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Epidemiological and ecological characteristics of past dengue virus infection in Santa Clara, Peru

Michael H. Reiskind[1], Kathy J. Baisley[2], Carlos Calampa[3], Trueman W. Sharp[3], Douglas M. Watts[3] and Mark L. Wilson[1]

1 *Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan, USA*
2 *Hammersmith Medicines Research, Central Middlesex Hospital, Park Royal, London NW10 7NS, UK*
3 *Ministry of Health, Iquitos, Peru; United States Naval Medical Research Institute Detachment (NAMRID), Lima, Peru*

Summary To determine risk factors associated with dengue (DEN) virus infection among residents of Santa Clara, Peru, a rural Amazonian village near Iquitos, a cross-sectional serological, epidemiological and environmental survey was conducted. Demographic, social and behavioural information was obtained by standardized questionnaire from 1225 Santa Clara residents (61.3%) aged 5 years or older. Additional data were obtained on the environmental variables and immature mosquito species and abundance surrounding each household ($n = 248$). Sera that had been collected previously by the Peruvian Ministry of Health from residents were tested by an enzyme-linked immunosorbent assay (ELISA) for DEN virus IgG antibody. Antibody identity was verified as DEN by plaque reduction neutralization test. Data on individuals were analysed by univariate and multivariable methods, and independent sample *t*-tests. Spatial clustering was evaluated by comparing distances among DEN positive households. Overall, antibody prevalence was 29.4% and more than doubled from the youngest to the oldest age groups, but did not differ by sex. Curiously, length of residence in Santa Clara was negatively associated with DEN virus antibodies. More frequent travel to Iquitos was positively associated with seroprevalence. Residents who obtained water from a river source rather than a local well also had significantly higher antibody prevalence. None of the environmental variables measured at each household corresponded to the patterns of antibody distribution. Of the larval mosquitoes found around residences, all were determined to be species other than *Aedes*. No evidence of spatial autocorrelation among antibody-positive households was detected. These results strongly suggested that recent DEN virus transmission did not occur in the village and that most infections of residents of this rural village were acquired while visiting the city of Iquitos.

keywords dengue fever, virus antibody serology, *Aedes aegypti*, spatial pattern, vector ecology, Peru

correspondence Mark L. Wilson, Department of Epidemiology, The University of Michigan, 109 Observatory Street, Ann Arbor, MI, 48109-2029, USA. E-mail: wilsonml@umich.edu

Introduction

Dengue (DEN) has recently re-emerged in the Amazon region of Peru, especially in and around Iquitos, a town of 300 000 people, and capital of Loreto province (Phillips *et al*. 1992). The first published laboratory confirmation of DEN in this area was in 1990, although DEN-like epidemics were reported since the 1950s (Hayes *et al*. 1996). A study following the 1991–1992 epidemic suggested that DEN transmission had occurred before this outbreak and currently was endemic (Hayes *et al*. 1996). That study also found a higher prevalence of DEN antibodies in urban and jungle dwellers than among those living in a rural or suburban setting.

Dengue fever, the disease caused by the DEN virus, afflicts 50–100 million people annually, and is a substantial cause of lost work time in the tropics (Gubler 1997). DEN virus is transmitted among humans primarily by *Aedes aegypti*, a mosquito whose survival and reproduction may depend on various microenvironmental factors around dwellings (Gubler 1997). Furthermore, the limited flight range of this vector suggests that conditions near people's

houses may affect risk of infection with DEN virus (Trpis & Hausermann 1986; Watts *et al.* 1987; Rodhain & Rosen 1997). Few studies, however, have systematically examined the household, environmental and behavioural factors that determine risk of DEN infection. Reports that investigated characteristics of individual houses often have been inconclusive (Koopman *et al.* 1991; Kuno 1995). To test the hypothesis that dengue transmission was being driven by environmental and behavioural factors in semirural Amazonian Peru, we undertook a retrospective study of DEN virus antibodies. An earlier community-based study of the epidemiology of Oropouche virus was conducted in 1996 in Santa Clara, a rural village outside Iquitos, and provided serum samples for this investigation (Baisley *et al.* 1998). Using these samples and epidemiological data, we measured other characteristics of the peridomestic environment and determined DEN virus antibody status to evaluate risk factors for previous infection.

