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High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua

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Summary To investigate the incidence of dengue virus (DENV) infection in Nicaragua, a 2-year prospective study was conducted in schoolchildren 4–16 years old in the capital city of Managua. Blood samples were collected before the rainy season in 2001, 2002 and 2003, and were assayed for DENV-specific antibodies. Participants were monitored for dengue-like illness, and acute and convalescent blood samples were collected from suspected dengue cases. In 2001 and 2002, 602 and 397 students were recruited, respectively, and paired annual serum samples were available from 467 and 719 participants in 2001–2002 and 2002–2003, respectively. The overall seroprevalence of anti-DENV antibodies was 91%, increasing from 75% at age 4 to 100% at age 16. The incidence of DENV infection was 12% in Year 1 and 6% in Year 2 ($P < 0.001$). During Year 1, four laboratory-confirmed dengue cases were detected, with one DENV2 isolate; during Year 2, there were six confirmed dengue cases, with one DENV1 isolate. These and additional circulating serotypes were confirmed by plaque reduction neutralisation test. This study demonstrates surprisingly high transmission of DENV in urban Nicaragua.

keywords dengue, schoolchildren, Nicaragua, seroprevalence, incidence of infection, prospective study

Introduction

Dengue is caused by any of the four serotypes of the mosquito-transmitted dengue virus (DENV1–4). DENV infection results in asymptomatic infection or a spectrum of acute febrile illnesses ranging from classic dengue fever, usually accompanied by headache, muscle and body aches, retro-orbital pain, and rash, to dengue haemorrhagic fever (DHF) – dengue fever with plasma leakage accompanied by haemorrhagic signs and thrombocytopenia – which when left untreated can lead to shock and sometimes death. In the past decades, as case management has improved, mortality rates due to dengue have decreased; nevertheless, the burden of disease and its economic impact is great.

Dengue has been documented in Southeast Asia and the Americas for over two centuries (Gubler 1998). Since the 1950s, the Asian region has been hyperendemic for dengue (all four serotypes circulating simultaneously), with DHF as the leading cause of paediatric hospitalisation (Halstead 1997). In the Americas, after the collapse of an intensive *Ae. aegypti* eradication programme in the 1970s, dengue caused by all four serotypes spread once again throughout the region (Pan American Health Organization 1994). DHF was first reported in the 1980s in several Latin American and Caribbean countries, although the incidence of DHF is less than that in Southeast Asia (Gubler 1997). In general, transmission of dengue in the Americas is thought to be lower than in Southeast Asia. However, studies in Nicaragua in 1998–2001 demonstrated that most dengue cases presenting to hospitals and health centres were secondary DENV infections, even in very young children (Hammond *et al.* 2005; Harris *et al.* 2000), suggesting a much higher incidence of infection with the virus than was previously thought. This study establishes symptomatic and asymptomatic rates of DENV infection using a prospective school-based cohort and demonstrates an unexpectedly high rate of DENV transmission in urban Nicaragua.

Materials and methods

Study population

The study population consisted of students aged 4–16 years old who attended 'El Centro Escolar Autónomo de Ruben Darío', a public primary and secondary school in Barrio San Luis, an urban region of Managua, Nicaragua. Recruitment occurred in May of 2001 and May of 2002. Informed consent was obtained from the parents/guardians of schoolchildren, and assent was obtained from all participants over the age of five. This study was approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley and the Ethical Review Committee of the Centro Nacional de Diagnóstico y Referencia of the Nicaraguan Ministry of Health (MOH).

