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Epidemiological studies on dengue virus type 3 in Playa municipality, Havana, Cuba, 2001–2002

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SUMMARY

Objectives: Recognizing the uniqueness of secondary dengue virus (DENV)-1/3 dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) cases at an interval of 24 years, we sought to estimate DENV infections as well as the ratios between mild disease and DHF/DSS by DENV infection sequence in Playa District (Havana, Cuba) during the 2001–2002 outbreak of dengue virus type 3 (DENV-3).
Methods: A retrospective seroepidemiological study was conducted in 2003 in Playa District. Blood samples were collected from a 1% random sample of residents and were studied for the prevalence of dengue neutralizing antibodies.
Results: DENV-3 was found to have infected 7.2% (95% confidence interval (95% CI) 6.0–8.4%) of susceptible individuals (the entire cohort), the majority of whom experienced silent infections. Virtually every individual who had a secondary infection in the sequence DENV-1 then DENV-3 became ill, with a ratio of severe to mild cases of 1:35 (95% CI 1:67–1:23). Secondary infections in the sequence DENV-2/3 were less pathogenic than DENV-1/3. Mild disease accompanying secondary DENV2/3 occurred at a ratio of 1:4.49 infections (95% CI 1:5.77–1:3.42) secondary infections.
Conclusions: The results obtained highlight the role of the infecting serotype and also the sequence of the viral infection in the clinical outcome of a dengue infection.

1. Introduction

During the past 50 years, dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) have emerged to become the major global arthropod-borne viral infectious disease. DHF, identified in Southeast Asia during the 1950s, was first noted in the Americas in 1981 and is now widespread throughout the hemisphere.[1,2] Studies in Thailand identified sequential infections by different dengue viruses as a fundamental risk factor for DHF/DSS.[3,4] A mechanism of pathogenesis postulated for this severe syndrome is antibody-dependent enhancement (ADE), in which complexes of dengue virus and non-neutralizing dengue antibodies infect mononuclear phagocytes with enhanced efficiency and increase the viral output per infected cell.[5,6] Many other factors, such as the infecting dengue virus (DENV) strain, the interval and sequence of infections, and the genetic background and chronic disease status of the individual, influence the final outcome of a DENV infection.[7,8]

Cuba is surrounded by dengue-endemic countries. DENV type 1 (DENV-1) was introduced to Cuba in 1977 and infected 44.5% of the population, resulting in more than 500 000 cases of non-hospitalized disease.[7,9,10] Four years later, in 1981, a DENV type 2 (DENV-2) Asian genotype was introduced, resulting in more than 400 000 cases, with 10 312 severe illnesses including 158 fatalities (101 children).[7,11] In 1997, following a long period of strong nationwide *Aedes aegypti* control, an American Asian DENV-2 was introduced in Santiago de Cuba, resulting in approximately 5000 cases, including 205 DHF/DSS cases with 12 deaths. Each of these epidemics was the subject of retrospective population-based seroepidemiological studies.[12,13] Important observations from the two epidemics involving infections in the sequence DENV-1/2 included the relative resistance of blacks to severe clinical expression during secondary dengue infections compared to whites,[12] the high risk of young children to vascular permeability

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accompanying a secondary DENV-2 infection,[14] a month-to-month increase in case fatality rates,[15] the increased severity of secondary DENV-1/2 infections (a DENV-2 infection in a previous immune DENV-1 individual) occurring at an interval of 20 years compared with 4 years,[16] and a correlation of this enhanced severity with a decrease in heterotypic DENV-1 neutralizing antibody titers with the passage of time.[17] These observations depended critically on our being able to relate serologically defined clinical dengue cases to total dengue infections, primary and secondary, in the affected populations. Epidemiological studies were made possible by the comprehensive Cuban dengue surveillance system, by universal access to health care for all patients, and to the sensitive and specific dengue neutralizing antibody method adopted for measuring dengue infections in open populations. As has been demonstrated repeatedly, neutralizing antibodies are sufficiently durable and specific to measure and identify primary and secondary dengue infections accurately.[12,18,19]

