original study after pooling data from the two Delhi and Hyderabad sites, assuming that populations in these sites were exposed to a similar risk of infection because of their geographical proximity (within a few hundred meters).

Force of infection and seroprevalence estimates

Dengue serostatus was considered a binary outcome variable, described by the IgG ELISA test result for each subject and assuming seroconversion is non-reversible. Assuming constant FOI over this 6-year age group, we estimated FOI (λ) using a catalytic model which predicts an increase in the proportion of seropositive individuals with age: [10, 16].

$$p_a = 1 - e^{-\lambda a}$$

where p_a is the proportion seropositive at age a. We estimated λ using a binomial regression model with a complementary log-log link, including seropositivity as the outcome variable and the natural logarithm of age as an offset, a parametrization in which the constant equals the log of average FOI [16, 17]. Separate estimates were made for each site; and for all sites combined. Clustering both at the national level, and for Delhi and Hyderabad where two sites were combined, was accounted for by relaxing the assumption of independence of observations within groups and generating robust standard errors.

Seroprevalence and its 95% confidence intervals for children aged from 1 to 10 years old were estimated from FOI using the formula above. We estimated the ages "a" at which prevalence "P" was 0.5 and 0.7, and their confidence intervals, using the same formula and by replacing "λ" with the estimated constant FOI from each site. The six years of age groups of observed seroprevalence data were grouped into 12, 0.5 year age categories. Mean seroprevalence for each group was graphed over the estimated seroprevalence, as shown in Fig. 1.

Force of infection and seroprevalence estimates

Based on Indian 2011 census data [18] and estimated annual seroconversion rates, we estimated the number of children aged < 11 years experiencing a primary dengue infection in 2011, assuming constant FOI from 2002 to 2011, according to the formula below:

$$\sum_{a=1}^{11} \delta_a (p_a - p_{a-1})$$

Where, δ_a represents the total size of the Indian population aged a years; and p_a is the proportion of the population seropositive by age a years.

Assessment of model fit

In our model, we assumed a constant force of infection. Goodness of fit was assessed by the Hosmer-Lemeshow test [19]. The predicted probabilities of being seropositive and seronegative were calculated for each individual and the data were grouped into deciles. The expected number of events, calculated as the sum of the predicted probabilities, was compared with observed events. Pearson's chi-squared test was applied to test the null hypothesis that the observed data approximates the fitted model under an assumption of constant FOI, with a p-value of > 0.05 applied to define an acceptable fit.

All analyses were conducted with Stata version 15.0 (Stata Corporation) and Microsoft Excel.

Results

Demographics of study subjects and observed seroprevalence

In total, the analysis included data from 2556 subjects, with between 301 and 649 children per site, with approximate equal age distributions (Table 1, see Additional file 1 for detailed age distributions). 52.6% of the subjects were female and the mean age of participants was 7.8 years (SD 1.6 years) with a range 5.0–10.0 years.

Estimated force of dengue infection

The overall annual FOI for all sites combined was 11.9% (95% CI 8.8–16.2%). It varied from a low of 3.5% (95% CI 2.8–4.4%) in Kalyani, West Bengal, to 21.2% (95% CI 18.4–24.5%) in Mumbai, Maharashtra (Fig. 1). Assuming constant FOI, the ages by which 50 and 70% of children were first infected were lowest in Mumbai, 3.3 and 5.7 years respectively (Table 2). In Kalyani FOI was

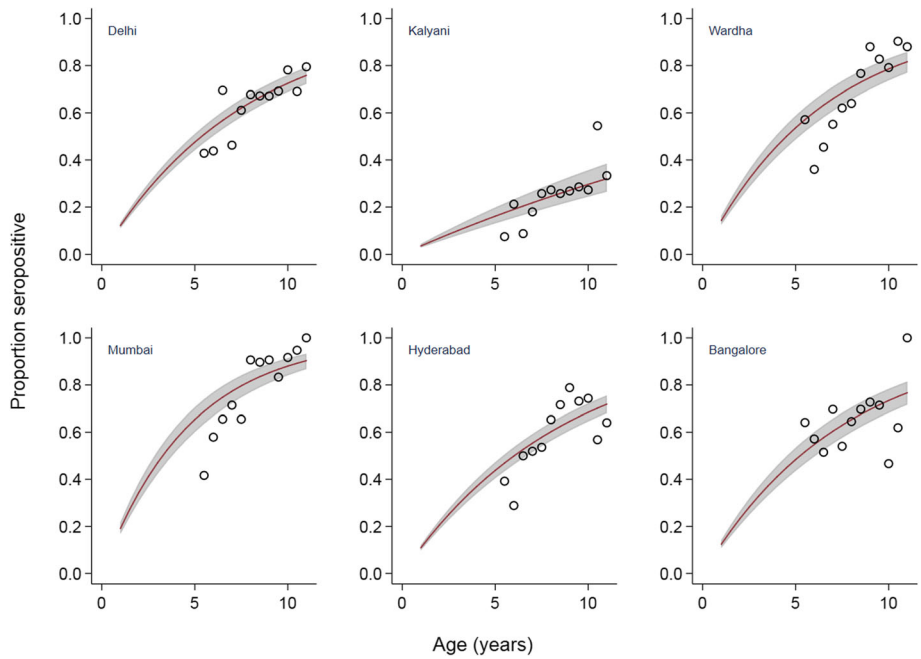

Fig. 1 Estimated seroprevalence (red lines), 95% confidence intervals (shaded areas) and observed seroprevalence (circles)*. *hollow circles indicate observed seroprevalence as measured in original study, divided into 6-month age categories [15]

Table 1 Number of subjects, mean age and overall seroprevalence by site [15]

Site name	Number of subjects enrolled	Mean age in years (standard deviation)	Seroprevalence (%) (95% CI)
Delhi (for 2 sites)	649	8.0 (1.7)	63.3 (59.6–67.0)
Kalyani	323	7.6 (1.4)	23.2 (18.7–28.2)
Wardha	323	8.0 (1.7)	69.0 (63.7–74.0)
Mumbai	301	8.0 (1.5)	80.1 (75.1–84.4)
Hyderabad (for 2 sites)	639	7.8 (1.6)	58.4 (54.5–62.2)
Bangalore	321	7.5 (1.5)	62.6 (57.0–67.8)
Total	2556	7.8 (1.6)	59.6 (57.7–61.5)

sufficiently low that we predicted < 50% of children would have been infected by the age of 11. For other sites, the median age of infection was between 3.3 and 6.0 years; 70% of children were estimated to have been infected by between 5.7 and 10.4 years of age. In the study population overall, 70% of children were estimated to have been infected at least once by the age of 10.1 years. Model goodness of fit as assessed by the Hosmer-Lemeshow test was acceptable for all sites except Wardha (*P*-value: 0.03), Hyderabad ($P = 0.01$) and for India overall (P = 0.01).

Estimated number of primary dengue infections

In 2011, India had a population of ~ 260,000,000 children aged < 11 years. We estimate that in 2011 17,013,527 (95% CI 14,518,438 – 19,218,733) children aged up to 10 years – 6.54% of the total population within this age group – were infected with dengue for the first time (see Additional file 2).