Study design

Annual blood samples were collected prior to the dengue season in May of 2001, May of 2002, and March of 2003 for serological testing for anti-DENV antibodies. Participants were monitored for dengue-like illness through a variety of methods. Firstly, school attendance of all study participants was taken by a study nurse, and motive of absence was determined. As school absenteeism was extremely high, resource constraints permitted the study nurse to visit the absent child's house only on the third day of absence. Secondly, a nurse's station at the school was made available to all schoolchildren, where study participants with a febrile illness were encouraged to seek medical attention and screening for dengue. Thirdly, all study participants received a special identification card to permit rapid access to a study doctor at the local health centre. Children with fever and dengue-like symptoms were identified as suspected cases of dengue. Acute (first day of identification) and convalescent phase serum samples were collected. Reverse transcriptase-polymerase chain reaction (RT-PCR) and virus isolation were performed on acute phase samples. Both acute and convalescent phase serum samples were processed for detection of DENV-specific antibodies.

Serological assays

Serum samples were frozen at −20 °C until serological testing was performed. Sequential annual samples from the same individual were processed side-by-side; only paired serum samples were evaluated. Two different assays were performed using cellular DENV antigen prepared from all four serotypes (Balmaseda *et al.* 2003): IgM antibody-capture ELISA (Kuno *et al.* 1991) modified as described by Balmaseda *et al.* (Balmaseda *et al.* 2003) and Inhibition ELISA (IE), which detects total anti-DENV antibodies (Balmaseda *et al.* 2003; Harris *et al.* 2000). The IE was performed as described (Fernandez & Vasquez 1990) with minor modifications. Briefly, 96-well polystyrene plates were coated with human anti-DENV immunoglobulins at a protein concentration of 10 μg/ml and incubated overnight at 4 °C. After washing the wells with phosphate buffered saline (PBS) plus 0.05% Tween 20 (PBS-T), 1% bovine serum albumin (BSA) diluted in PBS-T was added and incubated for 30 min at 37 °C. The mixture of the four DENV antigens was diluted 1:80 in PBS-T and added to each well, after which the plates were incubated for 1 h at 37 °C. After washing with PBS-T, the specimen (serially diluted in PBS-T with 0.4% BSA from 1:20 to 1:20 480) was added. The negative controls were added at a dilution of 1:20, and the positive controls were added at a dilution of 1:5120. The plates were then incubated for 1 h at 37 °C. After additional washes with PBS-T, HRP-conjugated human anti-DENV antibody diluted 1/6000 in PBS with 2.5% Normal Human Serum was added to each well. The plates were incubated for 1 h at 37 °C and washed with PBS-T, then the substrate tetramethyl-bencidine was added to each well. The plates were held at room temperature for 10 min, the reaction was stopped with 12.5% sulphuric acid, and the OD was read at 450 nm in an ELISA reader. All samples were processed in duplicate. The titre of each sample was calculated as the last dilution for which the per cent of inhibition (% I) was equal to or >50. The % I was calculated using the following formula:
$\%I = [1 - (\text{Absorbance of the sample}/\text{Average absorbance of the negative controls})] \times 100$.

Twenty-one per cent of samples were also processed by haemagglutination inhibition assay (HI) (Clarke & Casals 1958), using mouse brain DENV antigen (Instituto de Medicina Tropical 'Pedro Kouri', Havana, Cuba). Seropositivity was evaluated in 197 (21.2%) samples by HI, as compared with IE, resulting in a sensitivity of 98.9% and a specificity of 100% for IE. In addition, endpoint titration by HI was performed on 106 (11.4%) of the samples, with a Pearson's coefficient of 0.80 compared with IE results. Antibody titres determined by IE were approximately one dilution higher than HI titres, as noted previously (Harris *et al.* 2000). An additional 85 (7%) sample pairs were also processed by plaque reduction neutralisation test (PRNT). All PRNT testing was carried out by the US Naval Medical Research Institute Detachment (NAMRID), Lima, Perú (Morens *et al.* 1985) at a 1:30 and 1:60 dilution for DENV3 and DENV4 and 1:60 and 1:120 dilution for DENV1 and DENV2.

