The burden of dengue fever and chikungunya in southern coastal Ecuador: Epidemiology, clinical presentation, and phylogenetics from a prospective study in Machala in 2014 and 2015

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Abstract

Background: Dengue (DENV), chikungunya (CHIKV) and Zika (ZIKV) viruses are transmitted by the *Ae. aegypti* mosquito and present a major public health concern throughout the tropics and subtropics. Here we report the methods and findings from the first two years (January 1, 2014 to December 31, 2015) of an active and passive surveillance study conducted in the southern coastal city of Machala, Ecuador, where DENV is endemic.

Methodology/Principal Findings: Individuals whom presented at one of four sentinel clinics or the central hospital of the Ministry of Health with suspected DENV infections (index cases) were recruited into the study (n = 324). A subset of DENV positive index cases (n = 44) were selected, and individuals from the index household and four neighboring households within a 200-meter radius (associates) were recruited (n = 397). In 2014, 72.5% (132/182) of index patients and 35.6% (106/298) of associates had evidence of acute or recent DENV infections. In 2015, 28.3% (35/124) of index patients and 12.85% (11/86) of associates, had acute or recent DENV infections. For every case of dengue detected by passive surveillance, we detected an additional three infections in associates. Of associates with DENV infections, slightly more than half showed symptoms. The burden of symptomatic dengue was greatest in children under 10 years of age. The first CHIKV infections were detected in 2015 on epidemiological week 12. There were 50 index cases with acute CHIKV infections (50/122; 41%), including six with both acute CHIKV and acute or recent DENV infections. There were four associates with CHIKV infections (4/87, 4.6%), including one associate with both an acute CHIKV and recent DENV infection. No ZIKV infections were detected. Phylogenetic analyses of isolates of DENV from 2014 revealed genetic relatedness and shared ancestry of DENV1, DENV2 and DENV4 genomes from Ecuador with those from Venezuela and Colombia, as well as more than one introduction of the same serotype into Ecuador, indicating presence of viral flow between Ecuador and the surrounding countries.

Conclusions/Significance: The results of this active surveillance study provide a more accurate estimate of the symptomatic and subclinical burden of DENV and CHIKV infections and illness across age groups than has previously been detected through traditional passive surveillance.

Author summary

Dengue and chikungunya viruses are transmitted to people by *Aedes sp*. mosquitoes in tropical and subtropical regions. Human infections are underreported in traditional public health systems due to a high proportion of infections with mild or no symptoms. This results in an underestimate of the true burden of disease. In this study, we investigated dengue, chikungunya, and Zika infections in an urban center in southern coastal Ecuador in 2014 and 2015, an area known to be endemic for dengue. Patients with symptomatic dengue infections were referred from Ministry of Health sentinel clinics. We visited the households of patients and neighboring homes to identify additional people with infections. We found that the burden of illness due to dengue was greatest in children under 10 years of age. For every case of dengue detected by standard surveillance, we detected an additional three infections in the community. Of people in the community with dengue infections, slightly more than half showed symptoms. The first chikungunya infections were detected in March 2015. Genetic analyses indicate that there is movement of the dengue virus among Ecuador, Venezuela and Colombia. The results of this enhanced surveillance study provide a more accurate estimate of the symptomatic and subclinical burden of dengue and chikungunya infections across age groups than has previously been detected through traditional passive surveillance.

Key words: dengue fever, chikungunya, Zika fever, arboviruses, vector-borne diseases, *Aedes aegypti*, symptoms, phylogenetics, capacity strengthening, Ecuador, surveillance

Introduction

The region of the Americas is facing an unprecedented public health crisis of co-occurring epidemics of illness due to dengue virus (DENV), chikungunya virus (CHIKV) and Zika virus (ZIKV). These arboviruses cause acute febrile illness, and are transmitted to humans primarily by the female *Aedes aegypti* and *Aedes albopictus* mosquitoes.

Dengue disease is caused by infection by one of the four serotypes of the mosquito-borne dengue virus (DENV 1-4), RNA viruses belonging to the family *Flaviviridae* genus *Flavivirus*. Clinical manifestations range from mild disease (*i.e.*, fever, rash, and joint pain) to severe illness characterized by pathologic vascular permeability leading to hemorrhage, shock, and sometimes death [1]. Over the last three decades, the distribution, severity, and incidence of DENV has increased in Latin America, from 16.4 cases per 100,000 in the 1980's to 71.5 cases per 100,000 from 2000 to 2007 [2,3]. Current estimates of apparent DENV infection in the Americas range from 1.5 million [4] to 13.3 million [5] infections per year. In 2015, 2.35 million cases of DENV were reported in the Americas, leading to 10,200 cases of severe dengue and 1,181 deaths [6].

More recently, CHIKV and ZIKV have emerged, and are now causing major epidemics in the same populations in the Americas. The first cases of CHIKV (family *Togaviridae*, genus *alphavirus*) were reported in the Americas in 2013, resulting in approximately two million cases to date [7]. The first cases of ZIKV (family *Flaviviridae*, genus *flavivirus*) were reported in Brazil in 2015 [8,9]. To date, 774,668 suspected and confirmed autochthonous cases of ZIKV have been reported from 48 countries and territories (as of May 11, 2017) [10]

In Ecuador, DENV causes the greatest burden of mosquito-borne febrile illness. Historically, DENV was eradicated from Ecuador in the 1950s with support from the Rockefeller Foundation and the Pan American Sanitary Bureau, primarily through the use of DDT to control *Ae. aegypti*, the only known vector in Ecuador [11,12]. Following a weakening of the vector control program and the re-invasion of *Ae. aegypti* in the 1970s and 1980s, DENV1 re-emerged in Ecuador in 1988, and caused a major epidemic of classic dengue fever [13]. From 1993 to 1999 three serotypes circulated: DENV1, DENV2 (American strain), and DENV4**.** In 2000, DENV3 and DENV2 (Asian strain) were identified and the first cases of severe hemorrhagic dengue were subsequently reported [14].

