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Burden of Dengue Infection and Disease in a Pediatric Cohort in Urban Sri Lanka

Hasitha Tissera,* Ananda Amarasinghe, Aruna Dharshan De Silva, Pradeep Kariyawasam, Kizzmekia S. Corbett, Leah Katzelnick, Clarence Tam, G. William Letson, Harold S. Margolis, and Aravinda M. de Silva*

Epidemiology Unit, Ministry of Health, Colombo, Sri Lanka; Genetech Research Institute, Colombo, Sri Lanka; Public Health Department, Colombo Municipal Council, Colombo, Sri Lanka; Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina; London School of Hygiene and Tropical Medicine, London, United Kingdom; Pediatric Dengue Vaccine Initiative, International Vaccine Institute, Seoul, South Korea; Department of Zoology, University of Cambridge, Cambridge, United Kingdom; Saw Swee Hock School of Public Health, National University of Singapore, Singapore; El Paso County Public Health, Colorado Springs, Colorado; Dengue Branch, Centers for Disease Control and Prevention, San Juan, Puerto Rico

Abstract. Dengue is the most significant arthropod-borne viral infection of humans. Persons infected with dengue viruses (DENV) have subclinical or clinically apparent infections ranging from undifferentiated fever to dengue hemorrhagic fever/shock syndrome. Although recent studies estimated that the Indian subcontinent has the greatest burden of DENV infection and disease worldwide, we do not have reliable, population-based estimates of the incidence of infection and disease in this region. The goal of this study was to follow-up a cohort of 800 children living in a heavily urbanized area of Colombo, Sri Lanka to obtain accurate estimates of the incidence of DENV infection and disease. Annual blood samples were obtained from all children to estimate dengue seroprevalence at enrollment and to identify children exposed to new DENV infections during the study year. Blood was also obtained from any child in whom fever developed over the course of the study year to identify clinically apparent DENV infections. At enrollment, dengue seroprevalence was 53.07%, which indicated high transmission in this population. Over the study year, the incidence of DENV infection and disease were 8.39 (95% confidence interval = 6.56–10.53) and 3.38 (95% confidence interval = 2.24–4.88), respectively, per 100 children per year. The ratio of clinically inapparent to apparent infections was 1.48. These results will be useful for obtaining more accurate estimates of the burden of dengue in the region and for making decisions about testing and introduction of vaccines.

INTRODUCTION

Dengue is caused by four related mosquito-borne dengue viruses (DENV1–4), which are endemic to many tropical and subtropical regions of the world. Most persons infected with DENV are asymptomatic or show development of a febrile illness known as dengue fever, but disease can be more severe and progress to dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Infection with one DENV serotype confers protective immunity to future infections with that serotype only and these persons are susceptible to secondary infections with heterologous serotypes.¹ Persons with secondary DENV infections are at a greater risk of DHF/DSS than persons having their first infection.^{2,3}

Globally, an estimated four billion persons are at risk for DENV infection, and there are an estimated 390 million annual DENV infections and 96 million dengue cases.⁴ From prospective community and school-based cohort studies, it has been possible to obtain estimates for the incidence of DENV infection and disease for sites in southeast Asia^{5–10} and Latin America.^{11–13} Although dengue is emerging in the Indian subcontinent and is considered a major health issue, we do not have estimates of the true burden of DENV infection and disease in this region.

Dengue was first reported in Sri Lanka in the 1960s.¹⁴ Studies conducted during 1980–1984 showed a DENV seroprevalence of 50% among school children and an annual seroconversion rate of 10–15% among 5–7-year-old school children in Colombo, the capital of Sri Lanka.^{15,16} More

recently, analysis of age-stratified seroprevalence data indicated that the annual seroconversion rate among children < 12 years of age in Colombo is approximately 14%.¹⁶ In the past, most dengue cases have been reported from the Colombo district and other neighboring districts in the heavily urbanized southwestern region of the country. However, over the past 10–15 years, dengue has been reported from nearly all districts of the island, and over the past two decades, the number of reported DF and DHF cases has increased by more than 10-fold.¹⁷ This increase in cases has been attributed to introduction of new genotypes of DENV, as well as the expansion of the range of the virus on the island.^{17–19} In many ways, the changing epidemiology of dengue in Sri Lanka mirrors events in other parts of the subcontinent including India, which has also documented large increases in cases and appearance of new virus strains.^{20,21} We conducted a population-based study to determine the incidence of symptomatic and asymptomatic DENV infection among children living in Colombo, Sri Lanka, an urban setting of the Indian subcontinent.