Discussion

We conducted a secondary analysis of dengue seroprevalence data from pediatric populations in India. We found that among dengue-naïve children, 11.9% experience their first dengue infection every year. This means that 50% of children at these sites are infected by dengue at least once by the age of 5.8 years, and 70% of them are infected by the age of 10.1 years, although there was significant variation in FOI between sites. Our study was not the first to report estimates of dengue FOI in Indian populations. Imai et al. used data from 1988 to 89 to estimate FOI of 0.2% (95% CI: 0.1–0.7%) in the Andaman and Nicobar Islands [11, 13]. Rodriguez et al. estimated that the dengue FOI in Chennai from 2004 to 2011 was 23% (95% CI: 16–30%) [8]. The Andaman and Nicobar Islands are a unique geography; that study detected antibodies against only one of the four serotypes of dengue (dengue serotype- 2), and was conducted at a time when dengue endemicity was probably much lower than today. Rodriguez et al. sampled probabilistically from Chennai and found high FOI in pediatric populations. We identified similar FOI from Mumbai, a city with similar ecological conditions: both are coastal with similar ranges of temperature and high levels of unplanned infrastructure, construction sites and slum housing.

We assumed these sites experienced constant FOI for the 5 years prior to sample collection, representing the time period when study subjects were infected. A different approach would consider FOI to be time-varying, in which constant FOI is assumed only for a certain period [9]. Our assumption is broadly consistent with other studies that have found age-constant models adequately describe age-related seroprevalence data over a 6–9 year time horizon [8, 10]. The goodness-of-fit of our constant model provided some evidence that our assumption of constant FOI is valid for four of our six sites, but to more completely explore age-varying FOI, data from a larger age range of subjects would be needed. Further, a visual inspection of Fig. 1 suggests some deviation between the modelled values and the observed data especially at more extreme ages. This may be due to cyclical

Table 2 Annual FOI, goodness-of-fit statistics; and the ages by which 50 and 70% of children seroconverted

Site	Annual FOI, % (95% CI)	Goodness of fit Chi^2 statistic; P- value	Age of 50% population seroconversion, years (95% CI)	Age of 70% population seroconversion, years (95% CI)
Delhi (2 sites)	12.9 (11.1–15.0)	2.96; 0.94	5.4 (4.6–6.2)	9.3 (8.0–10.8)
Kalyani	3.5 (2.8–4.4)	4.72; 0.79	> 11	> 11
Wardha	15.4 (13.4–17.7)	16.9; 0.03	4.5 (3.9–5.2)	7.8 (6.8–9.0)
Mumbai	21.2 (18.4–24.5)	12.8; 0.12	3.3 (2.8–3.8)	5.7 (4.9–6.6)
Hyderabad (2 sites)	11.5 (11.2–11.8)	20.9; 0.01	6.0 (5.9–6.2)	10.4 (10.2–10.7)
Bangalore	13.2 (11.5–15.3)	10.2; 0.25	5.2 (4.5–6.0)	9.1 (7.9–10.5)
All sites combined	11.9 (8.8–16.2)	20.4; 0.01	5.8 (4.3–7.9)	10.1 (7.4–13.7)

dengue outbreaks in the respective geographies. For example, there were documented outbreaks in Mumbai in 2003, and in Wardha and Hyderabad in 2004 [20–22]. Children were disproportionately affected in Mumbai and Wardha which might provide an explanation for outliers in our observations i.e. higher seroprevalence in older children at these sites.

Similar dengue FOI has been estimated from seroprevalence data from dengue hyperendemic Southeast Asian countries. Prayitno et al. estimated the FOI in 1–18 year old Indonesian children in 2014 to be 14.0% [23]. Imai et al. estimated FOI in Thailand using data from 2000 to 01 in school children to be 15.7% [11]. Using 2008–09 data in children under 12 from Colombo, Sri Lanka, Tam et al. estimated the FOI to be 14.1% [10]. Consequently, and because reported dengue incidence rates in India are so low, we calculated the resulting number of primary dengue infections, estimating > 17 million primary infections in India, annually. Other researchers have estimated between 30 and 50% of primary infections are symptomatic [24] which would equate to ~ 5 – ~ 8.5 million cases annually in children aged < 11. When considering cases in other age groups, and following secondary or subsequent infections; these case numbers are broadly within the same range as those reported in Bhatt et al., that India suffers ~ 35 million symptomatic episodes per year, and provide additional evidence of a very significant level of under-reporting of dengue in India [6]. More detailed estimates of symptomatic episodes are limited by our lack of secondary infection history data; and more complex mathematical modeling was beyond the scope of our study.

This is the first study to estimate FOI in India using data from multiple geographies; urban and rural, and from multiple states. Our results point towards a high dengue FOI in children in India, which logically equates to a significant number of secondary infections and burden of symptomatic disease in this age group. With improved surveillance, we may begin to see incidence rates of dengue in India comparable to those seen in other hyperendemic countries. Longitudinal cohort studies, ideally incorporating fever surveillance and serological surveys, to more accurately describe the incidence of dengue and changing infection patterns with age, are needed [25].

Our study has several limitations. The original seroprevalence samples were collected in 2011–12 and the FOI we have derived corresponds to cumulative exposure experienced by study subjects in the years of their life before this time. Numbers of reported cases of dengue in India have increased significantly from 2011. [3] This can be attributed to several factors including population movement and increased exposure to the virus; improved dengue surveillance, increasing awareness among healthcare practitioners, availability of confirmatory diagnostics and improvement in access to healthcare resulting in increased reporting [4, 26]. As demonstrated by Rodriguez et al.in Chennai, it is also very likely that FOI has increased in India over recent decades [8]. Despite their geographical spread, study sites were not sampled to be representative of the whole of India and our extrapolation to the national level is a strong assumption which should be validated with more recent data from other sites. DNG10 also used convenience sampling for enrollment of subjects, a method which does not guarantee representativeness. We used IgG ELISA to ascertain infection history, an assay with known cross-reactivity to antibodies against other flaviviruses. However, dengue infection was confirmed by the plaque reduction neutralization test (PRNT) and > 97% of IgG positive samples were also positive by PRNT. Further, Japanese encephalitis (JE) seropositivity measured at the study sites using IgG ELISA, was 13.6% overall and with a similar trend at the site level as dengue seroprevalence (data not shown). Its confounding influence is therefore likely to be minimal. Because IgG ELISA is unable to distinguish primary from secondary infections we measured only the rate of primary seroconversion, and are unable to quantify the burden of secondary and subsequent dengue infections.

Conclusions

We demonstrate high dengue FOI in multiple Indian settings. Observed variations are likely reflective of dengue epidemiological variation in different parts of India. These data may be used for benchmarking the dengue endemicity in other areas in India, and to allow comparisons based on other epidemiological indicators.

Additional files

Additional file 1: Table S1. Number of subjects with IgG data available from DNG10 study according to age (% seropositive). (DOCX 20 kb)

Additional file 2: Table S2. Estimates of the number of children experiencing primary dengue infections in 2011, in India overall. (DOCX 18 kb)

Abbreviations

ELISA: Enzyme-linked Immunosorbent Assay; FOI: Force of Infection; IgG: Immunoglobulin G; JE: Japanese encephalitis; PRNT: Plaque reduction neutralization test

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Authors' contributions

AB and JN conceptualized the study. JN developed the statistical methods and model and wrote the statistical code with inputs from AFT and supervision from CT. AB, JN and SNC performed the data analysis. AB and JN drafted the manuscript. AB, JN, CT, SG, GRJ, AFT and SNC provided technical

feedback on methods, contributed in development of the manuscript and approved the final version for publication.

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Availability of data and materials
Data sufficient to replicate the current study are provided in Additional file 1. Subject-level data are available from the corresponding author on request.

Ethics approval and consent to participate
Study protocol for the original study (registered at ctri.nic.in: CTRI/2011/12/002243) was approved by ethics committees of all the participating study centers, and by the Indian Health Ministry's Screening Committee (HMSC). As this was secondary analysis of available data, consent from individual patients was not needed.