In 2000, a 3-month limited outbreak of DENV type 4 (DENV-4) was reported in Havana, resulting in 138 confirmed cases.[2] In June 2001, the nationwide dengue case surveillance system identified a DENV type 3 (DENV-3) genotype III outbreak in Havana that eventually expanded to involve 12 889 serologically confirmed cases, including 78 DHF/DSS and three fatalities (all in adults). All severe dengue cases were hospitalized at the Institute of Tropical Medicine, providing a unique opportunity for some of us to study the clinical features of primary DENV-3 and secondary DENV-1/3 and DENV-2 /3 disease.[20,21] No new confirmed DENV-3 cases were reported after April 2002.[22–24] Recognizing the uniqueness of secondary DENV-1/3 DHF/DSS cases in the literature and because no secondary DENV-3 infections have ever been studied at an interval of 24 years after DENV-1 and/or 20 years after DENV-2 infections, we thought it important to measure dengue infection in a population cohort using a retrospective seroepidemiological format. Accordingly, blood samples were collected from a 1% random sample of the population of Playa District of Havana City in late 2003. We report here estimates of DENV infections in Playa during the 2001–2002 outbreaks. Previous serological data from mild and DHF cases from the 2001–2002 outbreak allowed us to estimate the ratios between mild disease and DHF/DSS in different age groups by DENV infection sequence.[20,21]

2. Materials and methods

2.1. Study site

Havana, the capital city of Cuba, has 15 municipalities (districts), 2 193 848 inhabitants, and a population density of 3040/km^2. It is located in the northwest of the country, with a geographical expanse of 720.84 km^2 and an annual average temperature of 25 °C. Playa District, located in the northern sector of the city, has 182 964 inhabitants and a population density of 5057/km^2. At the time of the epidemic the district was served by 253 family doctors (one family doctor per approximately 720 inhabitants) located in nine health areas and comprising the primary health care system.[22–24] By order of the Ministry of Health, all patients with a provisional diagnosis of dengue who were thought sufficiently ill to require hospitalization were admitted to the hospital located within the Tropical Medicine Institute, "Pedro Kouri" (IPK). The Virology Laboratory at IPK performed serological tests on all reported suspected dengue patients during the 2001–2002 epidemic.

2.2. The 2001–2002 epidemic of DENV-3

At the end of June 2001, a confirmed dengue case was reported in Playa District. In spite of all vector control efforts, by week 29, at 4 weeks after transmission was detected, a second district reported confirmed dengue cases; by week 42, 14 of the 15 districts had reported dengue transmission. Once dengue virus transmission was detected, active case detection surveillance was established. As part of the active surveillance, healthcare workers actively visited every family daily looking for acute febrile cases and suspected dengue cases. Patients were followed up for 6–7 days both for clinical evolution as well as for serological study.[24] Acute and/or convalescent serum specimens from suspect cases were submitted to a laboratory network for testing, in accordance with a long-established system.[24] A total of 72 162 serum samples, collected 5–6 days after the onset of fever from febrile and suspected dengue cases seen in the primary health care system or emergency rooms of hospitals and polyclinics in the capital city, were serologically studied for dengue IgM detection. IgM studies were conducted first at the laboratory of the Centro Provincial de Higiene y Epidemiologia de Ciudad Habana (CPHE-CH) by ultramicro-ELISA for dengue IgM detection.[24] Positive samples were confirmed at the national reference center, the Tropical Medicine Institute (IPK), by an IgM antibody capture ELISA (MAC-ELISA).[25–27] Viral isolation and PCR studies identified DENV-3 genotype III as the etiological agent of the epidemic.[28] Dengue syndromes were classified according to the guidelines for control and prevention of dengue and DHF in the Americas.[25] We believe that almost all clinically overt dengue cases were reported during this outbreak. This conclusion derives from the known strength of the active dengue surveillance system and the requirement that health facilities submit serum from all clinical cases for serological and virological study. A total of 12 889 serologically confirmed cases (suspected dengue cases with a positive dengue IgM as determined by MAC-ELISA) were identified; 7063 presented with undifferentiated fever, 5748 with DF, and 78 with DHF, including three fatalities.[22–24] Among confirmed cases, 1660 (12.9%) were children younger than 15 years of age and 11 229 were adults (87.1%); 52.4% were female and 47.6% were male. Dengue transmission in Playa occurred over a period of 26 weeks, with 1826 confirmed cases reported, including 20 DHF/DSS (Table 1). A strong campaign of sanitation and vector control was initiated in January 2002, and the epidemic was under control by the end of March 2002, well before the expected end of the 2002 transmission season of September.[23,24] In this article, DF and undifferentiated fever cases have been combined and are referred to as 'mild' disease.