MATERIALS AND METHODS

Ethical approval for study. Ethical approval for this research was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka, and the Institutional Research Board of the International Vaccine Institute, Seoul, South Korea. Only children whose parents or legal guardians provided written informed consent were enrolled in the study.

Study site. The study was conducted in the city of Colombo, the commercial capital of Sri Lanka, which has a population of 647,100 and is the most densely populated area in the country; there are 17,353 persons/km².²² The city is divided into 47 municipal wards. Ward 33 was selected for the study because of its stable population, which reflects the socio-economic status and demographics of the entire municipal

*Address correspondence to Aravinda M. de Silva, CB # 7292, Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, E-mail: desilva@med.unc.edu or Hasitha Tissera, Epidemiology Unit, Ministry of Health, 231, De Saram Place, Colombo 10, Sri Lanka, E-mails: dr_korelege@yahoo.co.uk or chepid@sltnet.lk

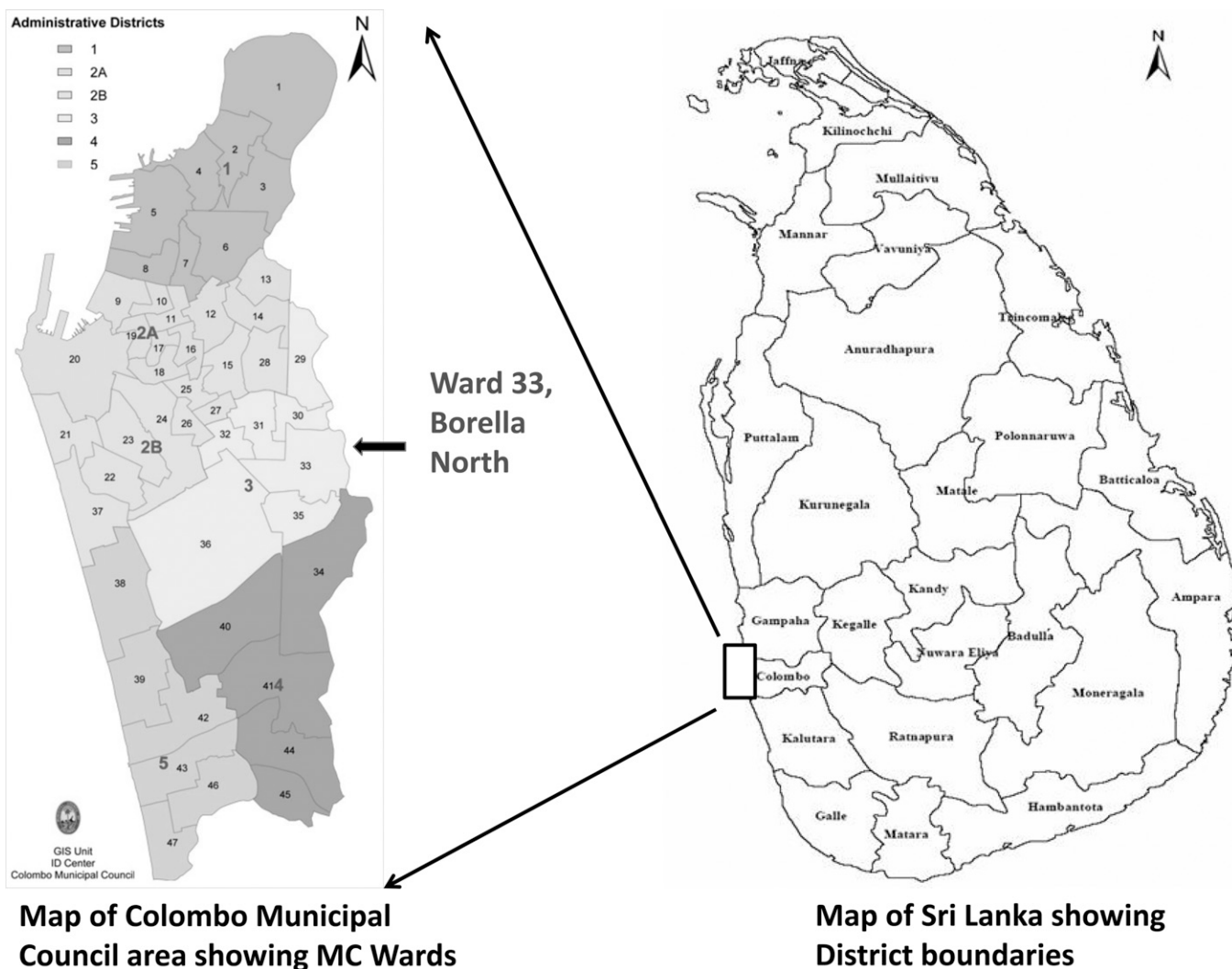


FIGURE 1. Location of study site (Ward 33) in relation to the Colombo Municipal Council (MC) and Colombo District, Colombo, Sri Lanka.

area. Dengue is endemic to this ward, and the catchment population seeking healthcare in the tertiary care institution situated within its boundaries (Figure 1).

Study population. A prospective cohort study was conducted during November 2008–January 2010. The protocol for the study is described in detail elsewhere.²³ In brief, a house-to-house census was conducted by research assistants to determine the size and obtain sociodemographic information and health-seeking patterns of the permanent resident population of children ≤ 12 years of age. A representative sample of 800 children ≤ 12 years of age was recruited for the current study. The sample size calculation was based on the ability to detect an annual incidence of dengue of 10% with an absolute precision of 3%. The estimated sample size was then inflated by 30% to allow for possible loss to follow-up during the study period. All children were enrolled in the study after written informed consent was obtained from parents/legal guardians and consent from children > 7 years of age. Each participating household was given a thermometer and each child was given an identification card with a unique study number. A febrile illness was defined as a temperature $\geq 38^{\circ}\text{C}$ lasting ≤ 7 days in any child in the study cohort documented by a parent, research assistant, or health care profes-