Consent for publication
All study subjects signed informed consent indicating these data would be published.

Competing interests
AB was and JN and AFT are employees of Sanofi Pasteur which manufactures a dengue vaccine, SG and GRJ were part of DNG10 study which was sponsored by Sanofi Pasteur. CT and SNC declare no conflicts of interest.

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Publisher's Note

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MAJOR ARTICLE

Dengue Endemicity, Force of Infection, and Variation in Transmission Intensity in 13 Endemic Countries

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Dengue endemicity varies but comparative, multicountry data are extremely limited. An improved understanding is needed to prioritize prevention, including vaccination, which is currently recommended only under specific epidemiological conditions. We used serological study data from 46 geographical sites in 13 countries to estimate dengue force of infection (FOI, the proportion of children seroconverting per year) under assumptions of either age-constant or age-varying FOI, and the age at which 50% and 80% of children had been infected. After exclusions, 13 661 subjects were included. Estimated constant FOI varied widely, from 1.7% (Singapore) to 24.1% (the Philippines). In the site-level analysis 44 sites (96%) reached 50% seroconversion and 35 sites (75%) reached 80% seroconversion by age 18 years, with significant heterogeneity. These findings confirm that children living in dengue-endemic countries receive intense early dengue exposure, increasing risk of secondary infection, and imply serosurveys at fine spatial resolutions are needed to inform vaccination campaigns.

Keywords. dengue; epidemiology; seroprevalence; vaccines; endemicity; infectious disease transmission.

Dengue viruses infect approximately 400 million people annually at frequencies that vary according to environmental, ecological, and behavioral factors [1, 2]. Disease burden estimates have historically been unreliable but more recent studies using more comprehensive data synthesis and systematic methods estimate that dengue viruses likely result in 24–130 million symptomatic episodes, 10 000–50 000 deaths, and costs of US $4–19 billion, annually [2–4]. Dengue represents a significant source of morbidity in affected regions and 50% of the global population is at risk of infection, with local variations caused by geographical, microclimatic, and ecological factors at the subnational and local levels [2, 5, 6].

Following infection, the likelihood of suffering mild or more serious symptoms depends on immunological or other factors that predispose individuals to more severe disease, particularly during chronological and/or immunological windows of enhancement caused by antibody-dependent enhancement or other mechanisms arising from infection with heterologous viral serotypes [7–9]. The risk of suffering a symptomatic episode is therefore a complex function of ecological and immunological factors with time-varying risk windows, determined by the underlying transmission intensity. A result is that the age distribution of symptomatic dengue disease is dependent on the epidemiological setting, with more intense transmission resulting in a younger median age of cases [10]. Infection frequency may be constant or may be shaped by individual events such as outbreaks or changes in human behavior that affect the risk of exposure [11].

Because most dengue infections are asymptomatic, prospective measurements of infection rates require longitudinal studies with blood samples at multiple time points, which are resource intensive and generally conducted in single study sites with limited geographical representativeness [12]. However, dengue seroprevalence at a given age is an alternative measure of endemicity, which can be measured relatively efficiently from age-stratified cross-sectional surveys [13]. Assuming transmission intensity is constant over time, the rate at which seroprevalence increases with age can provide a measure of the force of infection (FOI) or the rate at which susceptible (seronegative) individuals acquire infection [14]. Under assumptions of constant or varying endemicity, FOI can be used to estimate seroprevalence at a given age, an approach which can complement empirical seroprevalence measurements [15, 16].

Reliable estimates of age-stratified dengue seroprevalence are particularly important when considering immunization, because the efficacy of the world's first dengue vaccine (CYD-TDV, Dengvaxia; Sanofi Pasteur) is dependent on an individual's infection history [17]. World Health Organization (WHO) guidelines recommend vaccination only after individual screening for dengue antibodies or, if this is not feasible,

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where seroprevalence in 9 year olds exceeds a threshold of 80% [18]. Dengue seroprevalence is therefore an important determinant of the impact and cost-effectiveness of dengue vaccination.

Here, we used age-stratified dengue seroprevalence data from healthy children in 13 countries involved in dengue vaccine clinical trials and epidemiological studies to describe dengue endemicity across a wide range of geographical regions, using FOI as an indicator. We estimated the age at which seroprevalence reached 50% and 80% in countries overall and at each of 46 geographically distinct sites, to inform the feasibility and optimum age for efficient dengue vaccination strategies, aligned with WHO Strategic Advisory Group of Experts on Immunization recommendations.

METHODS

Ethical Approval

This is a secondary analysis of anonymized data. Ethics approval for analyses and publication of study data were secured from relevant ethical committees prior to the collection of any data (all approval numbers are in Supplementary Table 1).

Study Design

We analyzed data from cross-sectional, age-stratified serological surveys in 13 countries collected over 6 years. Data and blood samples originated from baseline measurements of clinical trials before any vaccine or placebo were administered, or from dedicated cross-sectional seroprevalence surveys.

Study Population

Eligible subjects were participants in Sanofi Pasteur dengue vaccine clinical trials or epidemiological studies, which collected dengue serological data from healthy, asymptomatic, unvaccinated individuals (Table 1). Enrollment methods in these studies varied and included investigators directly recruiting subjects under their care or following informational events at primary health care centers, schools, or community centers, depending on the local health care system and community organization. For phase 2b or phase 3 vaccine efficacy studies, which provided 29% of data, subjects were typically recruited following school-based (in Asia) or community-based (in Latin America) meetings held in proximity to participating hospital study sites, during which parents were informed about upcoming dengue vaccine studies and implications of participating [19, 20]. Recruitment for epidemiological studies, which provided approximately 42% of data, was conducted at multiple sites selected to provide geographical variability across countries. After school/community educational events, families wishing to participate presented to local health care facilities for recruitment [21, 22]. The remaining subjects were enrolled in earlier-phase clinical trials and were typically recruited directly from medical facilities by study investigators.

Data from all studies were combined into a single database and categorized according to the geographical district

Table 1. Site Description and Data (Country-Level Analysis)

Country	Sites	No. of sites	Studies	n	Age Range, y	First Enrollment	Last Enrollment
India	Delhi, Pune, Ludhiana, Bangalore, West Bengal, Wardha, Mumbai, Hyderabad	8	CYD47,[a] DNG10[b]	2562	5–18.7	Jan 11	Oct 12
Indonesia	Bali, West Java, Jakarta, Aceh, North Sumatera, West Sumatera, Jambi, Lampung, Banten, Central Java, East Java, East Kalimantan, South Sulawesi, Southeast Sulawesi	14	CYD14,[c] DNG26[b]	3539	1–18.9	Jun 11	Nov 14
Malaysia	Kuala Lumpur, Penang, Ipoh (Perak), Seremban (Negeri Sembilan), Kuching	5	CYD14,[c] CYD32[a]	547	2–14.8	Dec 10	Sep 11
Philippines	San Pablo (Region IV-A)	1	CYD08,[a] CYD14[a]	820	0.9–14.9	Jan 10	Jul 11
Singapore	Singapore	1	CYD28[a]	384	2–18	Apr 09	Oct 09
Thailand	Ratchaburi, Kamphaeng Phet	2	CYD14,[c] CYD23[c]	637	2–14.8	Feb 09	Nov 11
Vietnam	Long Xuyen (An Giang), My Tho (Tien Giang)	2	CYD14,[c] CYD22[a]	607	2–18.7	Mar 09	Oct 11
Brazil	Nordeste, Espirito Santo, Goias, Mato Grosso Sud	4	CYD15,[c] CYD30[a]	450	8.9–16.9	Aug 10	Nov 11
Colombia	Santander, Quindio, Cundinamarca, Meta, Casanare, Cali	6	CYD13,[a] CYD15,[c] CYD29[a]	1518	0.9–16.9	Oct 09	Mar 12
Honduras	Tegucigalpa	1	CYD13,[a] CYD15[c]	455	9–16.9	Oct 09	Sep 11
Mexico	San Luis, Veracruz, Morelos, Yucatan, Guerrero, Nuevo León	6	CYD13,[a] CYD15,[c] CYD33[a]	1213	0.6–16.9	Nov 09	Jul 12
Peru	Peru	1	CYD24,[a] CYD29[a]	671	0.9–11.9	Sep 08	Mar 12
Puerto Rico	Puerto Rico	1	CYD13,[a] CYD15[c]	258	9–16.9	Nov 09	Oct 11

[a]Clinical trial.