2.3. Population-based seroepidemiological study

In November 2003, a representative sample of families in Playa District was selected using family doctor household registries. For the analysis it was assumed that all individuals were independent. Using an equal probabilistic random sampling method, a sample size was calculated, assuming a DENV-3 antibody prevalence of 15%, with a statistical power of 99% ($\beta = 1$) and 95% ($\alpha = 0.05$) of confidence; 440 families were chosen from 253 family doctor offices in Playa. A demographic and health questionnaire was completed for each member of the selected families. From the 440 families, blood samples were collected from 1758 individuals of whom 58.8% ($n = 1034$) were female, 41.2% ($n = 724$) were male, 64.2% ($n = 1128$) were white, 21.1% ($n = 371$) were mixed race, and 14.7% ($n = 259$) were black. In the 2000 census report for Playa District the population was 43.3% male, 62.1% white, 24.3% mixed race, and 13.6% black. Distributions between sample and census data did not differ.

Using an institutional review board (IRB) sanctioned protocol approved by the Institute of Tropical Medicine Human Use Committee and following the precepts established by the Declaration of Helsinki, trained survey teams made one or two

Table 1
Mild dengue and DHF/DSS cases by age group in the Playa District DENV-3 epidemic, 2001–2002

Age group (years)	Dengue cases, Playa municipality[a]		Playa population
	Undifferentiated and dengue fevers	DHF/DSS	
2–9	95	0	42 298
10–19	277	0	
20–25	237	0	13 865
26–29	134	3	93 792
30–39	429	10	
40–49	245	4	
50–59	223	2	
60–69	127	1	33 009
70–79	29	0	
≥80	10	0	
Total	1806	20[b]	182 964

DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; DENV-3, dengue virus type 3.
[a] Serologically confirmed cases.
[b] Nineteen DHF/DSS cases experienced a DENV-1/DENV-3 infection and one a tertiary DENV-1/DENV-2/DENV-3 infection.[21]

visits to each selected household. Signed consent was obtained for each household participant; parental consent was obtained for children. A questionnaire was completed for every family member recording general demographic data and history of dengue-like illnesses during the 2001–2002 epidemic. Blood was collected by finger-prick on two Nobuto type A filter papers (Toyo Roshi International, Inc., Tokyo, Japan), in accordance with the manufacturer's instructions.

2.4. Serological tests

Following the manufacturer's instructions, blood on the filter paper was eluted at 1:10 dilution in phosphate-buffered saline (PBS) after overnight incubation at 4 °C. Filter paper eluates were screened by ELISA inhibition method (EIM)[20,26] to determine the presence of anti-dengue IgG antibodies. Briefly, polystyrene plates (Costar) were adsorbed with human anti-dengue IgG at 10 μg/ml concentration, in coating buffer, and incubated overnight at 4 °C. The plates were blocked and 100 μl of DENV-3 antigen diluted 1/40 in PBS plus 0.05% Tween 20 was added. After incubation, 100 μl of eluates at 1:20 dilution was added. After washing, 100 μl of human IgG anti-dengue conjugate diluted in PBS plus 0.05% Tween 20 and 2% fetal bovine serum (FBS) was added. Substrate was added and the test was read at 492 nm. The inhibition percentage was calculated using the following equation: inhibition % = [1 − (OD sample/OD negative control)] × 100. Samples with ≥50% inhibition were considered positive for anti-dengue IgG. All EIM-positive samples were tested by plaque-reduction neutralization test (PRNT).[26,29–31] Neutralizing antibody to the four dengue serotypes was measured using the single dilution (1:30) method employed previously to identify dengue neutralizing antibodies after the 1981 and 1997 epidemics.[12,13,31,32] All eluates were assayed for neutralizing antibodies on BHK-21 clone 15 cells in triplicate wells, and mean plaque counts were calculated.