Although West Nile Virus (WNV) has not yet been detected in Nicaragua, 82 study samples were analysed by IgM MAC-ELISAs with DEN or WNV antigen (CDC, Fort Collins, CO, USA) in parallel, and the results suggest that our population is naïve to WNV infection (data not shown). PRNT on a similar set of 100 serum samples from 6-year-old children in Managua, performed at Mahidol University, Thailand, also indicated that Nicaraguan children had not been exposed to WNV (S. Yoksan, Mahidol University, Bangkok, Thailand, pers. comm.).

Definitions

A positive DENV infection was defined as a ≥4-fold increase in IE titre. An asymptomatic DENV infection was defined as a positive DENV infection with no documented DENV disease episode. A symptomatic dengue case was defined as a febrile illness wherein serum samples yielded DENV-specific IgM antibodies, a ≥4-fold increase in IE titre from acute to convalescent phase sera, or detection of DENV via RT-PCR or virus isolation. The symptomatic infection rate was calculated as the number of cases of dengue divided by the population under study in a 1-year period, whereas the rate of asymptomatic infection was calculated as the number of DENV infections with no documented dengue disease divided by the study population during a 1-year period. A primary or secondary infection was defined as a positive DENV infection whose first annual sample was seronegative (titre < 1:20 by IE) or seropositive (titre ≥ 1:20), respectively.

Statistical analysis

All data were analysed in Epi-Info 6.0.2 (CDC, Atlanta, GA, USA) via chi-squared analysis. Determination of Pearson's coefficient was performed in Microsoft Excel.

Results

Study population

In May 2001, 602 children were enrolled in the study; 396 children were newly recruited prior to the second sample collection in May 2002. Paired annual samples for 2001–2002 and 2002–2003 were available from 467 (78%) and 719 (84%) participants, respectively. Three hundred and ninety-eight children remained enrolled for the entire duration of the study. The vast majority (>95%) of those who did not complete the study were those who withdrew from the school, many of whom moved out of the area. The average age was 8.8, 9.4, and 10.4 years old at the time of sample collection in 2001, 2002 and 2003, respectively. An equal male to female ratio was maintained throughout the 2 years of the study.

To characterise the overall health status of the population, haematocrit, white blood cell (WBC) count, weight and height were monitored at the time of the annual blood sample collection. The percentage of participants with anaemia diminished slightly from 2001 to 2003 (from 19% to 14%), while the percentage of low-weight children (approximately 13%) was stable over the study period. The prevalence of leucocytosis (WBC > 10 000/mm^3) was high among participants, and was found to steadily increase over the 2 years, from 16% to 24%.

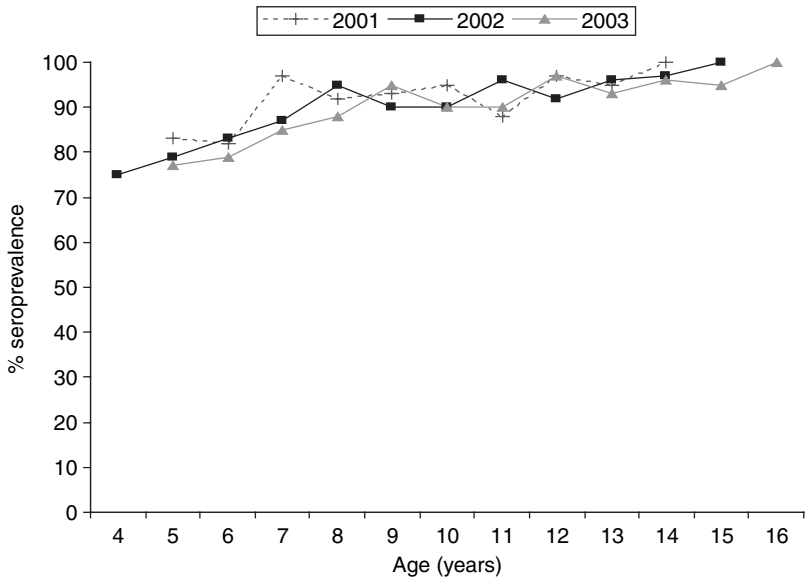
Seroprevalence and incidence of DENV infection