Materials and methods

Study site and population

Santa Clara is a suburban community of the city of Iquitos, Department of Loreto, Peru, 10 km from the city centre. The population of Santa Clara was approximately 2400, spread over an area of about 6 km^2. Principal occupations were farming and fishing. The surrounding area was primarily cultivated land and small, secondary growth forests. Iquitos (3°40′ S, 73°10′ W) has a tropical climate with a mean temperature of 27.5 °C and rainfall of 2.7 m per year.

Study cohort

The study cohort included individuals > 5 years of age who participated in a Ministry of Health serosurvey during 1996 to determine the relative importance of infectious diseases in the Santa Clara community. Only those residents who agreed to provide written informed consent were enrolled in the dengue antibody survey by a trained interviewer. For individuals less than 18 years of age, written consent was obtained from their guardians. The survey was carried out under a protocol approved for the use of humans by the US Naval Medical Research Institute Detachment, Peruvian Ministry of Health and the Yale University School of Medicine.

Blood samples and analysis

A venous blood sample was collected from each participant by a trained phlebotomist. A needle and evacuated tube was used to draw 7 ml of blood from adults and 5 ml from children. At the end of each day of collecting (June–September 1996), the blood was centrifuged and the serum fraction was transferred to sterile one-dram vials. These were stored at –20 °C until anonymously tested for DEN viral IgG antibody.

Serological analysis

Sera were tested with in a few months of collection, using an enzyme-linked immunosorbent assay (ELISA) at a dilution of 1 : 100. DEN 1 Hawaii infected and uninfected Vero cell cultures were used as antigens. They were coated onto a 96-well plate at 4 °C overnight. To decrease non-specific binding, plates were blocked with 5% skim milk in phosphate-buffered saline (PBS) for 30 min at 37 °C. Serum was diluted in 5% skim milk/PBS solution. Each dilution was added to four wells, two coated with infected cell antigen, diluted in PBS with 0.1% Tween-20, two coated with uninfected cell antigen, also diluted in PBS with 0.1% Tween-20, and allowed to incubate for 1 h (Ansari *et al.* 1993). After the plates were washed, horseradish peroxidase-conjugated mouse antihuman IgG was added to each well and allowed to incubate for 1 h at 37 °C. The wells were washed again and the substrate (ABTS, Kirkegaard and Perry, Gaithersburg, MD, USA) was added. Following a 30-min incubation period (37 °C), the plate was read at 410 nm on a microplate spectrometer. Optical density readings were calculated by subtracting the OD reading of uninfected wells from infected wells. A positive result was determined if the mean adjusted OD reading was three standard deviations above the antibody negative control sera.

As the ELISA IgG antibody assay does not identify flaviviral specific antibody, a sample of sera with ELISA IgG antibody reactive for dengue viral antigen was tested by a plaque reduction neutralization test (PRNT) as described previously (Morens *et al.* 1985). Serum samples were diluted to 1 : 10 in Eagle's minimum essential medium (EMEM) supplemented with 2% foetal calf serum (FCS) heat treated at 56 °C for 30 min. After incubating each dilution with approximately 20–30 plaque forming units (PFU) of DEN 1 (Hawaii strain) and/or DEN 2 (New Guinea strain) viruses, aliquots of the mixtures were inoculated into a suspension of BHK-21, clone 15 cells, 0.5 ml of an estimated 3×10^{-5} cells per ml per each well of a 12 well plate. Cells and inoculum were incubated at 37 °C for 2–4 h, and then carboxymethyl cellulose liquid medium was added to each well, 1 ml per well. After incubating for 5–7 days, cells were stained with napthanol blue black, and PFUs were counted 30 min later to estimate percent reduction in PFUs. Samples were considered antibody positive for a single DEN virus serotype or

for both DEN 1 and 2 serotypes, if the viral dose was reduced by ≥70%.