Today the burden of DENV is greatest in the coastal lowland region of Ecuador, the site of the current study, where the disease is hyper-endemic and DENV 1-4 co-circulate. Over a five-year period (2010 to 2014), 72,060 cases of dengue were reported in Ecuador, with an annual average of 14,412 cases [15]. Prior studies in southern coastal Ecuador indicate that DENV transmission is highly seasonal, with the greatest incidence of disease and density of mosquito vectors during the hot, rainy season from February to May, and lower transmission throughout the rest of the year [16,17]. DENV epidemics in the region are associated with El Niño climate events that cause increased rainfall and warmer air temperatures [16]. Local social-ecological risk factors for DENV infections and *Ae. aegypti* proliferation include poor housing conditions, interruptions in the piped water supply in the urban periphery, lack of knowledge of DENV transmission, and water storage behavior [17–19].

The first cases of CHIKV were reported in Ecuador at the end of 2014, resulting in a major epidemic in 2015, with over 33,000 cases reported. The first cases of ZIKV were confirmed in Ecuador on January 7, 2016. A total of 5,302 suspected and confirmed cases of ZIKV have been reported to date (as of May 4, 2017), including two cases of congenital syndrome associated with ZIKV, which were first reported in early May 2017 [10].

In Ecuador, suspected and confirmed cases of DENV, ZIKV, and CHIKV infections require mandatory notification to the Ministry of Health (MoH). The MoH in Ecuador follows the 2009 WHO dengue diagnostic guidelines. The national surveillance system is based on passive surveillance of cases from MoH clinics and hospitals, which provide free healthcare to the population. Most reported cases are diagnosed clinically. A subset of cases are diagnosed for DENV using NS1 and IgM ELISAs in local diagnostic laboratories operated by the MoH, and some cases are diagnosed for DENV, CHIKV, ZIKV using quantitative PCR at the national reference laboratory of the National Institute for Public Health Research (INSPI) of the MoH. Positive cases trigger focal vector control interventions in the infected home and surrounding homes by the MoH (i.e., fogging, indoor residual spraying, source reduction, and larvicide application).

There have been prior enhanced surveillance studies to estimate the burden of dengue fever in Asia [20–23] and Latin America [24–29], with study designs ranging from pediatric to adult cohorts, tracking of school-based absentees, use of sentinel clinics, and community-based cluster investigations. In general, these studies found that enhanced surveillance methods identified a greater number of dengue infections, especially mild and subclinical infections, compared to traditional passive surveillance systems. Enhanced surveillance studies generate high-resolution information on the spatial and temporal distribution of infections and illness across the population. This is especially important in settings and in subgroups with low-health care seeking behavior or limited access to health centers. These data allow the public health sector to more accurately estimate the social and economic burden of the disease, allowing for more informed decision-making regarding the allocation of scarce resources. These studies can also inform the design and implementation of interventions targeted at high-risk groups, such as vaccination campaigns or vaccine trials.

The aim of this study was to characterize the epidemiology, clinical presentation, and viral phylogenetics of suspected DENV infections in the city of Machala, Ecuador, in 2014 and 2015. Patients with acute DENV infections (index cases) were recruited from sentinel clinics and the central hospital. Index cases triggered active surveillance of DENV, CHIKV and ZIKV infections in individuals (associates) living within 200 meters of the index patient. We focus specifically on: (1) characterization of DENV infections in index cases and associates (i.e., symptoms, serotypes, serology), (2) prevalence of DENV infection and expansion factors (EF) from clusters of homes around the index home, (3) detection of the emergence of CHIKV in Machala in 2015 and ZIKV surveillance, (4) multivariate models of symptoms associated with DENV and CHIKV infections, and (5) phylogenetic analysis of DENV circulating in 2014,. This study contributes to an ongoing collaboration with the MoH of Ecuador to strengthen febrile vector-borne disease surveillance in southern coastal Ecuador, providing high resolution epidemiological information for the region [30].

Materials and Methods

Ethics Statement.

This protocol was reviewed and approval by Institutional Review Boards (IRBs) at SUNY Upstate Medical University, Cornell University, the Human Research Protection Office (HRPO) of the U.S. Department of Defense, the Luis Vernaza Hospital in Guayaquil, Ecuador, and the Ecuadorean Ministry of Health. Prior to the start of the study, all participants engaged in a written informed consent or assent process, as applicable. In the event the participant was unable to participate in the informed consent or assent process, a recognized health-care proxy represented them in the process and documented consent. Children aged 7 to 17 signed an assent statement and parents signed an informed consent. Parents signed an informed consent on behalf of children under the age of 7 years to > 6 months. The study population included children (> 6 months) to adults who were evaluated in sentinel clinics or the hospital with a clinical diagnosis of DENV illness, and children (> 6 months) and adults who resided in homes within 200 meters of the index household.

Study Site.

Machala, Ecuador, (population 280,694, capital of El Oro Province) is a mid-sized coastal port city located along the Pan American Highway, 70 kilometers north of the Ecuador-Peru border (Fig 1). Machala has among the highest incidence rates of DENV in Ecuador, and prior studies reported the highest *Ae. aegypti* densities compared to sites from 10 countries in Latin America and Asia [17,31,32]. In 2014 and 2015, 1,196 and 2,791 dengue cases, respectively, were reported from Machala (mean annual incidence 42.6 and 99.4 cases per 10,000 people) [33]. The first cases of CHIKV were reported by the MoH in May of 2015, and the first cases of ZIKV were reported in February of 2016. Based on the high volume of people and goods moving across the border and the high incidence of DENV historically, Machala is a strategic location to monitor and investigate DENV and now CHIKV and ZIKV transmission dynamics.