Seroprevalence of anti-DENV antibodies, as determined by IE on paired serum samples, increased with age (Figure 1). By age five, 80% of the study population had been exposed to DENV, and over 90% of children >7 years old showed evidence of exposure to DENV. The overall seroprevalence of anti-DENV antibodies in the study population over the 3 years was 91%. Incidence of infection was determined by a ≥4-fold increase in IE titre in sequential annual samples; analysis of 85 annual paired serum samples by PRNT compared with IE yielded high sensitivity and specificity. A significant reduction in incidence of infection was detected over the 2 years, dropping from 12% to 6% (Table 1). The primary infection rate (DENV infections in seronegative persons) was 18% and 6% in Years 1 and 2, respectively. The secondary infection rate (DENV infections in seropositive persons) was 11% and 6% in Years 1 and 2, respectively.

Incidence of disease

In Years 1 and 2, respectively, 14 and 30 children were suspected of dengue, of whom four (29%) and six (20%) children were laboratory-confirmed dengue cases, representing a symptomatic infection rate of 8.5/1000 and 8.3/1000 (Table 1). Two cases were positive by RT-PCR and virus isolation, one in each year of the study. The majority of cases displayed a ≥4-fold increase in IE titre and a seroconversion of DENV-specific IgM antibodies between acute and convalescent phase samples. In Year 1 of the study, one child with laboratory-confirmed DENV infection was hospitalised and treated for DHF.

Early case detection through monitoring of attendance was not effective, as absenteeism rates were extremely high. Analysis showed that when the duration of absence was 1, 2 or 3 days, the percentage of absence because of febrile illness was 0.07%, 6.2% and 24%, respectively. Therefore, children were visited at their homes after 2 days



2001	*	40	45	66	60	55	73	60	36	22	8		
2002	32	33	60	67	92	105	107	106	87	57	32	11	*
2003		32	34	46	60	84	98	99	103	81	52	21	7

Figure 1 Age-stratified seroprevalence of participants. Seroprevalence was determined using the Inhibition ELISA assay, which detects total anti-dengue virus (DENV) antibodies, to analyse paired annual serum samples. The *n* for each year of age in 2001, 2002 and 2003 is presented in the table below the graph. The data for 4-year olds in 2001 and 16 years olds in 2002 were not included because of low numbers ($n = 2$–3), as indicated by an asterisk.

Table 1 Incidence of symptomatic and asymptomatic dengue virus (DENV) infections according to immune status in Years 1 and 2

	Year 1: 2001–2002			Year 2: 2002–2003		
	Primary	Secondary	Total	Primary	Secondary	Total
Infections*	7 (18)	48 (11)	55 (12)	5 (7)	36 (6)	41 (6)
Cases†	0	4	4 (0.85)‡	0	6	6 (0.83)‡
Total population§	39	428	467	70	649	719
DENV isolates		DENV2			DENV1	

No. of infections (% of total).
* ≥4-fold increase in Inhibition ELISA titre (symptomatic or asymptomatic infection).
† Febrile episode with laboratory-confirmed DENV infection (symptomatic infection).
‡ The percentage is calculated as cases divided by the total population under study.
§ The total 'primary population' began the year seronegative for anti-DENV antibodies, whereas the total 'secondary population' began the year seropositive for anti-DENV antibodies.

of absence. Although a small percentage of suspected cases were detected through house visits, all confirmed cases were detected when the ill subject sought medical attention from either the study nurse at the school or the study physician at the corresponding health centre.

Circulating serotypes

One DENV2 infection and one DENV1 infection in symptomatic dengue cases were detected by RT-PCR and virus isolation during Years 1 and 2, respectively. PRNT performed on 85 pairs of sequential annual serum samples confirmed that DENV2 and DENV1 were the predominant circulating serotypes for Years 1 and 2, respectively (Figure 2). PRNT also revealed the circulation of DENV1, DENV2 and DENV4 in both years and confirmed the predominance of DENV3 before the beginning of the study.