sional. After assessment and treatment by a designated physician, the research assistants interviewed the patient and parent by using a standard case investigation form.

Sample collection. Blood samples were obtained from all children at enrollment (during November 2008–January 2009) and one year after enrollment (during November 2009–January 2010) by finger prick and stored as blood spots on protein saver cards (Whatman, Piscataway, NJ and Id Biological Systems, Greenville, SC).^{24,25} From children with a documented fever, whole blood was collected by venipuncture and placed in tubes containing EDTA. Some of the whole blood was used to prepare dried blood spots for serologic testing. The remaining blood was centrifuged and the plasma used for molecular diagnostic testing. Ten or more days after recovery from fever, convalescent-phase samples were collected by finger prick and stored as blood spots on protein saver cards.

DENV strains used for laboratory assays. The World Health Organization reference DENV strains (i.e., DENV1 West Pac 74, DENV2 S-16803, DENV3 CH54389, and DENV4 TVP-360) were used for preparing antigen and infectious stocks for serologic assays. These reference viruses were initially obtained from Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD). Infectious stocks of

virus were prepared by using the C6/36 mosquito cell line, and dengue antigens were harvested from Vero cells as described.²⁶

Molecular detection of DENV. Plasma obtained during the acute phase of the febrile illness was tested by reverse transcription–polymerase chain reaction (RT-PCR) to detect and serotype DENVs as described.¹⁷

Detection of IgM and IgG against DENV in dried blood spots. Antibodies were eluted from dried blood spots by submerging the filter paper in phosphate-buffered saline and incubating at 37°C for 2.5 hours. The final volume of the eluted antibody was adjusted to obtain a 1:50 or 1:100 dilution of the original blood volume applied to the filter paper. A recent report validated the use of dried blood spots for dengue serologic analysis.²⁷