[b]Epidemiological study.

[c]Efficacy trial.

or comparable administrative unit (hereafter, "site") of each participating study center, on the advice of local Sanofi Pasteur staff with expertise on local geography. The analysis was restricted to subjects aged 7 months to < 19 years on the day of blood sampling, as data on older subjects were limited and indicated minimal variation in seroprevalence in older age groups. A sensitivity analysis was conducted on the entire population aged > 7 months for India, Singapore, and Vietnam, the only countries where subjects > 19 years had been enrolled, to assess the impact of excluding older adults from the analysis. Data from areas that are not dengue endemic (Australia, United States, and Mexico City) and from subjects with inconclusive dengue serological results were excluded from analysis (Figure 1). A country-level analysis was followed by a site-level analysis, in which sites with < 10 subjects and those that enrolled only subjects aged < 2 years were removed.

Data Collection and Laboratory Analyses

Baseline serum samples, drawn before any vaccine was administered from both vaccine and placebo arm subjects of clinical trials, were collected between September 2008 and November 2014 (Table 1). Dengue exposure for each subject was ascertained in clinical trials by 50% plaque reduction neutralization test ($PRNT_{50}$) with a lower limit of quantitation titer of 10 (reciprocal dilution) as described previously [23]. For epidemiological studies DNG10 and DNG26, serostatus was determined by IgG enzyme-linked immunosorbent assay (ELISA; Panbio and Focus Diagnostics); >97% of a positive subset were confirmed by $PRNT_{50}$ providing confidence in the specificity of IgG ELISA assays [21, 24]. As the youngest subject was aged 0.6 years, we assumed declining maternal antibodies had little or no impact on our analyses.

Statistical Methods

FOI was estimated using catalytic models in which seroprevalence is assumed to increase exponentially with age:

$$P_a = 1 - e^{-\lambda a}$$

Here, P_a is the seroprevalence at age a years, and λ represents FOI or the annual risk of seroconversion among initially seronegative individuals. The parameter λ can be estimated through a generalized linear model with complementary log-log link:

$$ln\left(-\ln\left(1 - p_a\right)\right) = \ln\left(\lambda\right) + ln(a)$$

where the logarithm of each subject's age is included as an offset with a coefficient constrained to 1. This model implicitly assumes that FOI is constant throughout the age range and that transmission intensity is stable over time.

FOI estimates were derived for each country and site with uncertainty described through generation of exact binomial 95% confidence intervals (CIs). Intersite variability in countries

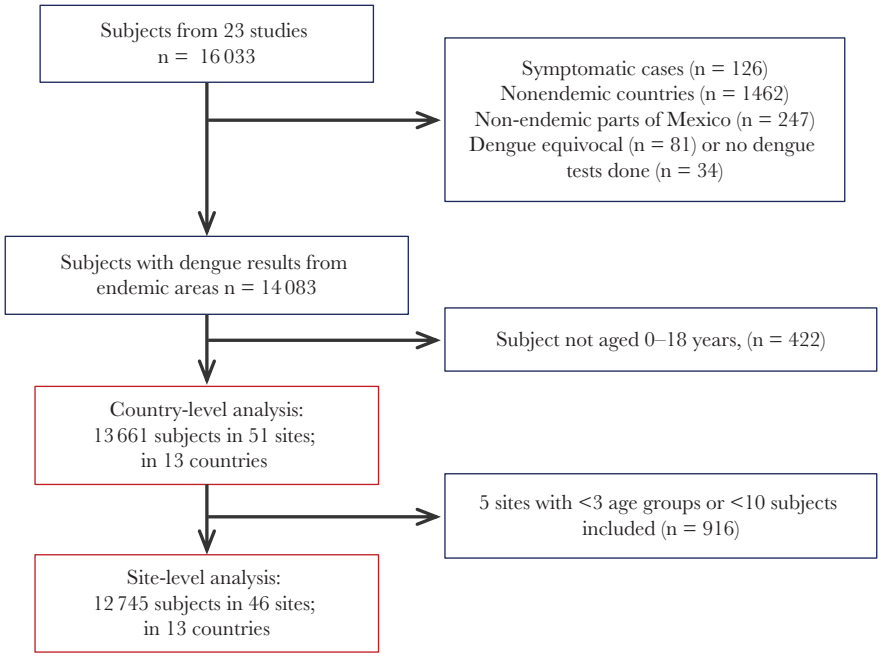

Figure 1. Study flow chart.

with > 1 site was accounted for by generating robust standard errors, assuming sites were independent clusters.

To describe possible changes in FOI within large age strata, we also generated age-varying FOI estimates for specific age groups using linear piecewise models. We fitted log-binomial models with 2 age terms:

$$-\ln(1 - P_a) = \lambda_1 a_1 + \lambda_2 a_2$$

For each study site, we determined the optimal age-varying FOI model by sequentially varying the age breakpoint for each whole year of data with at least 2 adjacent data points (eg, for countries with data starting in 3-year-old children the first possible breakpoint was age 5 years) and identifying the model with the lowest value for Akaike's information criterion. For each country, we determined whether constant or age-varying models fit the data better by 10-fold cross-validation, taking a random 10% of the sample, and selected the model (constant or age varying) with lower root mean squared error. Seroprevalence by age, per country, was estimated from the resulting models. Graphs of estimated constant and age-varying seroprevalence were developed for each country, overlaid with observed seroprevalence and their 95% CIs grouped by year, using robust variance estimates for countries with > 1 site to account for clustering.

We estimated the age at which 50% and 80% ($p = 0.5$ or 0.8) of children seroconverted in each country and site from the optimal model for each site, using the following formula in case of constant FOI or if the threshold was reached before the breakpoint:

$$a = \frac{-\ln[p]}{\lambda}$$

or if after the observed breakpoint for age-varying models, by:

$$a_2 = \frac{-\ln[p] - \lambda_1 a_1}{\lambda_2}$$

All analyses were conducted using Stata version 15.0.

RESULTS

Dataset

Our database contained information from 16 033 subjects participating in 23 clinical trials and epidemiological studies. After exclusions, 13 661 subjects from 15 studies were eligible in 13 country-level analyses and 12 745 in 46 site-level analyses, with a mean of 268 subjects per site (Table 1 and Figure 1). Countries with the highest number of sites and subjects were Indonesia, India, Colombia, and Mexico. The range of subject ages was narrowest in Brazil (7.9 years) and widest in Indonesia (17.9 years).