Fig 1: Map of the study site: A. Location of Ecuador in the Americas. B. Location of El Oro Province in Ecuador, the city of Machala indicated as a red dot. C. The city of Machala, showing the five Ministry of Health clinical sites/hospital: 1. Mabel Estupiñan Clinic, 2. Teofilo Davila Hospital, 3. Brisas del Mar Clinic, 4. El Paraiso Clinic, 5. Rayito de Luz Clinic. The location of meteorological stations are indicated by A-E as follows: A. Puerto Bolivar, B. Los Esteros, C. Mabel Estupiñan; D. Florida; E. Crucitas.

Sentinel clinics operated by the MoH in Machala were selected based on the number of reported DENV cases and the resources that they were able to offer for coordinating and supporting the methods of this surveillance study. Of the twenty-three MoH clinics in Machala, four were selected. These included the clinics Brisas del Mar, Rayito de Luz, Mabel Estupiñan, and El Paraiso. In addition, the Teófilo Dávila Hospital of the MoH was included, because it is the principal public hospital of the province, where the MoH clinics refer patients with severe DENV infections.

Passive and active surveillance study design.

Hospitalized or clinic visit patients with a clinical diagnosis of an acute DENV infection, as determined by MoH physicians, were referred to our study technician or nurse at the hospital. All patients that were referred to the study team were invited to participate in the study. These individuals are referred to as index patients or cases. Informed consent was obtained and the

following data were collected using a customized database on an Ipad (FileMaker Pro Advanced 13.0v5): demographic information including home address, primary reason for hospitalization, date of onset of fever, symptoms within the last seven days, medications, and aural temperature. Data were uploaded daily and stored in a secure cloud-based server (GoZync). At the time of clinical evaluation a 20 ml blood specimen (adjusted for age and weight by the National Institute of Health criteria) was obtained by venipuncture from each participant. Samples were processed at our diagnostic laboratory at the hospital. Serum samples were used to test for acute dengue infections using NS1 rapid strip tests (PanBio Dengue Early Rapid Test; sensitivity: 91.89%, specificity: 98.39%). Additional serum, cells and plasma were separated via centrifugation and aliquoted in multiple tubes and stored at -80°C.

Each week, up to four index patients who were confirmed to be positive for DENV by NS1 rapid strip test were randomly selected and invited to participate in the study. The study team visited the household of the index patient, and invited the patient's family members to participate. The study team then invited individuals to participate who resided in the nearest neighboring homes in each of the four cardinal directions within a 200-meter radius of the index household, the typical flight range of the *Ae. aegypti* mosquito. The neighboring homes plus the index home are referred to as a cluster. Investigations in clusters were initiated within two days of the index patient entering the study. The diagnostic tests and clinical assessments described above for index patients were repeated for all associates. The location (latitude, longitude) of each home was recorded using handheld Garmin GPS units. Passive and active surveillance study designs were optimized in a prior study by the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Kamphaeng Phet Province, Thailand [23].

Diagnostic assays.

Additional diagnostic testing for DENV was conducted using serum samples and commercial ELISA kits (Panbio) to test for NS1 (Dengue Early ELISA), IgM (Dengue Capture IgM), and IgG (Dengue Capture IgG). Participants were classified as having "primary" infection if the IgM to IgG ratio was ≥ 1.8 and "secondary" infection if the ratio was < 1.8 [23,34,35].

Specimens were shipped to SUNY Upstate Medical University for testing by qualitative real-time reverse transcriptase (RT)-PCR assays for DENV1-4 and ZIKV / CHIKV. All samples from 2014 and 2015 were screened for DENV1-4 and CHIKV. Samples from 2015 were tested for ZIKV, and if a positive sample was detected, then samples from 2014 were screened. All analyses were performed on a BioRad DNA Engine Chromo 4 System with MJ Opticon Monitor Analysis Software. For DENV1-4 analysis, total RNA was extracted from 140 µL of human serum specimens using the QIAamp® Viral RNA Mini Kit (QIAgen, Cat# 52906) according to the manufacturer's suggested protocol and resuspended in 50 µL of buffer. Ten (10) µL of RNA (or the equivalent of 28 µL of serum) was used ina 20 µL reverse transcriptase reaction, of which 5 µL of the resulting cDNA was used for the PCR reaction. All samples and controls were analyzed in duplicate in a multiplex RT-PCR reaction for 45 cycles using SuperScript III Platinum One-Step qRT-PCR System (Life Technologies Cat# 11732-020) based on the CDC DENV1-4 Real Time RT-PCR Assay (CDC, Catalog number KK0128) and a published assay [36] (primers and probes in Supplemental Table 1). Samples were classified as positive according to a suggested C(t) value of ≤ 37.00, which coincides with a cutoff based on CDC recommendations for identifying positive DENV samples. For ZIKV and CHIKV analysis, total RNA was extracted from human serum specimens using the QIAamp® Viral RNA Mini Kit (QIAgen, Cat# 52906) according to a modified assay developed at the Walter Reed Army

Institute of Research (WRAIR), Viral Diseases Branch. All samples and controls were analyzed in duplicate in a multiplex RT-PCR reaction using TAQMAN Fast Virus 1-Step Mix,(Life Technologies Cat# 4444432). The CHIKV primer/probe set (HEX reporter) was adapted from Armed Forces Research Institute of Medicine Sciences (AFRIMS) protocol, Set 3, which was designed specifically for the Asian genotype CHIK strain currently in the Caribbean and verified using Synthetic CHIKV RNA control (ATCC, Cat# VR-3246SD). The ZIKV primer/probe set (FAM reporter) was based on the AFRIMS protocol that was adapted from a published assay [37] and verified using RNA extracted from ZIKV culture fluid (ZeptoMetrix Corp., Cat# 0810092CF). Both primer/probe sets were specific for their respective viral target and did not detect other viruses (DENV1-4, YFV, and JEV). Samples were classified as positive based on the same cutoff value used for DENV (C(t) value of ≤37.00). Primers and probes for DENV, CHIKV, and ZIKV are shown in Supplemental Table 1.