We performed dengue IgM capture enzyme-linked immunosorbent assay (ELISA) as described,²⁸ except that monoclonal antibody (MAb) 4G2 against flavivirus, followed by enzyme-conjugated goat anti-mouse IgG to detect captured DENV antigen. In brief, 96-well plates were coated (overnight at 4°C) with 100 µL/well (1 ng/µL) of goat anti-human IgM (Sigma, St. Louis, MO) at a concentration of 0.1 mol/L in carbonate buffer, pH 9.6. Plates were washed three times in Tris-buffered saline containing 0.2% Tween 20 (TBST) and blocked with 200 µL/well of 1 × Tris-buffered saline containing 0.05% Tween 20 and 3% nonfat dry milk. Paired serum samples were tested on the same plate. Diluted serum (1:50) was loaded in duplicate and incubated (37°C for 1 hour) to capture IgM. Unbound antibody was washed, and wells were successively incubated with DENV antigen (mixture of serotypes DEN1–4), mouse anti-flavivirus 4G2 MAb, and human-absorbed alkaline phosphatase (AP)–conjugated goat anti-mouse IgG (Sigma). Optical density (OD) was measured at 405 nm after final incubation with AP substrate.

Dengue IgG ELISA was performed as described.²⁹ Plates were coated overnight (4°C) with 100 µL/well of mouse anti-flavivirus 4G2 MAb at a concentration of 0.1 mol/L in carbonate buffer, pH 9.6, and then washed three times in TBST. Plates were then blocked with standard diluents and successively incubated (37°C for 1 hour) with DEN1–4 antigen, diluted serum (1:100) in duplicate wells, and AP-conjugated goat anti-human IgG (Fc portion). Three washings with TBST were conducted between incubations. Plates were read at 405 nm after a final incubation with AP substrate (15 minutes at room temperature in the dark).

Measurement of DENV neutralizing antibodies. The presence of DENV neutralizing antibody was determined by using a flow cytometry–based neutralization assay with U937 monocytic cells stably transfected with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin as described.³⁰ This flow cytometry–based neutralization test has comparable specificity to the conventional dengue plaque-reduction neutralization test and does not detect antibodies against Japanese encephalitis virus (Broadwater A, de Silva AM, unpublished data).³⁰ Blood samples were serially diluted (four-fold dilutions starting at 1:40 and ending at 1:10,240) and the serum dilution that neutralized 50% of the viruses was calculated by nonlinear, dose-response regression analysis with Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

Determination of dengue serostatus at study enrollment. DENV serostatus (DENV naive or immune) at enrollment was determined by using a dengue IgG immunoassay. An OD value ≥ 0.3 were considered dengue antibody positive.

During the study period, primary infections were defined as dengue-naive children at enrollment who had had an infection. Secondary infections were defined as dengue-immune children at enrollment who had had an infection.

Laboratory criteria for diagnosis of new DENV infections.

A laboratory-confirmed clinically apparent dengue case was defined as a child who had a febrile illness and showed positive results in at least two of the three diagnostic assays (PCR, increasing levels of IgM and/or IgG in paired acute-phase and convalescent-phase blood samples). In the few cases in which there was only an acute-phase blood sample, the diagnosis was based on RT-PCR testing only. To detect clinically inapparent infections, paired baseline and end-of-year blood samples from all children were tested by IgG immunoassay. Dengue-naive children at enrollment (391 children) who seroconverted over the study year (baseline sample seronegative and end-of-year sample seropositive [≥ 0.3 OD units]) were classified as primary infections. Classification of these cases as primary infections was confirmed by neutralization testing of selected samples. The DENV-immune children at enrollment (441 children) who had increasing levels of IgG against DENV by the end of the year (≥ 0.1 OD units) were initially classified as secondary infections. However, neutralization testing indicated that when samples were collected 12 months apart, the IgG ELISA had poor specificity for detecting secondary infections (Corbett KS and others, unpublished data). Therefore, we tested paired baseline and end-of-year samples from all children who were dengue immune at enrollment for the presence of neutralizing antibodies against each of the four serotypes.