Discussion

This 2-year study of schoolchildren is the first to prospectively investigate seroprevalence and symptomatic and asymptomatic incidence of DENV infection in a healthy

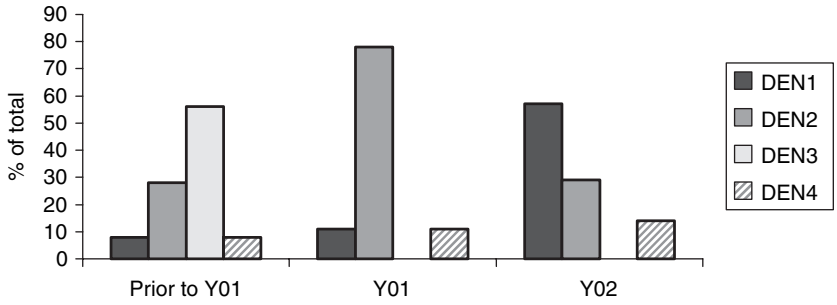


Figure 2 Circulating DENV serotypes between annual routine blood samples, as determined by PRNT. Prior to Year 1 of the study, circulating DENV serotypes were inferred from 2001 serum samples containing neutralising antibodies to a single serotype, or containing titres of neutralising antibodies to one serotype >2-fold higher than the titres against other serotypes. Circulating DENV serotypes during Years 1 and 2 were determined from paired sera in which a monotypic seroconversion was observed, or in which the titre against a single serotype that was not present in the pre-infection sample increased >2-fold. The numbers of paired sera for which the infecting serotype could be determined were limited, as the majority of the DENV infections were secondary, with cross-reacting neutralising antibodies, and endpoint titrations by PRNT were not performed. Prior to Year 1, $n = 25$ sera; Year 1, $n = 9$ paired sera; Year 2, $n = 7$ paired sera.

population in Central America. An unexpectedly high seroprevalence of anti-DENV antibodies was documented at very young ages. The incidence of symptomatic and asymptomatic infection varied significantly between the 2 years, as did the predominant circulating DENV serotype.

In 1985, the prevalence of anti-DENV antibodies in a small cross-sectional study in Nicaragua was 20% across all ages and 13% in children 5–15 years old (G. Huelva Boniche and A. Gonzalez, Ministry of Health, Managua, Nicaragua, pers. comm.). In 1997, serological screening demonstrated an overall seroprevalence of anti-DENV antibodies of 77%, ranging from 66% in children to 81% in adults (A. Balmaseda and J. de los Reyes, unpublished results). The present study determined the seroprevalence of DENV-specific antibodies in children in Managua ages 4–16 to be 91%, with a steady increase from 75% at age 4 to 100% by age 16. Hence, we document a dramatic increase in seroprevalence over the 20-year period since dengue was first reported in Nicaragua.

Few studies in the Americas have investigated seroprevalence of anti-DENV antibodies in a healthy population. In general, the data available from large urban centres in the Americas – Iquitos, Perú (Hayes *et al.* 1996), Salvador, Brazil (Teixeira *et al.* 2002) and Rio de Janeiro, Brazil (da Cunha *et al.* 1995) – indicate lower seroprevalence levels (66–83%) than in Managua. However, in Santo Domingo, Dominican Republic, the seroprevalence in older ages is similar to that in Nicaragua, although the increase in seroprevalence by age is less steep (Yamashiro *et al.* 2004).