Statistical analysis.

A participant was considered to have an acute DENV infection if s/he tested positive by NS1 rapid test, NS1 ELISA or RT-PCR. If the person was negative for those three tests, but had anti-dengue IgM antibodies, they were classified as having a recent DENV infection. Individuals who were negative for all of the tests were classified as uninfected with DENV. Individuals who tested negative for all of the tests except for the presence of IgG antibodies were not classified. Individuals who tested positive for CHIKV or ZIKV by RT-PCR were classified as having an acute CHIKV or ZIKV infection.

We calculated expansion factors (EF) for DENV, which provide a more accurate estimate of the burden of disease from case reports, by creating a correction factor for underreporting. For a disease such as dengue, where the rate of symptomatic infections varies, and the degree of severity plays a role in the decision to seek hospital care, EF estimates are similarly varied. In this case, we sought to explore how many infections and symptomatic cases were present in a cluster for each index case. There are a variety of methods used to calculate EFs in the literature [4,38–40], using different data sources and study designs, from cluster-based small cohort methods to large scale (national-level) surveillance data corrections. Here we use a local EF estimate from our cluster study, as described in the following paragraph.

The DENV expansion factor (EF) is the ratio of the best estimate of DENV infections (often from active surveillance) to the number of reported cases (often from passive surveillance) [40]. An EF = 1 reflects 100% reporting of DENV infections, and EF > 1 indicates underreporting [40]. In this instance, we treat the index cases in the cluster (plus any associates who recently sought medical care) as ‘reported’ and the associates with acute and recent dengue infections as the ‘best estimate of DENV infections’. Data from the clusters were used to estimate the weekly and cluster-level dengue infection expansion factor (EF), by dividing the total number of acute or recent dengue infections in associates by the number of initiating acute index cases, plus reporting associates. As the purpose of deriving this EF was to correct MoH reported cases, associates (n=7) who had sought medical care for DENV infections in the past two weeks were added to the index case, as it is possible that they would have been captured by the MoH surveillance system. Although dengue is a mandatory notifiable disease and all suspected cases are supposed to be reported to the MoH, the actual percent that are reported is unknown. Thus, our cluster level calculated EF is:

$$\mathrm{EF} = (\#\ \text{positive in the population tested}) \div (\text{index case} + \#\ \text{reporting associates})$$

We also calculated symptomatic EFs using the number of associates who had dengue symptoms a positive DENV infection in the numerator. We calculated cluster-wise estimates of EF, and present average (SD) and a range values. We assume that the tested population is representative of the larger population, but acknowledge there may be unknown bias due to correlations between likelihood of infection and participation, in either direction.

In addition, we estimated the attributable symptomatic DENV infection rate (ASIR) for associates, where:

$$\text{ASIR} = \frac{\#\ \text{symptomatic DENV positive}}{\#\ \text{DENV positive}} - \frac{\#\ \text{symptomatic DENV negative}}{\#\ \text{DENV negative}}$$

Statistical analyses were conducted using SAS 9.4. Student's t-test was used to determine differences in continuous variables, and Chi-square or Fisher's exact test were used for proportions. Multivariate logistic regressions were developed using proc logistic and backwards selection to identify symptoms correlated with DENV and CHIKV infections in index cases only.

Sequencing and consensus assembly.

Samples from 2014 that were DENV positive by RT-PCR were sent to Walter Reed Army Institute of Research (WRAIR), Viral Diseases Branch, for full-length sequencing. Samples were extracted using a QIAGEN QIAamp viral mini RNA extraction kit in accordance with manufacturer's protocols. Full genome was amplified on Fluidigm Access Array system using dengue serotype specific primers and the Life Technologies SuperScript TM III One-Step RT-PCR system with Platnimum® Taq High Fidelity polymerase, followed by cDNA quality check using Agilent Bioanalyzer DNA7500 kit and RT-PCR product purification. Purified RT-PCR products were quantified using the Invitrogen Quant-iTTM PicoGreen dsDNA Reagent and Kit following the manufacturer's protocols. MiSeq library preparation included: dilution of purified amplicons products to 0.2ng/µL, tagmentation using 5 microliters of each dilution stock as input DNA, neutralization of each Nextera® XT Tagmentation reaction using 5µl NT buffer, PCR amplification using index primers from Nextera XT Index kit version 2 set C, PCR clean up using 25 microliters per PCR reaction of Beckman Counter AMPure XP beads, and library normalization using applicable reagents provided in the Nextera XT® DNA Library Preparation kit. After normalization, each library was pooled and sequenced using the Illumina MiSeq reagent kit (version 2, 500 cycles) and Illumina MiSeq next generation sequencer in accordance with Illumina protocols.

Construction of consensus genomes was performed using ngs_mapper v1.2.4 in-house developed pipeline (available on github, http://dx.doi.org/10.5281/zenodo.46716). Briefly, raw fastq data were stripped of barcodes and adapters and subjected to read filtering using a quality threshold of Q25. Remaining reads were further end-trimmed using a quality threshold of Q25 using Trimmomatic [41]. Trimmed reads with quality >Q25 were initially mapped to a set of reference sequences to determine the best reference fit for each of the samples. Following reference determination, reads from each of the samples were re-mapped to their closest related reference genome, to maximize the number of mapped reads. Reference mapping was performed using the BWA-MEM algorithm [42]. Assemblies were further processed using samtools version 0.1 [43] and an in-house developed python program called *basecaller.py* to produce an adapted VCF for each segment, in parallel, which incorporates genomic ambiguity inherent in RNA viruses into the final consensus genome for that sample based on thresholds set by the investigator. Threshold for consensus genomic reconstruction for ambiguity incorporation was

set at 20% for this analysis, meaning if any site contained a different nucleotide call that was present at 20% or greater in the dataset (taking quality of call into account) the site was given an ambiguous base call (according to IUPAC conventions). Consensus sequences for all samples were constructed, in parallel, from the adapted VCF output. All consensus sequences were further manually quality-checked. Statistics and graphics illustrating read depth and quality of mappings for each sample across each segment produced by the pipeline were done using matplotlib [44].