To establish criteria for defining secondary infections by using paired samples obtained 12 months apart, we used a test set of eight symptomatic secondary cases (detected by PCR and serologic analysis performed on samples collected within one month of acute infection). Only half (4 of 8) of the cases had a ≥ 4 -fold increase, and all (8/8) of the cases had a ≥ 2 -fold increase in neutralizing antibody levels when paired baseline and end-of-year samples from these children were tested. Therefore, children who had ≥ 2 fold increases in levels of neutralizing antibodies to one or more serotypes by the end of the year were designated as secondary infections. Samples were tested at least twice and only paired samples that displayed a reproducible two-fold increase were included as new secondary infections. Inapparent cases were defined as children who had not been identified as a clinical dengue case over the study year but still seroconverted by IgG immunoassay (primary infections) or had increasing levels of neutralizing antibodies (secondary infections) by the end of the study year.

RESULTS

During November 2008–February 2009, a total of 800 children between 0–12 years of age were enrolled in the study from ward 33 (Figure 1); these children were representative of the age and demographic distribution of the 2,527 children known to be permanent resident of the ward. Only one child was lost to follow-up during the study period.

The dengue seroprevalence at enrollment was determined by testing all children by ELISA for IgG against DENV (Table 1). To confirm that IgG seropositivity was caused by dengue infection and not exposure to related Japanese encephalitis virus, which is also present in Sri Lanka, all IgG-positive

TABLE 1
Age-specific dengue seroprevalence in children, Sri Lanka*

Age group (years)	No. children	No. seropositive by IgG ELISA	No. seropositive confirmed by dengue neutralization test	Seroprevalence (%)
< 1	51	12	11	21.57
1–3	196	71	67	34.18
4–6	191	105	100	52.36
7–9	225	150	145	64.44
10–12	136	103	101	74.26
Total	799	441	424	53.07

* Dengue seroprevalence at the beginning of study (baseline) was determined by screening all serum samples by dengue IgG enzyme-linked immunosorbent assay (ELISA) and then testing the positive samples by dengue neutralization test.

samples were tested for DENV-neutralizing antibodies. Most (96%) of IgG-positive serum samples also neutralized DENV, indicating that in this population the results of the IgG ELISA reflected dengue seroprevalence. The overall dengue seroprevalence was 53% at enrollment (Table 1). The age specific seroprevalence steadily increased with age from a low of 22% in the youngest (< 1 year) age group to a high of 74.26% in the oldest (10–12 years) age group (Table 1).

A total of 681 fever episodes were detected among the 799 children, indicating that on average each child reported 0.9 fever episodes over one person-year of follow-up (Table 2). Dengue was laboratory confirmed for 3.9% (27 of 681) of the fever episodes (Table 2); no child had more than one episode of dengue fever over the course of the study. Therefore, the incidence of clinically apparent dengue in the cohort was 3.38 cases/100 children/year (Table 2). When combined with the clinically inapparent infections, the total incidence of DENV infection was 8.39 cases/100 children (Table 2). The highest incidence of infection and disease was in children 1–3 years of age. The overall ratio of inapparent to apparent infections was 1.48. We used a two-fold instead of a four-fold increase in neutralizing antibody levels to identify new secondary infections because a four-fold increase had poor sensitivity for paired samples collected 12 months apart. It is possible that the two-fold increase reduces specificity and leads to a modest overestimation of true incidence.

Of the 67 new apparent and inapparent DENV infections, 35 were primary infections and 32 were secondary infections (Table 3). As might be expected, the ratio of primary to secondary infections decreased with age from 3 in the youngest (< 1 year) age group to 0.75 in the oldest (10–12 years) age group. The ratios of inapparent to apparent infections were not statistically significantly different ($P > 0.1$) between primary and secondary infections (Table 3).

The prevalence of each DENV serotype during the study year was estimated by identifying the serotypes responsible

TABLE 3
Dengue disease outcome in children exposed to primary or secondary infections, Sri Lanka

Infection, no.	Disease		
	Inapparent	Apparent	Inapparent:apparent ratio
Primary, 35	20	15	1.33
Secondary, 32	20	12	1.67
Total, 67	40	27	1.48

for primary infections (Supplemental Table 1). DENV2 was the most common serotype (49%) followed by DENV3 (27%), DENV1 (23%), and DENV4 (3%). The number of persons with monospecific neutralizing antibodies to each serotype at enrollment into the study was used to estimate the prevalence of each serotype in this population before the start of our study (Supplemental Table 1). This analysis showed that DENV2 (43%) and DENV3 (29%) were more common than DENV1 (9%) and DENV4 (12%) in the period preceding the study.