Dengue Force of Infection

Under the assumption of constant FOI, dengue FOI varied between countries from a low of 1.7% (95% CI, 1.4–2.2) in Singapore, increasing to 24.1% (95% CI, 21.8–26.5) in the Philippines (Table 2). FOI was lower than 10% in Singapore, Mexico, Peru, and Puerto Rico. In most countries, constant and age-varying models predicted similar seroprevalence at most ages; constant models fit data better in 8 countries (Table 2 and Figure 2). In all countries except India and Brazil, age-varying FOI was higher in younger children than older children, indicating a decreasing rate of first infection as children aged. The highest FOI estimates occurred in very young Filipino children, with an annual seroconversion risk of 43% up to the age of 2 years. Estimated dengue seroprevalence increased with age in all scenarios, except for the age-varying Singapore model where estimated seroprevalence declined at age 4 years. In a sensitivity analysis, the impact of including adults aged > 19 years from India, Singapore, and Vietnam was minimal (constant FOI changed from 11.9 [95% CI, 8.7–16.2] to 11.5 [95% CI, 8.4–15.6]; 1.7 [95% CI, 1.4–2.2] to 2.0 [95% CI, 1.7–2.3]; and 11.4 [95% CI, 10.2–12.8] to 11.4 [95% CI, 10.3–12.7], respectively). At the site level, the age-constant FOI was > 10% per year at 31 of 46 sites and constant models fit observed data better at 36 of 46 sites. FOI estimates at the site level are provided in Supplementary Table 2.

Age at 50% and 80% Seroconversion Thresholds

According to the best-fitting model, the estimated age at which 50% of children had seroconverted was < 10 years in 12 of 13 countries in our analysis; the youngest was in the Philippines (1.6 years; 95% CI, 1.4–3.1, Table 3). In Singapore, a seroprevalence of 50% was not reached within the range of our observed data, by age 18 years (Table 2). An 80% seroprevalence threshold was reached by the age of 18 years in 10 countries, 3 of which reached this threshold by the age of 9 years (Philippines, Colombia, and Honduras).

Forty-six sites were included in the site level analysis. We estimated 80% of children had been infected by age 18 years (ie, within the range of our observed data) at 35 (76%) sites and by age 9 years at 14 (30%) sites (Figure 3). The youngest estimated age at which 80% of children seroconverted was 5.3 years, observed at Casanare, in Colombia. At least 50% of children were estimated to have seroconverted by the age of 18 years at 44 (96%) sites, and at all 15 sites in Latin America (Supplementary Figure 1). In Kalyani (West Bengal, India), median seroprevalence was not reached. Seroprevalence at age 9 was also high at other sites, notably across Indonesia (Supplementary Table 2). Within countries, there was considerable variation between sites in the age at which 50% and 80% of children seroconverted (Figure 3).

Table 2. Constant and Age-Varying FOI Estimates for Each Country

Country	Constant FOI (95% CI)	Age-Varying FOI (95% CI)	Corresponding Age Range, y	Better Fit[a]
India	11.9 (8.7–16.2)	10.7 (6.9–14.4) 20.0 (10.7–29.4)	5–6 7–18	Constant
Indonesia	14.7 (12.8–16.9)	15.1 (13.1–17.1) 4.1 (−3.9 to 12.2)	1–13 14–18	Constant
Malaysia	8.6 (6.7–10.9)	12.2 (11.3–13.1) 4.7 (−0.2 to 9.6)	2–3 4–14	Constant
Philippines	24.1 (21.8–26.5)	42.6 (35.4–49.7) 13.5 (10.1–16.9)	0–1 2–14	Age-varying
Singapore	1.7 (1.4–2.2)	6.6 (4.0–9.2) −0.8 (−2.0 to 0.3)	2–3 4–18	Age-varying
Thailand	14.8 (13.7–16.0)	16.4 (15.0–17.9) 8.7 (6.6–10.9)	2–6 7–14	Age-varying
Vietnam	11.4 (10.2–12.8)	17.3 (16.5–18.1) 5.4 (3.8–7.0)	2–3 4–18	Constant
Brazil	10.7 (7.7–14.8)	9.2 (5.6–12.9) 23.1 (20.3–25.9)	8–10 11–16	Constant
Colombia	18.4 (14.2–23.7)	18.7 (13.1–24.3) 9.9 (−25.9 to −45.8)	0–12 13–16	Constant
Honduras	17.5 (15.6–19.7)	17.8 (14.8–20.8) 15.1 (1.8–32.1)	9–10 11–16	Constant
Mexico	7.1 (5.1–9.8)	7.7 (4.8–10.5) −0.2 (−8.6 to 8.1)	0–11 12–16	Age-varying
Peru	9.2 (8.0–10.7)	13.2 (10.2–16.2) 4.5 (1.7–7.2)	0–2 3–11	Constant
Puerto Rico	8.4 (7.2–9.9)	8.8 (7.1–10.5) 2.8 (−13.3 to 18.8)	9–13 14–16	Age-varying

Abbreviations: CI, confidence interval; FOI, force of infection; RMSE, lower root mean squared error.

[a]For each country the better model fit was assigned based on the lower RMSE.

DISCUSSION

We analyzed data from over 13 000 children to describe dengue transmission intensity at 46 geographically distinct, endemic sites in 13 countries in Asia and Latin America. Study subjects were in age groups likely to seroconvert, providing the necessary variation in seroprevalence to estimate FOI. Dengue serological status was confirmed with gold-standard diagnostics and consistent analyses were used to make comparisons across countries and sites.

Across the age ranges sampled, children at most sites were at high risk of dengue infection, with FOI exceeding 7% in all countries except Singapore. Countries with higher levels of transmission included the Philippines, Colombia, Honduras, Indonesia, and Thailand, in which ≥ 14% of seronegative children were infected each year. In these countries, intense dengue exposure results in a steep reduction in dengue-naive individuals early in life, providing a large pool of individuals at risk of secondary infection. While these data describe the force of primary (ie, first) dengue infections, these transmission intensities would translate, at the population level, to a significant burden of secondary infections, which are more likely to be symptomatic and severe. Malaysia and Singapore had lower transmission than other Asian countries, which could be an indication of improvements in dengue control measures.

Age-varying models were developed to assess whether clear variation in infection risk was observed as children aged. Strong evidence for this variation was lacking; seroprevalence estimates from constant and age-varying models were broadly similar and differences in cross-validation errors from different models were small (Supplementary Table 3). However, in age-varying models transmission intensity was more frequently (11 out of 13 countries) higher in younger children, perhaps indicating their increased exposure to infectious mosquito bites. In Singapore, FOI declined for a significant proportion of the study sample (children aged > 3 years), a finding which is biologically counter intuitive. This is possibly a consequence of intensive and effective vector control activities and behavior that minimizes exposure to infectious bites (eg, use of air conditioning) resulting in low seroprevalence throughout childhood. Singapore also tends to experience severe, cyclical epidemics and a recent large outbreak could result in higher seroprevalence in younger than older children. For example, there was a large outbreak in late 2005, approximately 4 years before study subjects were bled, and if young children were disproportionately infected this could give the impression of declining FOI [25].