Phylogenetic analyses.

The five sequenced full genome DENV1 samples were aligned to a set of full genome DENV1 reference sequences obtained from GenBank using MEGAv6 [45]. The 131 reference genomes were selected to represent: i) all DENV1 genotype lineages, for accurate genotype determination, ii) wide sampling time periods, with a focus on the most recently sampled genomes (2009-2016), iii) most geographical regions, with a focus on Central and South America. In addition, the top 20 genomes matching the five genomes from Ecuador through Basic Local Alignment Search Tool (Blast) [46] were added to the reference dataset. A set of 140 full genome DENV2 reference sequences was obtained from GenBank following the same criteria as for DENV1, and aligned to the 27 DENV2 sequenced genomes from Ecuador. Likewise**,** a set of 100 full genome DENV4 reference sequences was obtained from GenBank following the same criteria as for DENV1, and aligned to the single DENV4 sequenced genome from Ecuador. We were unable to sequence DENV3 due to limited sample volume.

The best-fit models of evolution for DENV1, DENV2 and DENV4 datasets were determined using jModelTest v2.1.7 and chosen based on Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) [47]. Maximum Likelihood (ML) phylogenetic trees for each of the DENV1, DENV2 and DENV4 datasets were inferred using Phyml v 4.9.1 [48,49]. The model of evolution used for the full genome tree inferences was GTR+I+Γ (general time reversible with empirically estimated proportion of invariant sites and gamma distribution of among-site variation, 4 categories), for all three dengue serotypes. The tree space was searched heuristically using the best of NNI (Nearest Neighbor Interchanges) and SPR (Subtree Pruning and Regrafting). Node confidence values were determined by aLRT (approximate Likelihood Ratio Test) using the nonparametric Shimodaira-Hasegawa approach. Node confidence values of >0.75 are considered good support. The resulting trees were rooted by the KR919820 sylvatic reference genome [50] for DENV1, and by the sylvatic genotype outgroups for DENV2 and DENV4.

Results

From January 1, 2014, through December 31, 2015, a total of 324 index cases with suspected DENV infections were recruited from the sentinel clinics and the hospital in Machala, Ecuador (194 index patients in 2014, 130 in 2015) (Table 1, Fig 2). We randomly selected 44 index cases as initiates of clusters, from which 397 associates were recruited into the study (310 associates in 2014, 87 in 2015). In 2014 and 2015, DENV transmission began in January and February, peaked in May, and tailed off in September and October (Fig 3). CHIKV was first identified in our study on epidemiological week 12 in 2015, and transmission followed a similar seasonal curve as DENV. No ZIKV infections were detected through either passive or active surveillance.

Table 1 shows the diagnostic results from 2014 and 2015. There were some individuals who did not have enough information to categorize as DENV positive or negative, for example, an individual who was negative for an NS1 rapid test and PCR, but did not have any ELISA or serology test results. To account for these discrepancies, prevalence estimates include people for whom dengue test results were available, as indicated by the denominators in the diagnostic results section of the table.

Fig 2. Study design. DENV surveillance study design in Machala, Ecuador, in 2014 and 2015.

Fig 3. Weekly DENV and CHIKV infections in 2014 and 2015. (A) Acute or recent DENV infection, (B) Non-DENV infections, (C) CHIKV infections, and (D) non-DENV, non-CHIKV infections. Note: no surveillance was conducted in week 30 of 2014.

Passive surveillance of index cases

In 2014, 132 of 182 (72.5%) index cases were positive for an acute or recent DENV infection (Table 1). In 2015, 35 of 124 (28.3%) index cases had an acute or recent DENV infections, and 50 of 122 (41%) had an acute CHIKV. One index case was positive for both acute DENV and acute CHIKV infections, and five index cases were positive for recent DENV infections and acute CHIKV infections. In addition, there were 45 index cases that were negative for DENV and CHIKV in 2014, and 38 in 2015 (Supplemental Table 4).

Fig 2. Study design. DENV surveillance study design in Machala, Ecuador, in 2014 and 2015.

In 2014, all four DENV serotypes were detected in index cases, including one individual positive for DENV1 and DENV2 (Table 2). Most infections in 2014 were DENV2 (43/51, 84.3% of serotyped index patients) and were secondary DENV infections (73/122, 59.8%). In 2015, DENV1 and DENV2 were detected. Most infections were DENV1 (13/22, 59.1% of serotyped index patients) and were primary DENV infections (21/35, 60.0%). In index cases, young adults aged 21 to 30 years had the highest prevalence of primary DENV infections, and adults aged 31 to 40 years had the highest prevalence of secondary DENV infections (Fig 4).

Fig 4: Age specific prevalence of index and associate cases. (A) Acute or recent DENV infection, (B) acute CHIKV infection, (C) primary versus secondary DENV infections.

In index cases, children and adolescents (< 20 years) accounted for 57.9% of all febrile acute or recent DENV infections (Table 3). Children aged 11 to 20 years had the highest prevalence of febrile acute or recent DENV infections (57/88, 64.8% of index cases 11-20 years of age), and the highest prevalence of combined symptomatic and subclinical DENV infections (Fig 4). In contrast, the prevalence of febrile and afebrile acute CHIKV infections increased with increasing age (Table 3, Figure 4). Index subjects aged 51 to 60 years had the highest prevalence of febrile acute CHIKV infections (6/8=75% of index subjects 51-60 years). Individuals with CHIKV infections were significantly older (34 years, SD=18.0, N=36) than those with DENV infections (21 years, SD = 14.0, N=161) ($p<0.0001$), excluding individuals with both acute CHIKV and acute/recent DENV infections. Adults (>20 years) accounted for 73% of febrile acute CHIKV infections (Table 3).