DISCUSSION

Over the past two decades, dengue has emerged as a major health problem in the Indian subcontinent.^{20,21} Dengue epidemics in the region have been linked to more intense transmission, expansion of the range of the virus, and introduction of new strains.^{17–21} Most estimates of dengue incidence in the region are based on hospital-based studies and nationally reported cases, which grossly underestimate the true burden of disease and infection.³¹ We followed a cohort of 799 children in Colombo, Sri Lanka, and estimated the incidence of infection and disease to be 8.39 and 3.38 cases/100 children, respectively. The ratio of clinically inapparent to apparent infections was 1.48, which indicated that for every apparent infection there were approximately 1.5 inapparent infections in children. This high intensity of transmission was also supported by the seroprevalence data, which showed a gradually increasing prevalence with age that ranged from 22% in the youngest age group to 74% in the oldest age group. The four DENV serotypes were circulating in this population before and during our study; serotypes 2 and 3 were more prevalent than serotypes 1 and 4 (Supplemental Table 1). Our study establishes a rigorously measured, accurate burden of dengue infection and disease among children living in an urban area of Sri Lanka.

Recently, we used dengue seroprevalence data from this cohort to model the rate of primary infections in dengue-naive children.¹⁶ Using a catalytic model, we estimated the incidence of primary infection to be 14.1% per year

TABLE 2
Age-specific fever episodes and incidence of dengue infection and illness in children, Sri Lanka*

Age (years)	No. children	Fever episodes		New dengue infections			Incidence of infection per 100 children	Incidence of disease per 100 children
		Total	Mean/child	Total	Inapparent	Apparent		
< 1	51	31	0.6	1	0	1	1.96 (0.05–10.45)	1.96 (0.05–10.45)
1–3	196	252	1.3	27	15	12	13.78 (9.28–19.41)	6.12 (3.20–10.45)
4–6	191	174	0.9	13	11	2	6.81 (3.67–11.36)	1.05 (0.13–3.73)
7–9	225	137	0.6	15	6	9	6.67 (3.78–10.76)	4.00 (1.85–7.46)
10–12	136	87	0.6	11	8	3	8.09 (3.25–16.03)	2.21 (0.46–6.31)
Total	799	681	0.9	67	40	27	8.39 (6.56–10.53)	3.38 (2.24–4.88)

* Values in parentheses are 95% confidence intervals. Clinically apparent dengue infections were diagnosed by performing paired serologic analysis and polymerase chain reaction on acute-phase and convalescent-phase serum samples obtained from febrile children. Total dengue infections over the study year were detected by testing paired baseline and end of year samples by IgG enzyme-linked immunosorbent assay and neutralization test. Children who were infected but not identified as having apparent cases were designated as having inapparent cases.

(95% confidence interval [CI] = 12.7–15.6%), which is higher than the incidence of 8.39% (95% CI = 6.56–10.53%) observed for total infections (primary and secondary infections). A more legitimate comparison would be to compare the model based estimate for primary infections with the incidence of primary infections in our cohort. The incidence of primary infection noted in the current study was 9.8% (95% CI = 6.80–12.80%), which is closer to the model-based estimate of 14.1% per year (95% CI = 12.7–15.6%). A previous study of school children in Colombo over a five-year period during 1980–1985 estimated the incidence of new infections to range between 10.78 and 18.54 cases/100 children in different years.¹⁵ Collectively, these findings demonstrate intense transmission of dengue among children in Colombo.