Briefly, BHK-21 clone 15 cells were grown at 37 °C in complete medium E-MEM (minimum essential medium with Earle's balanced salts) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 10% L-glutamine, and sodium bicarbonate to adjust the medium to pH 7.4–7.8. For antibody titration, 100 μl of serum dilution was incubated for 1 h at 37 °C with 100 μl of the virus working dilution, calculated to give 10–20 plaque-forming units (pfu)/50 μl of the final volume of virus–serum mixture. After incubation, 50 μl of virus–serum mixture was added to the cell suspension in triplicate. After 4 h incubation at 37 °C in a CO_2 atmosphere, each well received 0.5 ml of 3% medium viscosity carboxymethyl cellulose made up in Earle's minimum essential medium without phenol red (MEM) with 10% heat-inactivated FBS, 1% glutamine 2 mM, and 100 U of penicillin plus 100 μg/ml streptomycin. Infected cells were incubated for 5–9 days in the same conditions as above depending on the serotype. After incubation, plates were rinsed under tap water and stained with a solution of naphthol blue black and acetic acid. Calculations of 50% endpoint plaque reduction neutralization titers were made using log probit paper.[32] According to criteria previously used to study known introductions of DENV-1 followed by DENV-2, serum that reduced DENV plaques by ≥50% at a 1/30 serum dilution for a single DENV was considered a prior primary infection; serum that neutralized ≥50% plaques of two DENV were considered cases immune to two viruses.[12,13,20,21,33] Finally, neutralization by three or four viruses was scored as prior infection by more than two dengue infections.

The following strains were employed in the neutralization tests: DENV-1, Angola (1988), DENV-2 A15/81, Cuba (1981), DENV-3 116/00, Cuba (2000), and DENV-4, Dominica (1981). DENV-1 and DENV-4 strains were obtained from the late Dr Robert Shope, University of Texas Medical Branch.

To eliminate uncertainty about the extent of DENV-1 infections among 20–25-year-olds who were young infants during the 1977–1979 epidemic and possibly shielded from exposure to mosquitoes, this age group was omitted from the analysis of infections in the sequence DENV-1 then DENV-3. Secondary dengue infection rates were estimated using the age group 26–59 years.

2.5. Statistical analysis

Tests of statistical significance for nominal and ordinal qualitative variables used to compare the hypothesis of independence between variables with the homogeneity test. A simple binomial confidence interval, as has been calculated in various cases, assumes that all sampled individuals are independent.

Percentages of the prevalence of infections among the sampled individuals were calculated with their 95% confidence intervals (95% CI). Data determined by Alvarez et al. were used for the calculation of the ratios of infection of DF and DHF cases.[20,21]

3. Results

In December 2003, of 1758 individuals sampled, 41.4% (95% CI 39.1–43.7%) had neutralizing antibodies to one or more of the four DENV serotypes (Table 2). Given extensive data documenting the universal and lifetime longevity of DENV neutralizing antibodies following infection,[17,34,35] these antibodies are taken to reflect the cumulative infection experience among the residents of Playa District from known dengue virus introductions in the 1940s and in 1977–1979, 1981, 2000, and 2001–2002.

DENV-3 antibodies were detected in 126 (7.2%, 95% CI 6.0–8.4%) individuals in the entire cohort. According to history of dengue-like illness recorded during the sample collection interview, 13.5% (95% CI 11.9–15.1%) of the total individuals with DENV-3 antibody developed a symptomatic infection. Considering these figures, and applying a multiplication factor derived from the total and sample population (182 964/1758 = 104), an estimated 13 104 (7.16%, 95% CI 7.0–7.2%) individuals were infected in the whole district, with approximately 1769 clinical cases (13.5%, 95% CI 12.9–14.0%). This figure is very close to the total clinical cases (n = 1806) reported in Playa municipality during the epidemic (Table 1).