According to WHO guidelines, an overall population benefit of dengue vaccination with CYD-TDV dengue vaccine can be expected in very high transmission settings, as defined by

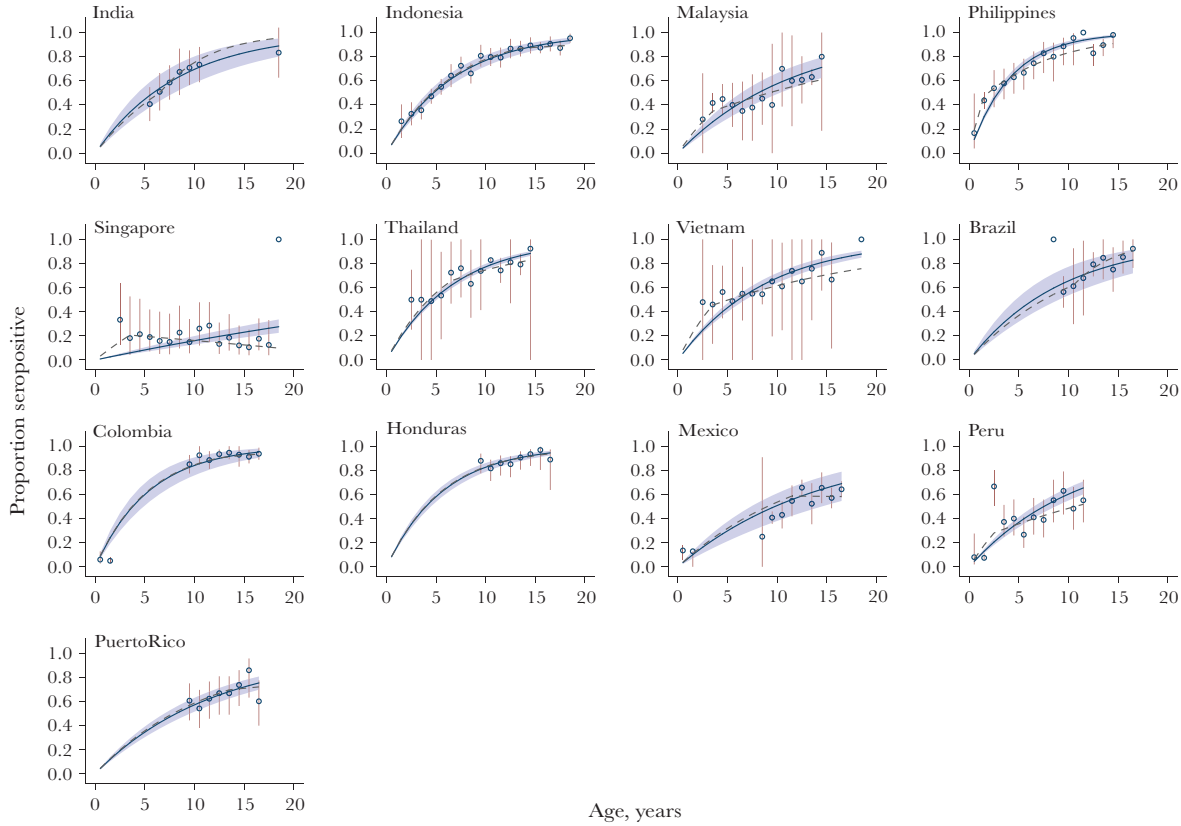

Figure 2. Observed seroprevalence by age (circles) and 95% confidence interval (CI), adjusted for clustering (spikes) and estimate seroprevalence assuming constant force of infection (FOI) (solid line) and 95% CIs (shaded area). Dotted lines correspond to estimated seroprevalence under an assumption of age-varying FOI. (Country-specific seroprevalence estimates provided in Supplementary Table 4)

seroprevalence of ≥ 80% in subjects aged 9 years of age or older, noting that such areas are rare [18]. Here, we estimated that 14 of 46 (30%) sites met this criterion: 1 in Brazil, 1 in Honduras, 4 in Colombia, 1 in India, 6 in Indonesia, and at the only site included from the Philippines. These data represent transmission levels when blood samples were drawn, several years ago, but indicate several sites may benefit from dengue vaccination at the population level.

Another objective of this analysis was to understand variability in endemicity within countries because few multisite dengue seroprevalence studies have been conducted. We identified significant variability within the same country: for example,

Table 3. Ages at Which 50% and 80% of Children Become Dengue Seropositive, Per Country, Using Constant or Age-Varying Models

	Median Age at Seroconversion, y (95% CI)		80th Percentile Age at Seroconversion, y (95% CI)	
Country	Constant Model	Age-Varying Model	Constant Model	Age-Varying Model
India	**5.8 (5.5–6.1)**	6.5 (6.0–6.9)	**13.5 (12.8–14.3)**	11.1 (8.8–17.4)
Indonesia	**4.7 (4.5–4.9)**	4.6 (4.4–4.8)	**11.0 (10.5–11.5)**	10.6 (10.1–11.2)
Malaysia	**8.1 (7.1–9.2)**	9.2 (5.9–>18)	**18.8 (16.6–21.3)**	>18
Philippines	2.9 (2.6–3.2)	**1.6 (1.4–3.1)**	6.7 (6.1–7.4)	**8.7 (6.6–12.2)**
Singapore	>18	**>18**	>18	**>18**
Thailand	4.7 (4.2–5.2)	**4.2 (3.7–4.9)**	10.9 (9.8–12)	**12.7 (0–40.2)**
Vietnam	**6.1 (5.5–6.8)**	5.1 (4.3–6.5)	**14.1 (12.7–15.7)**	>18
Brazil	**6.5 (5.8–7.3)**	7.5 (6.4–9.1)	**15.1 (13.4–17.0)**	13.3 (11.9–17.4)
Colombia	**3.8 (3.5–4.1)**	3.7 (3.4–4.1)	**8.8 (8.1–9.5)**	8.6 (7.9–9.4)
Honduras	**4.0 (3.5–4.5)**	3.9 (3.3–4.7)	**9.2 (8.2–10.3)**	9.0 (7.7–10.9)
Mexico	9.8 (8.8–10.9)	**9.0 (8.0–10.4)**	>18	**>18**
Peru	**7.5 (6.5–8.7)**	10.6 (6.5–28.8)	**17.4 (15.1–20.2)**	>18
Puerto Rico	8.2 (7.0–9.6)	**7.9 (6.6–9.8)**	>18	**>18**

Best-fitting models are highlighted in bold.

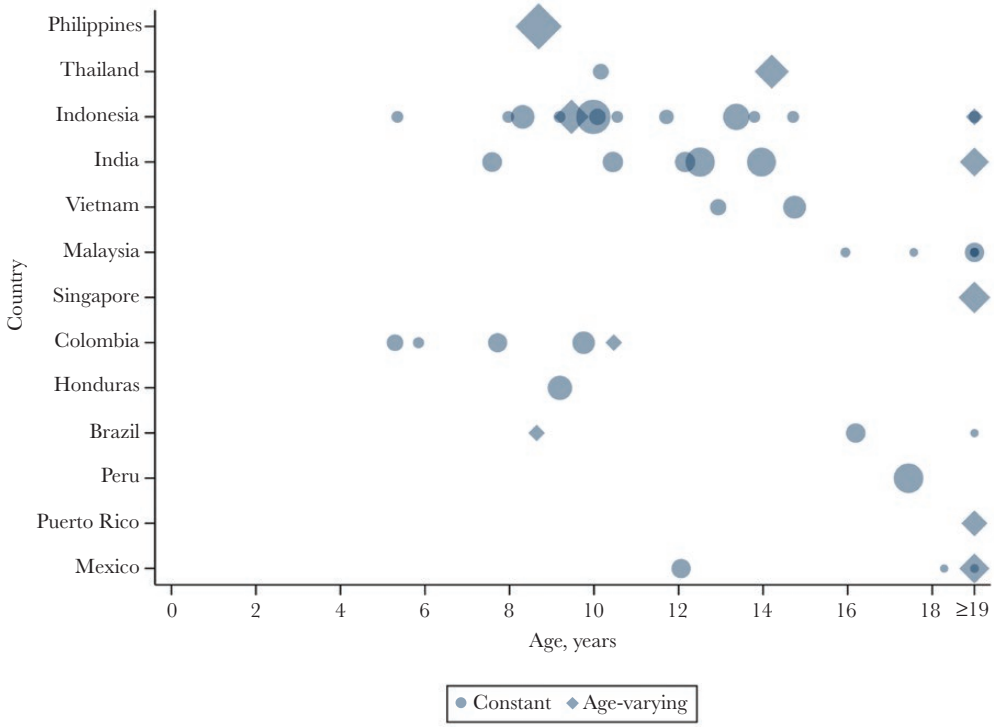

Figure 3. Age of 80% seroconversion by site, estimated from best-fitting constant (circles) or age-varying (diamonds) models. Symbol size corresponds to frequency weights. ≥19 signifies estimates were outside the range of our data.