Questionnaire and mosquito samples

A standardized questionnaire was administered in Spanish to all participants concurrent with the blood drawing (June–September 1996). Parents answered for children under the age of 10. Information concerning age, gender, the number of people living in the house and recent medical history of febrile illness was collected. Participants also were asked about the frequency of their travel to the jungle, to other rural villages, up the Nanay river and to Iquitos. Limited data on house construction and peridomestic vegetation were recorded.

Household surveys

A follow up study collecting detailed information on risk factors for DEN was performed in the summer of 1998. All extant houses inhabited by previously enrolled participants were surveyed, after verbal permission was obtained from an adult member of the household. Detailed data were collected on house construction, number and type of animals kept, number and volume of water storage containers, and within a 20 m radius of each house the number and size of mosquito breeding sites and vegetation type and amount. All containers holding water within this area or inside each house were examined for mosquito larvae. When larvae were found, they were collected and reared to adulthood for species identification. When feasible, all individual larvae were collected and reared. In cases of large numbers of larvae, a sample was collected and reared.

Statistical analysis

All statistical analyses were made using SAS™ for Windows (SAS Institute, Cary, NC, USA). Univariate comparisons of frequencies used χ^2 tests, and comparisons of means were performed using Student's *t*-test. Multivariable analysis employed logistic regression, using the SAS procedure Proc Logistic. Cuzick and Edwards nearest neighbour tests and Monte Carlo simulation distributions to test for spatial clustering were performed using Gamma™ and Stat!™ softwares (Biomedware Inc., Ann Arbor, MI, USA).

Results

Sample population

Among the estimated 2000 residents in Santa Clara during 1996, serum samples were available from 1225 persons for tests of DEN antibody. Of those, 551 were males and 674 were females. These participants lived in 320 houses (78.4% of the 408 houses in the village). The mean age for sampled males was 26.7 (range 5–87) years and for females 24.8 years (range 5–83). Participants had lived in Santa Clara an average of 16.5 years and 58% of those surveyed had lived in Santa Clara their entire lives.

In the subsequent 1998 household study, 248 of the 320 houses (60.8%) were surveyed for environmental and entomological characteristics. There were 994 (81.1%) residents in the 248 houses surveyed.

Confirmation of DEN virus antibody

Among the 361 IgG antibody reactive serum samples by the ELISA, a random sample of 210 were selected and tested by the PRNT for DEN 1 and 2 antibodies. Of the 210 samples tested, 97.6% ($n = 205$) were antibody positive for either one or both DEN virus serotypes. At this rate, no more than 10 would have been negative by the PRNT, which would have produced a minor decrease in the over all prevalence from 29.5 to 28.7% by the PRNT. Comparison of the results based on ELISA *vs.* the PRNT antibody prevalence indicated no differences in the conclusions of our study.

Antibody prevalence

Overall antibody prevalence for DEN virus IgG antibody was 29.5%. There was a strong linear association of increasing prevalence with age (χ^2 test for trend = 120.8, $P < 0.001$) (Table 1). The difference in antibody prevalence between females (27.5%) and males (31.7%) was not significant ($P < 0.10$) (Table 2).

Risk factors

Numerous individual variables were significantly associated with the presence of DEN IgG antibody in a univariate analysis (Table 2). People who had ever travelled to Iquitos were more likely to have DEN IgG (OR = 1.59, 95% CI = 1.18, 2.15). Source of water was also significantly associated with DEN IgG antibody, with those people who obtained their water from a river having a higher prevalence of DEN antibody (OR = 1.43, 95% CI = 1.08, 1.89). Length of residence in Santa Clara was negatively associated with DEN IgG antibody, with those resident for <5 years more likely to be antibody positive than those resident for longer (5–15 years resident: OR = 0.72, CI = 0.52, 0.99; >15 years: OR = 0.33, 95% CI = 0.23, 0.46). Being a farmer was associated with a higher probability of having DEN IgG antibody positive *vs.* all

Table 1 Age specific DEN virus antibody prevalence among residents of Santa Clara, Peru