From antibody prevalence in the cohort, we calculated primary DENV-3, secondary DENV-1/3 and DENV-2/3, tertiary DENV-1+2/3 and DENV-2+4/3, and the quaternary infection rate occurring during 2001–2002 in Playa District. A primary DENV-3 infection

Table 2
Prevalence of different combinations of dengue neutralizing antibodies in the sampled population resident in Playa District, Havana; December 2003

Age group, years	Dengue viruses															Negative	Studied sample
	Monotypic				Combined												
	1	2	3	4	1+2	1+3	1+4	2+3	2+4	3+4	1+2+3	1+2+4	1+3+4	2+3+4	1+2+3+4		
	n	*n*	*n*	*n*	*n*	*n*	*n*	*n*	*n*	*n*	*n*	*n*	*n*	*n*	*n*		
2–19	0	0	17	0	0	0	0	0	0	0	0	0	0	0	0	292	309
20–25	10	2	2	1	3	0	0	4	1	0	1	0	0	0	0	113	137
26–39	62	25	5	4	39	1	0	5	6	0	11	4	0	1	1	489	892
40–59	89	32	7	5	67	5	1	3	10	0	13	2	0	0	5		
≥60	41	117	4	2	52	0	1	10	16	0	17	9	0	4	10	137	420
Total	202	176	35	12	161	6	2	22	33	0	42	15	0	5	16	1031	1758

rate for the 2001–2002 outbreak was calculated from the 17 monotypic DENV-3 antibodies observed in the sera from 309 individuals aged 2–19 years, who were born after the outbreak of DENV-2 in 1981 (17/309 = 5.5%, 95% CI 4.4–6.6%). Applying this infection rate to the total Playa population aged 2–19 years, of 42 298, there were 2326 primary DENV-3 infections in Playa, among which were 372 mild cases (Table 1). As evidence of the focal and limited nature of the DENV-4 introduction of 2000, only scattered monotypic DENV-4 antibodies were observed in individuals in the 26–59 years age group and none were observed in individuals under the age of 19 years.

Although data are shown, dengue infection rates were not estimated for individuals aged ≥60 years because in this age group we could not differentiate DENV-2 antibodies from infections that may have been acquired before or during the World War II period from those infections that might have occurred in 1981.

Using DENV neutralizing antibody prevalence in the age group 26–59 years (Table 2), we calculated primary, secondary, and tertiary DENV-3 infection rates. Secondary DENV-1/3 infection rates were calculated from the number of individuals with DENV-1 + DENV-3 antibodies in the survey. This number was divided by estimated pre-outbreak monotypic DENV-1 immunes, comprising monotypic DENV-1 immunes observed in 2003 and DENV-1 + DENV-3 immunes (151 + 6), or 6/157 = 3.8% (95% CI 2.9–4.7%). A similar strategy was used to estimate the secondary DENV-2/3 infection rate, 8/65 = 12.3% (95% CI 10.8–13.8%), and tertiary and quaternary infection rates. The combined DENV-3 infection rate in heterotypic immunes was 45/381 = 11.8% (95% CI 10.3–13.3%).

Applying an expansion factor derived from dividing the district total by the serologically sampled Playa population aged 26–59 years (93 792/892 = 105.1), primary, secondary, tertiary, and quaternary dengue infections were estimated for Playa District during the 2001–2002 DENV-3 outbreak (Table 3).

As the ratios of overt disease in residents of Playa District attributed to different DENV infection events had previously been measured by Alvarez et al.,[20] we extended these data to the 1031 individuals in this age group with mild illness (Table 1). This analysis provides estimates of 32 primary DENV-3 infections, 625 secondary DENV-1/3 infections, 187 secondary DENV-2/3 infections, and 187 tertiary DENV infections. From this partition of infection sequence among overt cases we used our estimates of DENV-3 infection rates to calculate attack rates for mild and DHF/DSS cases by infection sequence and parity in Playa District (Table 3). The ratio of mild disease to total primary infections with the DENV-3 strain in individuals aged 26–59 years was calculated from the number of overt mild cases with primary DENV-3 infections divided by the estimated total primary DENV-3 infections (Table 3). There were 1261 estimated primary DENV-3 infections in individuals aged 26–59 years among which were 32 reported cases, or one mild case for every 39.4 infections (2.45%, 95% CI 1.6–3.4%). Employing a similar analysis, it was estimated that nearly all secondary DENV-3 infections in the sequence DENV-1/3 were overt (Table 3). This conclusion is based on the estimate that 631

Table 3
Observed primary and estimated secondary, tertiary, and quaternary DENV-3 infections in individuals aged 26–59 years in the study cohort, with projections of total cases in Playa District (population 93 792) during the 2001–2002 DENV-3 epidemic, Havana, Cuba. Clinical cases reported from Playa District are described in Table 1