We found significant differences in DENV symptoms by age group and by primary versus secondary infections (Table 4). Symptoms that were less common in the 0 to 10 year age group included diarrhea for all infections, muscle/joint pain for all infections, retro-orbital pain in secondary infections, and drowsiness/lethargy in secondary infections ($p<0.05$).

Overall, we identified more severe illness in secondary DENV infections than in primary infections (Supplemental Table 3). Vomiting (45/82=54.9% vs. 15/43=34.9%, p=0.04) and hospitalization (33/75-44.0% vs. 4/37=10.8%, p=0.001) were significantly more common among individuals with secondary infections. Bleeding (12/82=14.6% vs. 3/42=7.14%) and diarrhea (25/82=30.5% vs. 10/43=23.3%) were also more common in individuals with secondary infections, although the differences between primary versus secondary infections were not statistically significant ($p>0.05$). Fever (temperature measured >38°C) was significantly less common among secondary than primary infections (7/79=8.86% vs. 10/39=25.6%, p = 0.02). We did not find significant differences in symptoms between DENV1 and DENV 2, the predominant serotypes detected in this study (Supplemental Table 2).

Multivariate analysis of symptoms in index cases

Multivariate logistic regression analysis was used to identify the symptoms of index patients associated with (1) DENV vs. non-DENV infections (excluding CHIKV infections), (2) CHIKV versus acute or recent DENV infections, and (3) CHIKV versus non-CHIKV infections (excluding DENV) (Table 5).

The best model to explain DENV vs. non-DENV infections indicated that the presence of rhinorrhea was associated with decreased odds of DENV infection (Adj OR=0.28, 95% CI: 0.14-0.55, p=0.0003). Diarrhea was predictive of DENV in both years, but more so in 2014 than 2015 (year*diarrhea interaction p=0.0255). Abdominal pain was predictive of dengue in both years, but more so in 2015 than in 2014 (year*abdominal pain interaction p=0.0254) (Table 5). These results are consistent with bivariate analyses of symptoms in 2014 and 2015 (Supplemental Table 4).

The best model to explain CHIKV infections versus acute or recent DENV infections included age (Adj OR=1.05, 95% CI: 1.03-1.08, $p<0.0001$), rash (Adj OR=2.66, 95% CI: 1.08-6.52, p=0.03), and absence of cough (Adj OR=0.33, 95% CI: 0.11-0.99, p=0.048) (Table 5). Bivariate analyses of index patients with DENV versus CHIKV infections indicated that DENV patients were more likely to present with abdominal pain (p=0.04), and patients with CHIKV were more likely to present with muscle or joint pain (p=0.004) (Supplemental Table 5).

The best model to explain CHIKV versus non-CHIKV infections (excluding DENV infections) included muscle or joint pain (Adj OR=18.41, 95% CI: 2.29 – 154.19, p=0.007), rash (Adj OR=4.48, 95% CI: 1.4 – 14.28, p=0.005), and rhinorrhea (Adj OR=0.19, 95% CI: 0.06-0.61, p=0.005) (Table 5).

Active surveillance of associates

In each cluster of homes, approximately nine associates were recruited into this study per index case. The distance between the households of associates and the respective index households ranged from 2.2 to 164 meters, with an average of 39 meters (SD=29 m). Most associate households (95.4%) were within 100 meters of the index household. Associates recruited into the study were more likely to be female ($p<0.0001$) and were older ($p<0.0001$) than index cases (Table 1).

In 2014, 106 of 298 (35.6%) associates had evidence of acute or recent DENV infections (Table 1). As in index cases, the prevalence of DENV disease decreased in 2015, with 11 of 86 (12.85%) associates with acute or recent infections. In 2015 there were four of 87 associates with CHIKV infections (4.6%), including one associate with both acute CHIKV and recent DENV infections. There were 16 associates with a febrile illness that was neither DENV nor CHIKV (symptoms presented in Supplemental Table 6).

In 2014, DENV1, DENV2, and DENV3 were detected in associates (Table 2). As in index cases, most infections were DENV2 (10/18, 55.6%). A similar proportion of primary (38/106, 35.8%) and secondary (43/106, 40.6%) infections were detected. In 2015, DENV2 was detected in one associate, and the majority of infections were primary infections (21/35, 60.0%).

Slightly more than half of the associates with acute or recent DENV infections (excluding one with an acute CHIKV infection) reported dengue-like symptoms (63/115, 56.3%), i.e., fever, rash, muscle or joint pain, abdominal pain or tenderness, bleeding, drowsiness or lethargy within the last seven days. The ratio of DENV positive associates with dengue symptoms to those without was 1:0.7. The overall attributable symptomatic DENV infection rate (ASIR) was 0.02 in 2014 and -0.45 in 2015. The decline in the DENV ASIR from 2014 to 2015 was due to the emergence of CHIKV in 2015. Overall, few associates with acute or recent DENV infections sought medical care (6.5%, 7/106 in 2014 and 0%, 0/10 in 2015) (Table 2).

Associate children aged 0 to 10 years had the highest prevalence of febrile acute or recent DENV infections (3/23=13% of associates 0-10 years of age). However, when afebrile infections were considered, children aged 11-20 had the highest overall prevalence of DENV infections (Fig. 4). The prevalence of primary DENV infections peaked at 11-20 years (Fig 4c). There was no clear peak age class in secondary associates, likely due to the small sample size. There were no associates who were febrile and positive for acute CHIKV.