Age group (years)	No. (% of total) persons tested	No. (% of tested) antibody positive
5–10	272 (22.2)	22 (8.1)
11–20	300 (24.5)	62 (20.7)
21–30	225 (18.4)	86 (38.2)
31–40	171 (14.0)	65 (38.0)
41–50	96 (7.8)	40 (41.7)
51–60	76 (6.2)	39 (51.3)
61–70	60 (4.9)	35 (58.3)
71–87	25 (2.0)	12 (48.0)
Total	1225 (100)	361 (29.5)

χ^2 for trend = 120.8, $P < 0.001$.

other occupations (OR = 2.60, 95% CI = 1.81, 3.74). In addition, previous infection with Oropouche virus also was associated being DEN IgG positive (OR = 2.09, 95% CI = 1.63, 2.69). Other variables, such as travel to surrounding villages, travel up the Nanay River, type of sanitary facilities, fever in the past 6 months, and gender, were not significantly associated with DEN antibody in the univariate analysis (Table 2).

Comparisons of means of continuous variables demonstrated additional significant differences between persons with and without DEN antibody (Table 3). Positive individuals were older, had lived in Santa Clara longer, had a longer duration of illness with fever in the past 6 months, had a higher frequency of travel to Iquitos and travelled

Table 2 Dengue virus antibody prevalence and univariate odds ratio estimates for various epidemiological characteristics among residents of Santa Clara, Peru

Variable	No. (%) tested	Number (%) antibody positive	Odds ratio	95% CI
Sex				
Female	674 (55.0)	186 (27.5)		
Male	551 (45.0)	175 (31.7)	1.23	0.96–1.57
Iquitos travel				
No	309 (25.3)	70 (29.3)		
Yes	914 (74.7)	290 (31.8)	**1.59**	**1.18–2.15**
Caserio travel				
No	834 (68.3)	233 (27.9)		
Yes	387 (31.7)	127 (32.8)	1.26	0.97–1.64
Rio Nanay travel				
No	1130 (93.2)	350 (31.0)		
Yes	82 (6.8)	26 (31.7)	1.12	0.68–1.82
Water source				
Well	923 (75.5)	255 (27.6)		
River	300 (24.5)	106 (35.3)	**1.43**	**1.08–1.89**
Time resident				
0–5 years	224 (18.3)	95 (42.4)		
5–15 years	526 (42.9)	102 (19.4)	**0.33**	**0.23–0.46**
15+ years	475 (38.8)	164 (35.5)	**0.72**	**0.52–0.99**
Fever				
Yes	234 (19.1)	65 (27.8)		
No	993 (80.9)	296 (29.8)	1.11	0.81–1.52
Sanitary				
None	526 (43.6)	168 (31.9)		
Latrine	681 (56.4)	185 (27.2)	0.79	0.62–1.02
Occupation				
Farmer	128 (10.4)	63 (49.2)		
Other	1098 (89.6)	298 (27.1)	**2.60**	**1.81–3.74**
Oropouche antibody				
Neg.	812 (66.3)	196 (24.1)		
Pos.	413 (33.7)	165 (40.0)	**2.09**	**1.63–2.69**

Boldface indicates an odds ratio significantly different from one at $P < 0.05$.

Table 3 Comparison of mean values for continuos variables among 1225 residents of Santa Clara, Peru, grouped by antibody status (two-sample *t*-test)

Variable	DEN antibody positives (mean)	DEN antibody negatives (mean)	*P*-value (*t*-test)
Age	34.5 years	21.9 years	0.0001
Time resident	18.5 years	15.6 years	0.0093
Days ill with fever	6.9 days	5.5 days	0.0132
Travel to Iquitos	6.6/month*	4.8/month*	0.0001
Travel to Caserios	3.0/month*	1.8/month*	0.0019
Travel up the Rio Nanay	1.2/month*	0.9/month*	0.7922
Travel to 'other' places	1.2/month*	0.9/month*	0.3939
No. persons in the house	6.96	7.07	0.5300
No. persons travelled	1.78	1.70	0.5327
No. families sharing house	1.45	1.39	0.1770
No. persons eligible for the survey in house	5.78 persons	5.93 persons	0.3615

* Average rate per month.

more to surrounding villages ('*caserios*'). There were no differences in rate of travel up the Nanay River, rate of travel to other outlying villages, number of people in the house, number of eligible persons, or number of people in the house who recently travelled anywhere (Table 3).