	Primary infections	Secondary infections		Tertiary infections		Quaternary infections
	DENV-3	DENV-1/3	DENV-2/3	DENV-1/2/3	DENV-2/4/3	DENV-1/2/4/3
Infections in cohort	12	6	8	24	1	6
Estimated DENV-3 infections, all Playa	1261.2	630.6	840.8	2522.4	105.1	630.6
Reported mild/DHF cases[a]	32/0	625/18	187/0	187/1		0/0
Mild/estimated DENV-3 infections	1:39.4	1:1	1:4.49	1:14.05		0
DHF/estimated DENV-3 infections	0	1:35	0	1:2627.5		0

DENV, dengue virus; DHF, dengue hemorrhagic fever.
[a] DF and DHF estimated ratios using data collected by Alvarez et al., 2006;[20] Alvarez et al., 2008.[21]

Table 4
Dengue antibody distribution by ethnic group and gender, Playa cohort, 2003

	Ethnic group[a]			Gender[b]	
	White, *n* (%)	Mixed, *n* (%)	Black, *n* (%)	Female, *n* (%)	Male, *n* (%)
Any dengue	467 (41.40)	143 (38.54)	117 (45.17)	432 (41.78)	295 (40.75)
Negative	661 (58.60)	228 (61.46)	142 (54.83)	602 (58.22)	429 (59.25)
Total	1128	371	259	1034	724

[a] $p = 0.52$.
[b] $p = 0.69$.

secondary dengue infections occurred in the sequence DENV-1/3. Almost the same number of mild and severe cases ($n = 644$) from Playa District were attributed to infections in the sequence DENV-1/3. Secondary DENV-2/3 infections were associated with mild illnesses, the case:infection ratio was 1:4.49 (95% CI 1:5.77–1:3.42), but no DHF/DSS cases were observed in the sequence DENV-2/3. Tertiary dengue infections produced mild disease and DHF infrequently, at 1:14 (95% CI 1:18–1:10) and 1:2627 (95% CI 1:3412–1:2115), respectively.

Table 4 displays dengue antibody prevalence observed in the serological cohort by ethnic group ($p = 0.52$) and gender ($p = 0.69$). No significant differences were observed in any group.

4. Discussion

The technical underpinning of this study is based upon three epidemiological attributes unique to Cuba. First, the introduction and propagation of only one DENV at a time, such that the sequence of the infecting serotypes is known. In other geographic settings, where multiple serotypes are circulating at the same time and continuously for several years/decades, it is difficult to predict the sequence of infection from a serosurvey due to the 'original antigenic sin' phenomena that might occur. Second is the comprehensive island-wide case reporting and surveillance system that permitted near universal serological confirmation of cases. Third is the evidence that sera from individuals who had unapparent DENV infections one or more years earlier, tested at a 1:30 dilution, circulate mono- or bivalent dengue neutralizing antibodies that reliably match previous infection experience.[7,12,13,20,24,33,36] The absence of DENV-1 and DENV-2 neutralizing antibodies in individuals aged 2–19 years confirms the fact that DENV-3 was the first dengue introduction since 1977 and 1981. A retrospective seroepidemiological study of the DENV-3 epidemic of 2001–2002 was undertaken in 2003 using a 1% random sample of the Playa District population, powered to measure a DENV-3 antibody prevalence of 15%. Playa had the highest number of reported dengue cases during the 2001–2002 epidemic.[23,24] There is sufficient congruence between the serologically studied sample and the general population of Playa (Table 1) to warrant projection of serosurvey data to the entire population of the municipality.

Among the population of Playa District, 7.2% suffered a DENV-3 infection, resulting in 13 104 infected individuals and 1806 clinical cases. The number of overt dengue cases as determined by the seroepidemiological study was quite similar to that reported to the dengue surveillance system. However, as both systems may potentially under-detect mild disease, it is possible that some clinically overt cases were not notified.

Similar to other reports, this study found a much higher incidence of asymptomatic than symptomatic dengue infections.[37] As participants of the seroepidemiological survey were interviewed almost 2 years after the epidemic, it is likely that some individuals may not have remembered a case of mild disease. However, this potential bias is diminished by the strength of the active dengue surveillance system to recover virtually all dengue cases.