At the household cluster level, prevalence rates varied by the DENV serotype of the index patient. In 10 of 44 clusters, the index case had a DENV1 infection. In these clusters, 20% of associate cases had acute or recent DENV infections (12/60; 95% CI: 11.8-31.8%), with a range of 0% to 57.1%. The index case had a DENV2 infection in 17 of 44 clusters. Among these clusters, a significantly greater proportion of associate cases (36.6%; 59/161; 95% CI: 29.6-44.3%) (p=0.02) had acute or recent DENV infections, with a range of 12.5% to 87.5% within spatiotemporal clusters. ,

The overall estimated expansion factor (EF), calculated as the ratio of all DENV infections in the clusters to the number of index and reported associate infections, was 3.16. Cluster estimates of expansion factors ranged from 1 to 10, with a median of 3 and a mean of 3.28 (SD=2.14). The mean EFs were 3.8 for 2014 and 1.92 for 2015. The symptomatic EF was 2.31 on average (SD=1.84), (cluster mean=2.84 overall, 3.38 in 2014 and 1.42 in 2015).

Phylogenetic analysis of DENV.

The best-fit models for the evolution of DENV1, DENV2, and DENV4, as determined by AIC versus BIC, agreed in all cases. ML phylogenetic tree demonstrated a clear distinction of DENV1 genotypes *I, II, IV* and *V,* and the sylvatic genotypes *III* and *VI* (Fig 5). The five genomes from Ecuador, all sampled in 2014, belonged to genotype *V* of DENV1 and were found in the sub-lineage containing mainly Central and South American genomes (i.e., Colombia, Venezuela, Argentina, Brazil and Puerto Rico). More importantly, sequences from Ecuador fell into two distinct clades within this sub-lineage; two Ecuadorian genomes more closely related to

genomes sampled in Argentina and Venezuela (Clade A), and three Ecuadorian genomes more closely related to a genome from Colombia (Clade B).

Fig 5. Maximum likelihood phylogenetic tree of DENV1 genotypes from Ecuador in 2014. Samples from Ecuador are colored magenta (dark and light). The two clades containing the genomes from Ecuador are marked in the tree (A and B). aLRT confidence values are shown next to the respective node. The tree is rooted on the sylvatic genotype *VI* sample. Some clades were collapsed in the tree to increase clarity. All collapsed clades were supported with high (>0.75) aLRT values and contained only genomes from a single country, indicated in the name of the clade. Colored taxa represent known genotype references.

The ML phylogenetic tree of DENV2 showed a clear distinction of DENV2 genotypes, including sylvatic, American, Cosmopolitan, Asian I, Asian II and Asian/American (Fig 6). The samples from Ecuador were found within the Asian/American genotype, making up a monophyletic cluster (Clade A) separated from the rest of the South American taxa with high support (aLRT = 1). Genomes clustering closest to the clade A from Ecuador were sampled in Colombia and Venezuela. Sequences from other neighboring countries, such as Peru and Brazil, were found further down in the Asian/American lineage and were separated from the clade A, and from sequences from Colombia and Venezuela, with high support (aLRT = 0.99).

Fig 6. Maximum likelihood phylogenetic tree of DENV2 genotypes from Ecuador in 2014. Samples from Ecuador are colored magenta in a monophyletic clade A. aLRT confidence values are shown next to the respective node. The tree is rooted on the sylvatic genotype outgroup. Some clades were collapsed in the tree to increase clarity. All collapsed clades were supported with high (>0.75) aLRT values and contained only genomes from a single country, indicated in the name of the clade. Colored taxa represent known genotype references.

The ML phylogenetic tree of DENV4 demonstrated a clear distinction of genotypes *I, IIA, IIB, III* and sylvatic (Fig 7). However, two taxa from India/1961-1962 clustered with genotype *I* with low support (aLRT=0.04), indicating their position in the tree was uncertain and they might belong to a different genotype. The single Ecuador sequence was located within the genotype *IIB* lineage (magenta in the tree). It was surrounded by sequences collected from Venezuela, Colombia and Brazil, indicating their common ancestry. However, the aLRT support for the Ecuador node was low (0.37) suggesting that its correct placement was uncertain.

Fig 7. Maximum likelihood phylogenetic tree of DENV4 genotypes from Ecuador in 2014. Sample from Ecuador is colored in magenta. aLRT confidence values are shown next to the respective node. Low aLRT values are highlighted in red. The tree is rooted on the sylvatic genotype outgroup. Some clades were collapsed in the tree to increase clarity. All collapsed clades were supported with high (>0.75) aLRT values and contained only genomes from a single country, indicated in the name of the clade. Colored taxa represent known genotype references.

Discussion

To date, this is one of the most comprehensive epidemiological studies of DENV, CHIKV and ZIKV in Ecuador, and the study is ongoing. The results of this enhanced surveillance study provide a more accurate estimate of the symptomatic and subclinical burden

of CHIKV and DENV infections across age groups than detected through traditional passive surveillance. We found that burden of symptomatic dengue was greatest in children under 10 years of age. For every case of dengue detected by standard surveillance, we detected an additional three infections in the community. Of people in the community with dengue infections, slightly more than half showed symptoms. Our results indicate that the relative contribution of DENV to symptomatic infections varied greatly from 2014 to 2015 due to the emergence of CHIKV. Genetic analyses indicate that there is movement of the dengue virus between Ecuador and neighboring countries, highlighting the importance of sentinel surveillance sites, such as Machala, in border regions.

Burden of disease and EF estimates.

On average over the two years of the study, 122 of 384 (31.8%) of associates were DENV positive, a higher prevalence than findings from similar studies in Asia. In Vietnam, studies found 18% DENV prevalence in 100 meter clusters around index patients, using PCR, NS1 ELISA, or serology [20]. In Thailand, cluster DENV prevalence ranged from 10.1% to 14.3% using PCR or serology [21,22]. One of possible explanations for the higher cluster prevalence in this study is the use of the NS1 rapid strip test. We found that the prevalence of DENV infections in clusters varied by DENV serotype (DENV1: 20.0%; DENV2: 36.6%). The higher cluster prevalence for DENV2 is consistent with prior studies that found greater infection rates for DENV2 compared to DENV1 [51].