in Indonesia, the median age of first infection varied between sites from 2.3 to 10.1 years, and in Brazil this varied from 3.7 to 11.4 years. We did not have data from multiple sites in all countries but this implies site-specific seroprevalence assessments would be needed prior to dengue vaccine introduction without prior serotesting, and such data would be useful to prioritize areas where vaccination would be most efficient [18]. However, few observed seroprevalence data points fell outside the confidence intervals of our estimated seroprevalence and statistical approaches such as this, accounting for uncertainty, could be considered complementary to empirical seroprevalence studies in endemic countries [13].

Our analysis was confined to exploring FOI and seroprevalence as a function of age but, because dengue is a cyclical, epidemic disease, calendar time is another, and perhaps more plausible, explanation for observed variation in FOI, as observed elsewhere [26]. Our study precluded detailed analysis of the effect of time on FOI; although data were available for more than 1 time point in some countries, samples for most countries were collected within a 2-year timeframe. While higher FOI in younger age groups is a finding compatible with higher transmission intensity in more recent years, we could not explore specific and granular cohort effects. Future studies would be needed to shed further light on the contributions of age, time, and geography to variations in dengue endemicity. Another limitation of our analysis is that many of our datasets were collected for the purposes of clinical research rather than as part of geographically representative surveys. These clinical trials, from which 58% of our data were collected, often targeted areas of high dengue endemicity, cannot be considered nationally representative, and, in countries where endemicity is heterogeneous, likely represent populations with higher-than-average exposure. This is especially relevant for Latin American countries where dengue is not endemic nationwide; for example, in Columbia and Mexico significant proportions of the population live in areas where the disease does not circulate [27].

The models used for FOI estimation impose certain constraints; notably, the power of age-varying models to detect a meaningful breakpoint is partly dependent on the age range of the data, which varies between countries. We confined the analysis to single breakpoints corresponding to whole years of age and to years with > 2 years of adjacent data, which is a simplistic design, prohibiting additional flexibility. Comparisons between countries on this point should therefore be made with caution. Akaike's information criterion was used to identify optimal (constant vs age varying) models for each country but in many cases both models fit the data well and we identified only weak evidence for age-varying effects.

All 4 dengue serotypes circulate in most of these countries [28] and we calculated only total (or average) dengue FOI, assuming this is relatively stable over time, without more granular or serotype specific variation. In fact, dengue epidemiology is

characterized by cyclical introductions of different serotypes giving rise to outbreaks, as elegantly demonstrated in an extremely thorough longitudinal serological analysis from the city of Iquitos in Peru [26, 29]. The authors demonstrated high attack rates following the introduction of new serotypes into naive populations of up to 89 infections/100 person-years and accompanying high, time-varying, serotype-specific FOI fluctuating across time and serotypes. From our samples, over 90% tested by $PRNT_{50}$ showed evidence of infection with > 1 serotype (possibly due to cross-reaction rather than true infection) and calculation of meaningful serotype-specific FOI estimates was therefore not possible without making unreliable assumptions from PRNT titers. Because total FOI has been shown to approximate the sum of serotype-specific FOI [10], we considered the approach was reasonable, and more complex modeling activities would be needed to further understand serotype-specific transmission dynamics. Our data should be considered representative of long-term average dengue exposure rates.

The infection history of around 40% of samples was determined by IgG ELISA. In concordance experiments, we found 97% of IgG-positive samples were $PRNT_{50}$ positive, providing a high level of confidence in the specificity of these tests but it was not possible from our dataset to assess sensitivity of the IgG vs PRNT.

We also did not consider the impact of other flavivirus infections but in dengue-endemic areas we considered positive dengue diagnostic results, confirmed by PRNT, to be definitive. Approximately 45% of samples came from India and Indonesia, countries in which large dengue seroprevalence studies had been conducted and the proportions of children in Asian studies were higher than in Latin America, which could affect statistical power.

Nonetheless, these data provide one of the largest dengue seroprevalence analyses performed, provide epidemiological information across endemic countries, can be used to guide public health decision-making, including the benefits/risks of vaccination, and inform health economic analyses.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Data availability. Qualified researchers may request access to patient level data and related study documents including the clinical study report, study protocol with any amendments, blank case report form, statistical analysis plan, and dataset specifications. Patient level data will be anonymized and study documents will be redacted to protect the privacy of trial participants. Further details on Sanofi's data sharing criteria, eligible studies, and process for requesting access can be found at: https://www.clinicalstudydatarequest.com.

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Potential conflicts of interest. J. N., A. B., M. C., C. F., L. C., and D. M. are or have been employees of Sanofi Pasteur, a company which makes a dengue vaccine. J. N., C. F., and D. M. own Sanofi shares. Sanofi Pasteur staff were involved in all aspects of data collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. C. C. T. has no conflicts of interest to declare. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Appendices

Appendix 1: list of all studies and clinical trial identifiers or related documents

Study	Clinical trials identifier/study approval code	Reference
CYD05	Eudra-CT number: 2014-001534-29	Capeding RZ, Luna IA, Bomasang E, et al. Live-attenuated, tetravalent dengue vaccine in children, adolescents and adults in a dengue endemic country: Randomized controlled phase I trial in the Philippines. *Vaccine*. 2011;29(22):3863-3872.
CYD08	NCT01064141	Crevat D, Brion JD, Gailhardou S, Laot TM, Capeding MR. First Experience of Concomitant Vaccination Against Dengue and MMR in Toddlers. *Pediatr Infect Dis J*. 2015;34(8):884-892.
CYD13	NCT00993447	Villar LÁ, Rivera-Medina DM, Arredondo-García JL, et al. Safety and Immunogenicity of a Recombinant Tetravalent Dengue Vaccine in 9–16 Year Olds. *Pediatr Infect Dis J*. 2013;32(10):1102-1109.
CYD14	NCT01373281	Capeding MR, Tran NH, Hadinegoro SRS, et al. Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. *Lancet*. 2014;384(9951):1358-1365.
CYD15	NCT01374516	Villar L, Dayan GH, Arredondo-García JL, et al. Efficacy of a tetravalent dengue vaccine in children in Latin America. *N Engl J Med*. 2015;372(2):113-123.
CYD22	NCT00875524	Tran NH, Luong CQ, Vu TQH, et al. Safety and Immunogenicity of Recombinant, Live Attenuated Tetravalent Dengue Vaccine (CYD- TDV) in Healthy

		Vietnamese Adults and Children. *J Vaccines Vaccin*. 2012;03(07).
CYD23	NCT00842530	Sabchareon A, Wallace D, Sirivichayakul C, et al. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. *Lancet*. 2012;380(9853):1559-1567.
CYD24	NCT00788151	Lanata CF, Andrade T, Gil AI, et al. Immunogenicity and safety of tetravalent dengue vaccine in 2-11 year-olds previously vaccinated against yellow fever: Randomized, controlled, phase II study in Piura, Peru. *Vaccine*. 2012;30(41):5935-5941.
CYD28	NCT00880893	Leo YS, Wilder-Smith A, Archuleta S, et al. Immunogenicity and safety of recombinant tetravalent dengue vaccine (CYD-TDV) in individuals aged 2-45 y: Phase II randomized controlled trial in Singapore. *Hum Vaccin Immunother*. 2012;8(9):1259-1271.
CYD29	NCT01436396	Lopez P, Lanata CF, Zambrano B, et al. Immunogenicity and safety of yellow fever vaccine (Stamaril) when administered concomitantly with a tetravalent dengue vaccine candidate in healthy toddlers at 12-13 months of age in Colombia and Peru a randomized trial. *Pediatr Infect Dis J*. 2016;35(10):1140-1147.
CYD30	NCT01187433	Dayan GH, Garbes P, Noriega F, et al. Immunogenicity and safety of a recombinant tetravalent dengue vaccine in children and adolescents ages 9-16 years in Brazil. *Am J Trop Med Hyg*. 2013;89(6):1058-1065.