A multivariable statistical model further elucidated the nature of risk factors associated with past DEN virus infection. Using variables that were significant in the univariate analysis and a stepwise selection method ($P < 0.15$ for retention in the model), a highly predictive multivariable model was produced (Table 4). The final model only included age (categorical), time resident, frequency of travel to Iquitos, water source and sex. For each 10-year increase in age, the likelihood of becoming DEN antibody positive increased 1.6-fold. Each additional year of residence in Santa Clara was associated with a slightly lower probability of being DEN antibody positive (OR = 0.98). The more frequently people travelled to Iquitos, the more likely they were to be DEN antibody positive. Similarly, people who took water from the river were 1.45 times more often found to have DEN antibody. Adding sex to the model had no effect on the significance of these other variables.

Table 4 Results of multivariable logistic regression model including stepwise, selected variables associated with DEN antibody among residents of Santa Clara, Peru. Criteria for variable retention in the model, $P < 0.15$ (except for gender)

Variable	Odds ratio	*P*-value
Age category (10 years intervals)	1.63	0.0001
Time resident (years)	0.98	0.0001
Rate of travel to Iquitos	2.16	0.0053
Water source (river *vs.* well)	1.45	0.0146
Gender (male *vs.* female)	1.12	0.41

Household factors

To test the importance of household characteristics, houses were classified as positive if at least one member of the household was DEN antibody positive, or negative if no members of the house had DEN antibodies. There were 159 (64.1%) positive houses *vs.* 89 (35.9%) negative houses. Positive houses had more household members than negative houses (4.4 *vs.* 3.3, $P < 0.0001$). Positive houses had significantly more dogs and cats than negative houses (0.55 dogs *vs.* 0.27, $P < 0.0034$; 0.69 cats *vs.* 0.29, $P < 0.0019$). However, when the number of people in a house was controlled for in a multivariable logistic model, the association with more cats was no longer present, although there still was a significant association with dogs. All other peridomestic variables that were measured, including number or volume of water storage containers and whether larvae were present, showed no association with the DEN antibody status of the house in either univariate comparisons, or the multivariable model.

Spatial analysis

No clustering of antibody positive individuals was found among houses in Santa Clara, nor was clustering of antibody positive houses detected. The spatial distribution of DEN antibody among Santa Clara residents appeared to be heterogeneous. Using a Monte Carlo procedure to generate a probability distribution, the observed pattern of individuals with DEN antibody was not significantly more clustered than expected ($P = 0.17$). A Cuzick and Edwards test of autocorrelation of case-control data was performed at the level of the house, with no significant results (first nearest neighbour analysis: $P = 0.32$). The results of the spatial analysis did not support the existence of clustering

either within households or among households within Santa Clara.

Mosquito larval survey

Each house ($n = 248$) was examined in order to find water containers or natural breeding sites such as tree holes containing water within a 20-m radius. All such potential breeding sites were carefully examined for larvae. A total of 172 houses (69.4%) had at least one site with standing water. Of these, 22 (8.9%) contained mosquito larvae, including several species in the genus *Culex*, and single representatives of individual species of *Psorophora, Limnatus, Toxorhynchites* and *Anopheles*. No *Aedes* larvae, and in particular no *Ae. Aegypti* larvae, were found.

Discussion

That the prevalence of DEN antibody among Santa Clara residents increased with age is consistent with longer-term, endemic transmission. Studies have suggested that DEN was being transmitted in the Iquitos area before the first laboratory confirmed outbreak in 1990 (Hayes *et al.* 1996). The observed increase in antibody prevalence with age in our study may have resulted from relatively stable transmission rates over decades. Alternatively, it may be that the 1990 epidemic and recent post-epidemic transmission affected proportionally more older people. However, studies of DEN epidemics do not suggest that older people are infected more often, and there are no reasons to believe that transmission in Santa Clara has been different (Hayes *et al.* 1996). Thus, the most parsimonious explanation is that endemic transmission has been ongoing for many years.