The overall incidence of DENV infection (symptomatic and asymptomatic disease) in our study population was 120/1000 in Year 1 and 60/1000 in Year 2, similar to those reported in Southeast Asia (Burke *et al.* 1988; Sangkawibha *et al.* 1984; Strickman *et al.* 2000). Comparison with the incidence of DENV infection in other Central American countries is difficult because such data are not available. The drop in incidence of DENV infection between the first and second years of the study may be due to various factors; one explanation could well be increased vector control efforts during Year 2, when an intensive campaign was conducted by the Nicaraguan MOH in Managua that consisted of source reduction, abatement and focal insecticide application. Larval indices in the school district dropped significantly from 9.3 and 1.3 (Breteau and container indices, respectively) in January 2002 to 4.2 and 0.7 in January 2003. The school grounds were also treated with insecticide and underwent a container destruction campaign in Year 2 of the study. This suggests that effective vector control measures may have a measurable impact on incidence of DENV infection, especially as Year 2 coincided with the introduction of a new serotype, DENV1, which would have been expected to result in a greater increase in infections because of the large pool of susceptibles in the population. In support of the hypothesis that control efforts were effective, the incidence of dengue cases in Managua reported to the MOH dengue surveillance system decreased by over half in Year 2 as well (J.J. Amador, unpublished data). The following year, when vector control measures diminished, a large epidemic spurred by the dissemination of DENV1 occurred (Balmaseda *et al.* 2006). To confirm this hypothesis, however, a more detailed analysis of entomological indices coupled to serological incidence data would be necessary, as year-to-year differences in dengue incidence can occur even in the absence of specific mosquito control programmes.

The incidence of symptomatic disease, 8.5/1000 in Year 1 and 8.3/1000 in Year 2, was significantly higher than that reported by the MOH dengue surveillance system for Managua (0.8/1000 in Year 1, and 0.3/1000 in Year 2). This indicates that the study surveillance system was at least 10-fold more sensitive than the dengue surveillance system, and suggests that the study surveillance system was significantly more effective in Year 2 of the study. The ratio of symptomatic to asymptomatic disease was 1:13 in Year 1 and 1:6 in Year 2. The change in this ratio is likely because of increased case detection in Year 2. One study in Thailand reported a similar ratio of symptomatic to asymptomatic disease (1:7) (Burke *et al.* 1988). Another found the ratio to be 1.0:0.9 (Endy *et al.* 2002). The difference in this last report is likely because of differences in study design; despite similar rates of absenteeism in the recent Thai and Nicaraguan studies, Endy *et al.*'s study (Endy *et al.* 2002) included house visits and sample collection from all febrile cases, with or without dengue-like symptoms, on the first day of absence. This strategy is likely to have detected numerous cases that would not have been reported as dengue-like illness 3 days after onset of fever, which is when house visits were conducted in the Nicaraguan study.

It should be noted that our measure of incidence of DENV infection, and therefore the ratio of symptomatic to asymptomatic dengue, may under-represent real infection rates for two reasons. Firstly, the interval between samples was 1 year, during which time the antibody concentration after an infection could have returned to pre-infection levels. Other studies have compared antibody titres in samples taken 3 months (Endy *et al.* 2002), 6 months (Burke *et al.* 1988) or 1 year apart (Graham *et al.* 1999; Sangkawibha *et al.* 1984; Thein *et al.* 1997). Secondly, we measured a change in overall DENV-specific antibody titre, and not a change in serotype-specific antibody titre, as is carried out with PRNT. However, given that analysis of 85 pairs of annual serum samples tested by both PRNT and IE yielded very similar results, the conclusions reported here are consistent with more traditional PRNT analysis.

Dengue epidemics in the Americas tend to be dominated by a single serotype, with low levels of non-dominant serotypes in circulation (Gubler 1997). During Year 1 of our study, DENV2 was isolated, whereas during Year 2, DENV1 was isolated. This DENV1 was the first isolate of this serotype in Managua from 1990. PRNT results from 85 pairs of samples not only confirmed the dominant serotypes, but also demonstrated the circulation of DENV1, DENV2 and DENV4 during both years of the study. PRNT data also confirmed the predominance of DENV3 prior to the study (Balmaseda *et al.* 1999; Harris *et al.* 2000).