From the 309 children aged 2–19 years, the data obtained suggest that 2326 children experienced a primary DENV-3 infection in the district. Consistent with laboratory-based surveillance studies of clinical cases reported from Havana during the past 20 years, no children had experienced any other DENV infection during their lifetime. Attack rates of mild disease among children and adolescents accompanying primary DENV-3 infections were relatively high, there being one mild case for every 6.25 primary DENV-3 infections (16%). Interestingly, primary DENV-3 infections in adults aged 26–59 years were milder than in children, with one mild case for every 39.4 DENV-3 infections in adults (2.5%). During the DENV-2 outbreak in Santiago de Cuba in 1997, very few mild dengue cases occurred in children, but mild disease did occur in 26–59-year-olds at a ratio of 1 case to 33 infections (3.0%), similar to the clinical expression of DENV-3 infections in this age group.[13] This confirms observations made previously in Thai children in whom primary DENV-2 infections were mostly unapparent, while primary DENV-3 infections were more severe.[38]

In previous seroepidemiological studies we observed that secondary dengue infection rates are higher than primary infection rates,[12] due to the probability that over a long period of time households either do or do not provide habitats for *A. aegypti*. Even after periods of complete control, when vector mosquitoes are re-introduced, households with permissive habitats again harbor *A. aegypti*. When DENV is introduced, residents of these infested households are at higher risk of infection than are the residents of non-infested households. The likely positive correlation between members of shared households will mean that the confidence intervals should in fact be wider than were obtained assuming independence. However for the analysis it was assumed that all individuals were independent, as some infections occur outside the home, and also very few households showed more than one member with anti-DENV-3 antibodies (data not shown).

In this study, the highest primary DENV-3 infection rate, measured in susceptible children, was 5.5%. This can be compared with the 11.8% DENV-3 infection rate in DENV immunes (Tables 2 and 3). An important observation made in 1997 was that when secondary DENV-2 infections occurred 18–20 years after primary DENV-1 infection, all such infections were clinically overt.[13] This was also true when DENV-3 infections occurred at an interval of 23–24 years after DENV-1 (Table 3). In Playa District, in addition to the 625 mild cases we estimated to have experienced this infection sequence, there were an additional 18 DHF/DSS cases, or a ratio of mild:severe disease of 1:35 (95% CI 1:67–1:23). During the 1997 outbreak, secondary DENV-2 disease was somewhat more severe than secondary DENV-3, the ratio of mild to severe cases being 22.8:1.[13]

Secondary infections in the sequence DENV-2/3 were not as pathogenic as DENV-1/3 infections. There were 187 mild cases detected without a single case of DHF/DSS. However, mild disease accompanying secondary DENV-2/3 occurred quite frequently, at a ratio of 1:4.49 (95% CI 1:5.77–1:3.42) secondary infections. Might these differences be related to the ability of polyclonal human DENV-2 antibodies to neutralize DENV-3? We plan to extend our previous study of heterotypic DENV-1 and DENV-2 neutralizing antibodies to DENV-3 viruses recovered from the 2001–2002 outbreak.[17]

In our study, tertiary infections were relatively common, there being an estimated 2627 individuals infected in this sequence, with mild disease estimated in 187 of these persons (7.1%), but only a single case of DHF/DSS. This high ratio of tertiary infections to overt severe dengue disease is consistent with a report describing the rarity of hospitalized disease accompanying a tertiary infection among Thai children.[39] We also failed to find overt quaternary infections.

Previous studies suggested that white individuals had a higher risk of developing DHF/DSS during DENV-1/2 infections than did blacks, while both whites and blacks had similar infection rates.[7,12,13,40–42] Again in Playa, DENV-3 infection rates were similar in whites and blacks (Table 4), while whites had higher attack rates of DHF/DSS than did blacks.[22]

In conclusion, we have studied the implications of DENV-3 infections accompanying first, second, or third sequential infections in individuals with DENV-1 and/or DENV-2 infections. Our results add new evidence of the complexity of dengue pathogenesis, highlighting the role of infecting serotype and also the

sequence of the viral infection on the clinical outcome of a dengue infection.

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Ethical approval: This work was done using an IRB sanctioned protocol approved by the Institute of Tropical Medicine Human Use Committee and following the precepts established by the Declaration of Helsinki.

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