Our current estimate for disease (3.38 cases/100 children < 12 years of age) is 14-fold higher than the reported national incidence of the disease (0.23/100 persons) among persons < 15 years of age in 2010 in Sri Lanka, which is based on the passive national surveillance system (Epidemiology Unit, Ministry of Health). Based on national surveillance for 2010, the disease estimates for children < 15 years of age in the Colombo district and the Colombo Municipality (study area) (Figure 1) were 0.64 and 1.61 cases/100 persons, respectively. Thus, as expected, the passive national program underreports the true burden of disease in Sri Lanka. Recent studies in Southeast Asia have estimated expansion factors of 8.9 and 9.6 when comparing national data with true disease incidence for Thailand and Cambodia, respectively.^{7,32} Moreover, a recent study of the global burden of dengue estimated that there are three times as many infections and dengue cases compared with cases reported to the World Health Organization.⁴

For most regions in the world with endemic dengue transmission, we do not have accurate estimates of infection and disease because of the cost and infrastructure required to conduct cohort studies. The most comprehensive cohort studies on dengue in Southeast Asia have been conducted in Thailand where the incidence of infection has ranged between 2% and 15% depending on the year and location of the study.^{5,6,8,33,34} Studies in Puerto Rico, Nicaragua, and Peru have documented incidences that range between 1.34% and 12% depending on the year and location.^{11,12} Thus, the intensity of dengue transmission among children in Colombo (8.14%) is similar to transmission documented in other cohort studies conducted in Southeast Asia and Latin America. The ratio of inapparent to apparent infections has also been estimated in different regions of the world. In southeast Asia, the ratio has ranged from 3:1 to 1:1, whereas in Latin America the ratio has ranged from 3:1 to 18:1, indicating that infections in Southeast Asia^{5,6,8} are more likely to lead to clinically apparent cases than infections in Latin America.^{11,12} The data we report indicate that the situation in Colombo Sri Lanka with a ratio of 1.5:1 is more similar to the situation in Southeast Asia.

The current study was not designed to test hypotheses about risk factors for DHF/DSS because our cohort of 799 children is too small to detect significant numbers of DHF/DSS cases. We did not observe any significant differences in the ratio of inapparent to apparent cases between primary and secondary cases. However, it is premature to conclude from these data that risk of developing a clinically apparent infection is the same in primary and secondary cases because the study was only powered to detect a large difference in risk after primary and secondary infection. Other studies have documented that

children exposed to primary and secondary infections have a similar risk of developing clinically apparent infections.⁶

It is well documented that the incidence of infection and disease can vary from year to year and our data were collected over a single year only. Our study was confined to a relatively small area and our results may not be generalizable to children in other parts of the city and country. Thus, studies such as this have to be conducted in other parts of the country over a longer period to understand how the incidence of infection and disease vary by location and over time. Dengue vaccines are currently being tested in different regions of the world.³⁵ It is important that trials also be conducted in the Indian subcontinent given the heavy burden of disease and the distinct strains of DENV circulating in the region. The results of the current study will be useful for designing vaccine trials in southern Asia and for making decisions about how best to introduce vaccines.

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Authors' addresses: Hasitha Tissera, Epidemiology Unit, Ministry of Health, Colombo, Sri Lanka, E-mail: dr_korelege@yahoo.co.uk. Ananda Amarasinghe, Epidemiology Unit, Ministry of Health, Colombo, Sri Lanka, and International Vaccine Institute, Pediatric Dengue Vaccine Initiative, Seoul, South Korea, E-mail: ana_amarasinghe@yahoo.co.uk. Aruna Dharshan De Silva, Genetech Research Institute, Colombo, Sri Lanka, E-mail: dslv90@yahoo.com. Pradeep Kariyawasam, Colombo Municipal Council, Public Health Department, Colombo, Sri Lanka, E-mail: munici@slt.lk. Kizzmekia S. Corbett and Aravinda M. de Silva, Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC, E-mails: kscorbet@email.unc.edu and aravinda_desilva@med.unc.edu or desilva@med.unc.edu. Leah Katzelnick, Department of Zoology, University of Cambridge, Cambridge, United Kingdom, E-mail: lck33@cam.ac.uk. Clarence Tam, London School of Hygiene and Tropical Medicine, London, United Kingdom, E-mail: clarence.tam@lshtm.ac.uk. G. William Letson, Center for Global Public Health, Colorado School of Public Health, Aurora, CO, E-mail: bwletson@gmail.com. Harold S. Margolis, Dengue Branch, Centers for Disease Control and Prevention, San Juan, PR, E-mail: hsm1@cdc.gov.

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