CYD32	NCT01254422.	HSS AS, Koh MT, Tan KK, et al. Safety and immunogenicity of a tetravalent dengue vaccine in healthy children aged 2-11 years in Malaysia: A randomized, placebo-controlled, Phase III study. *Vaccine*. 2013;31(49):5814-5821.
CYD33	NCT01411241	Melo FIR, Morales JJR, De Los Santos AHM, Rivas E, Vigne C, Noriega F. Immunogenicity and Safety of a Booster Injection of DTap-IPV//Hib (Pentaxim) Administered Concomitantly with Tetravalent Dengue Vaccine in Healthy Toddlers 15-18 Months of Age in Mexico: A Randomized Trial. *Pediatr Infect Dis J*. 2017;36(6):602-608.
CYD47	NCT01550289	Dubey AP, Agarkhedkar S, Chhatwal J, et al. Immunogenicity and safety of a tetravalent dengue vaccine in healthy adults in India: A randomized, observer-blind, placebo-controlled phase II trial. *Hum Vaccin Immunother*. 2016;12(2):512-518.
DNG10	ctri.nic.in: CTRI/2011/12/002243	Garg S, Chakravarti A, Singh R, et al. Dengue serotype-specific seroprevalence among 5- to 10-year-old children in India: a community-based cross-sectional study. *Int J Infect Dis*. 2017;54:25-30.
DNG26	Received local approvals for this epidemiological study (appendix 2)	Prayitno A, Taurel A, Nealon J, et al. Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children. *PLoS Negl Trop Dis*. 2017;11(6):e0005621.

Appendix 2: Ethics approvals for DNG10 and DNG26 studies in India and Indonesia

भारतीय आयुर्विज्ञान अनुसंधान परिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
अन्सारी नगर, पोस्ट बॉक्स 4911, नई दिल्ली - 110 029
ANSARI NAGAR, POST BOX 4911, NEW DELHI - 110 029

No. TDR/590/2012-ECD-II Dated: 04.07.2013

To

The Dean,
Maulana Azad Medical College,
Delhi-110002

Sub: The project entitled" Prospective dengue seroprevalence study in 5 to 10 year old children in India" under Dr. Suneela Garg, Maulana Azad Medical College, Delhi.

Sir,

This is with reference to the above mentioned proposal which was submitted for consideration in the Health Ministry Screening Committee Meeting for support from Sanofi Pasteur, Lyon, France. The meeting was held on **6th June, 2013** and the relevant portion of minutes is reproduced below:

"Approved for all centres"

You may now take necessary action at your end but kindly let us know the date of initiation of the project. You are also requested to submit 6 monthly progress report of the project to our office with a copy to International Health Division (IHD) of ICMR for necessary action.

It is requested to kindly send 5 copies each of duly filled and signed research proposal, project summary and check list in the format available at ICMR's is website for onward transmission to DST.

Yours faithfully,

(Dr. Nivedita Gupta)
Scientist 'D'
For Director-General

Copy to: Dr. Suneela Garg, Department of Community Medicine, Maulana Azad Medical College, Delhi-110002.

Komite Etik Penelitian Kesehatan
Fakultas Kedokteran Universitas Indonesia
Rumah Sakit Cipto Mangunkusumo
Health Research Ethics Committee
Faculty of Medicine Universitas Indonesia
Cipto Mangunkusumo Hospital
Jalan Salemba Raya No. 6, Jakarta Pusat 10430. Telp. 021-3157008. E-mail: ec_fkui@yahoo.com

Nomor : 462 /H2.F1/ETIK/2014

KETERANGAN LOLOS KAJI ETIK

ETHICAL APPROVAL

Komite Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Indonesia dalam upaya melindungi hak asasi dan kesejahteraan subyek penelitian kedokteran, telah mengkaji dengan teliti protokol berjudul:

The Ethics Committee of the Faculty of Medicine, University of Indonesia, with regards of the Protection of human rights and welfare in medical research, has carefully reviewed the research protocol entitled:

"Seroprevalens Dengue pada Anak Sehat di Perkotaan Indonesia".

Peneliti Utama : **Prof. Dr. dr. Sri Rezeki Hadinegoro, SpA(K)**
Principal Investigator

Nama Institusi : **Ilmu Kesehatan Anak FKUI/RSCM**
Name of the Institution

dan telah menyetujui protokol tersebut di atas.
And approved the above-mentioned protocol.

Jakarta, 14 JUL 2014
Ketua
Chairman

Prof. Dr. dr. Rianto Setiabudy, SpFK

***Ethical approval berlaku satu tahun dari tanggal persetujuan**
****Peneliti berkewajiban**
1. Menjaga kerahasiaan identitas subyek penelitian
2. Memberitahukan status penelitian apabila
   a. Setelah masa berlakunya keterangan lolos kaji etik, penelitian masih belum selesai, dalam hal ini *ethical clearance* harus diperpanjang
   b. Penelitian berhenti di tengah jalan
3. Melaporkan kejadian serius yang tidak diinginkan *(serious adverse events)*
4. Peneliti tidak boleh melakukan tindakan apapun pada subyek sebelum penelitian lolos kaji etik dan *informed consent*

Appendix 3: Email communication with all co-authors requesting permission to use co-authored works

From: Nealon, Joshua /FR
Sent: Monday, January 4, 2021 4:01 PM
To: Nealon, Joshua /FR
Subject: Permission to use our joint publications for PhD thesis

Dear collaborators,

It has been a while since I have been in touch with many of you, but I hope you have had a relaxing time with family and friends over the holiday season.

I have decided to apply for a PhD by research publication, at the University of Edinburgh, and am finalizing my thesis *"Epidemiological conditions to support dengue vaccine introduction in Asia using data from traditional and novel sources"*. The Phd "by publication" route requires applicants to submit published materials to which they have made major contributions. I plan to use ten papers we have written together which will form a thesis discussing dengue incidence, seroprevalence, force-of-infection and effectiveness study design; and how these epidemiological findings contribute to decisions about vaccine use.

As my co-authors, I would like to ask for your permission/agreement that I use the works listed in the table below (with my contributions described) as components of the PhD thesis. Could you please review this table and let me know if you have any objections or would like to discuss?

I apologize for emailing you in 'bcc' but we are a large group and I wanted to respect your privacy and prevent some of you from receiving many emails. For practical purposes, if I do not hear from you within 2 weeks I will assume you have no objections. Please do not hesitate to forward this message to co-authors you know have moved to new positions with new email addresses.

Thanks again for sharing these important projects together; I have learned from you all in this process and would not be in a position to submit this thesis without your support and expertise.

Wishing you all an enjoyable and productive 2021.

Josh.