To our surprise, length of residence in Santa Clara was negatively associated with prevalence of DEN antibody. Despite the fact that antibody positive people, on average, had lived in Santa Clara longer, this appeared to have been confounded by age. Older people were both more likely to have antibodies and to have lived in Santa Clara longer. When age was controlled for by a multivariable logistic regression, a 'protective' effect of living in Santa Clara appeared (Table 4).

Despite extensive efforts, no *Ae. aegypti* could be found in Santa Clara during the summer of 1998. Overall, 8.9% of houses had at least one species of mosquito larvae found within 20 m of the house, but none were known DEN virus vectors. This was consistent with reports from local entomologists who indicated that *Ae. aegypti* are not present in Santa Clara (Peruvian Ministry of Health, personal communication, 1998). The absence of DEN virus vectors in our survey is consistent with results suggesting that people were not infected in the village of Santa Clara. The lack of any associations between recognized household factors and DEN antibody prevalence also supports the hypothesis that transmission occurred elsewhere.

There was no significant spatial pattern of dengue antibody in Santa Clara, suggesting that transmission may not be occurring in the village. In addition, travel to Iquitos was associated with increased risk of having DEN antibodies. This association could have come about if predominantly older people travelled to Iquitos, and they are already at a higher risk of previous infection. However, the importance of travel to Iquitos was significant even after a multivariable logistic model had controlled for age. People at all ages who travelled to Iquitos more frequently had DEN antibodies. The urban setting of Iquitos appears ideal for intense transmission (Kuno 1995), and previous studies noted that antibody prevalence was roughly twice as high in Iquitos than the other rural and jungle locations tested (Hayes *et al.* 1996). In addition, *Ae. aegypti* is reportedly established in the city of Iquitos (Peruvian Ministry of Health, Iquitos, personal communication 1998). The importance of foci of transmission has been recognized, although scientific studies assessing such areas of high transmission are scant (Kuno 1995).

Santa Clara residents whose water came from the river were at a higher risk of having DEN antibodies than those who used well water. Even after age, travel to Iquitos, and years resident in Santa Clara were controlled, obtaining water from the river remained a significant risk factor. Studies in Asia have shown that water storage practices were associated with increased risk of dengue haemorrhagic fever (Shekhar & Huat 1992–1993). Other studies in Latin America and Australia also have noted that storing water for long periods of time provided breeding sites for *Ae. aegypti*, especially in larger containers (Koopman *et al.* 1991; McBride *et al.* 1998). Our household survey in Santa Clara showed no association between either number or volume of containers and DEN antibody prevalence. The reason for the increased risk of DEN antibody among those who obtain water from the river remains enigmatic. The preponderance of evidence in our study points to transmission occurring away from people's houses, most likely in Iquitos. However, the strength of water source as a risk factor, even after controlling for travel to Iquitos, suggests that water source is a separate risk factor. Interestingly, increased risk was associated previously with piped water in this region, even when location (rural, jungle, urban) was accounted for (Hayes *et al.* 1996). That observation was not explained, either.

The associations of both specific occupation and Oropouche antibody status with DEN disappeared when age, travel to Iquitos, length of time resident, gender and water

source were controlled for. One or more of these factors may have similar effects on risk of both infections, despite the evidence that Oropouche virus is transmitted by a biting midge (*Culicoides parensis*). The same may be true of occupation. When comparing farming to other occupations, there is a strong association with DEN antibody prevalence. However, this association is probably driven by demographic similarities, particularly in age, and not by any specific behaviour or exposure of farmers.

Our study strongly suggests that transmission of DEN virus in this region occurs primarily in the city of Iquitos, and not in the nearby village of Santa Clara. This may be true of other small villages in the region. Such information should be useful in directing control strategies. DEN prevention, and specifically *Ae. aegypti* control, would be inappropriate and wasted if local transmission was not occurring. A more detailed study of the locations where people visit in Iquitos may help locate transmission hot spots, and allow less expensive and more effective intervention.

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