In conclusion, the prevalence of anti-DENV antibodies in our study population and, in particular, the high seroprevalence at such young ages were unexpected in Nicaragua and resemble levels in Southeast Asia. Furthermore, we demonstrate the increased sensitivity of a prospective cohort study in comparison with passive surveillance.

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Séroprévalence élevée d'anticorps du virus de la dengue dans une étude prospective sur les écoliers au Managua, Nicaragua

Pour investiguer l'incidence de l'infection par le virus de la dengue (DENV) au Nicaragua, une étude prospective de deux ans a été conduite sur les écoliers de Managua âgés de 4 à 16 ans. Des échantillons de sang ont été collectés avant la saison pluvieuse en 2001, 2002 et 2003 et ont été soumis à un test d'anticorps spécifique pour le virus de la dengue. Les participants ont été examinés pour des maladies similaires à la dengue et des échantillons de sang ont été collectés chez les cas suspects de dengue en phase aiguë ou de convalescence. En 2001 et 2002, 602 et 397 écoliers ont respectivement été recrutés et des paires d'échantillons annuels étaient disponibles pour 467 et 719 participants pour les années 2001 et 2002 et pour les années 2002 et 2003 respectivement. La séroprévalence totale pour les anticorps du virus de la dengue était de 91%, allant de 75% à l'âge de 4 ans à 100% à l'âge de 16 ans. L'incidence de l'infection par le virus de la dengue était de 12% au cours de l'année 1 et de 6% au cours de l'année 2 ($P < 0{,}001$). Au cours de l'année 1, quatre cas de dengue confirmés au laboratoire ont été détectés avec une souche de sérotype DENV2. Au cours de l'année 2, six cas confirmés de dengue ont été détectés avec une souche de sérotype DENV1. Ces deux sérotypes et d'autres sérotypes courante ont été confirmés par test de réduction neutralisation sur plaque. Cette étude démontre la surprenante transmission élevée du virus de la dengue en milieu urbain au Nicaragua.

mots clés dengue, écoliers, Nicaragua, séroprévalence, incidence de l'infection, étude prospective

Alta prevalencia de anticuerpos contra el virus del dengue en un estudio prospectivo de niños en edad escolar en Managua, Nicaragua

Con el fin de averiguar la incidencia de infección por el virus del dengue (DENV) en Nicaragua, se llevó a cabo un estudio prospectivo de dos años en niños de edad escolar (4–16 años) en Managua. Se recolectaron muestras de sangre antes de la época lluviosa en el 2001, 2002, y 2003, que fueron analizadas, para anticuerpos específicos contra DENV. Se monitorizó la aparición de síntomas de dengue en todos los participantes y se les tomó muestras de sangre en casos sospechosos de dengue durante las fases agudas y convalescientes. Durante el 2001 y 2002 se reclutaron 602 y 397 estudiantes respectivamente, y se obtuvieron las dos muestras de suero de 467 y 719 participantes en el 2001–2002 y el 2002–2003 respectivamente. La seroprevalencia total de anticuerpos anti-DENV fue del 91%, aumentando del 75% a los 4 años hasta el 100% a los 16 años. La incidencia de infección por DENV fue del 12% en el año 1 y el 6% en el año 2 ($P < 0.001$). Durante el primer año se detectaron 4 casos de dengue confirmados en el laboratorio; uno de ellos con un aislado de DENV2. En el segundo año, se confirmaron 6 casos de dengue, uno de ellos con un aislado de DENV1. Tanto estos, como otros serotipos circulantes, se confirmaron mediante el método de neutralización por reducción en placas. Este estudio demuestra una alta transmisión de DENV en áreas urbanas de Nicaragua.

palabras clave dengue, escolares, Nicaragua, seroprevalencia, incidencia de infección, estudio prospectivo