We detected a high incidence of febrile dengue cases in associate children (<20 years) in clusters (10/86, 11.6%, 116 per 1,000 person-years, Table 3), which was much higher than estimates from pediatric cohort studies in Latin America, possibly due to a higher force of infection in areas near the index patient with dengue illness. For example in a pediatric cohort in Nicaragua rates were 16.1 per 1,000 person-years [52], door-to-door surveillance and school-based absentee surveillance in school children in Peru rates were 12.9 to 23.5 per 1,000 person-years [25], and in a school based cohort in Colombia rates were 4.9 to 5.9 per 1,000 person-years [27].

The expansion factor (EF) for DENV in Machala was estimated using the ratio of all infections and symptomatic infections to the number of medically-attended infections among the 44 clusters. Our overall estimate was 3.16 for all infections (cluster mean: 3.28 overall, 3.80 in 2014, 1.92 in 2015) and 2.31 (cluster mean: 2.84 overall, 3.38 in 2014, and 1.42 in 2015) for symptomatic infections, indicating that estimates of dengue incidence based on reporting from clinics and hospitals miss approximately 68% of infections. This EF is comparable to the low end of a range of previously reported EFs for the PAHO region [40]. In this study, the EFs were relatively stable over time, suggesting that even a few weeks of investigations can provide estimates for the season. Based on the MoH's estimate of an annual incidence of 4.3 per 1,000 person years in 2014 and 9.9 per 1,000 person years in 2015, the estimated actual annual incidences, including symptomatic and subclinical cases, are 16.3 per 1,000 person-years, and 17.6 per 1,000 person years in 2014 and 2015, respectively. Interestingly, we found that the EF was higher in 2014 than 2015, suggesting a higher force of infection in 2014, but with low symptomology. We temper this suggestion with caution, however, as our cluster sample size was smaller in 2015 (n=12) than 2014 (n=32). We found that the incidence of dengue infections in

this study was similar to previously reported estimates from active surveillance, including a pediatric cohort in Nicaragua (16.1 per 1,000 person-years) [10], enhanced community-based surveillance in Peru (23.5 per 1,000 person-years) [7], and enhanced laboratory-based surveillance in Puerto Rico (7.7 per 1,000 person-years) [8]. This suggests that the rapid surveillance methods developed in this study provide reliable estimates of the burden of disease, which can be applied to estimate the burden of other underreported febrile diseases, allowing the public health sector to more effectively and equitably conduct disease control interventions.

To our knowledge, most cluster-based dengue surveillance studies have been conducted in Asian countries. In Latin America, enhanced surveillance studies have focused on pediatric and adult cohorts, door-to-door community based surveillance, use of sentinel clinics, and enhanced laboratory diagnostics. Expansion factors estimates vary widely depending on the surveillance methods used, and the characteristics of the local population, including past exposure to DENV serotypes. In a pediatric cohort in Nicaragua, investigators detected 21.3 times more dengue cases than were reported to the national surveillance system [53]. A study in Peru compared passive surveillance of dengue to a cohort study and sentinel clinic surveillance, and estimated an EF of 5 for the cohort and an EF of 19 for the sentinel clinic surveillance [24]. They found that both sentinel and cohort surveillance methods detected an increase in dengue cases more rapidly than passive surveillance methods. In Puerto Rico, laboratory enhanced surveillance resulted in three times more cases registered than passive surveillance methods [26].

On average, index cases positive for DENV or CHIKV were more likely to be male than positive associates. These differences may reflect variation in exposure to infectious mosquito bites, a greater propensity for severe symptoms in men, gender differences in health-seeking behaviors, or a gender bias during the recruitment of associates (e.g., more women at home during the day). This could be a spurious result, although prior studies have reported a higher prevalence of DENV in men than in women [54,55].

One of the limitations of this study was that we surveyed the nearest neighbors of the index case, which are not necessarily representative of the population residing within 200 meters. Also, people may have been more willing to participate in the study if they or someone in their household had disease. Future studies could survey a greater number of households located randomly within the 200-meter radius for a more accurate measure of disease prevalence. Another limitation was that individuals who were positive for IgM and without dengue-like symptoms within the last seven days were classified as asymptomatic; however, the positive IgM could indicate an infection beyond the seven-day window. A more robust diagnosis would be based on the detection of a four-fold or greater rise in IgM antibody titer in acute and convalescent samples, which were not available for most subjects in this study.

Burden of CHIKV and other febrile illness:

In 2015, we found that 41% (50/122) of clinically diagnosed DENV infections were positive for CHIKV, higher than the proportion of laboratory-confirmed dengue cases (35/124=28.2%). We identified six index cases (6/122=4.94%) and one associate (1/87=1.1%) with evidence of both acute CHIKV and acute or recent DENV infections in 2015. There were also 96 individuals with undiagnosed febrile illness (non-DENV, non-CHIKV, non-ZIKV). The

burden of CHIKV is likely higher than reported here, since anti-body tests were not utilized. This highlights the difficulties of differential diagnosis in areas where DENV, CHIKV, ZIKV, and other febrile illnesses are co-circulating. These data also suggest that the large increase in DENV cases in 2015 reported by Pan American Health Organization (PAHO) and MoH in Ecuador (42,667 cases in 2015 versus 14,412 cases on average from 2010 to 2014 [15]) could be the result of other circulating arboviruses, including CHIKV.

We did not detect ZIKV in our surveillance system during the study period, consistent with MoH reports, which indicated that ZIKV circulated for the first time in Machala in February 2016. Although surveillance efforts were not focused specifically on clinical ZIKV infections, we suspect that the study would have detected some ZIKV infections if they were present in Machala due to the overlapping clinical presentations of DENV and ZIKV infections. However, more recent studies shown that Zika virus may be more readily detected in urine and whole blood, limiting our ability to detect ZIKV in serum samples by RT-PCR [56,57]. Although cases of Zika fever declined throughout much of Latin America in 2017, the Zika epidemic is still unfolding in Ecuador, with over 1,771 cases reported in 2017, representing 33% of all cases reported. Additionally, the first cases of congenital syndrome associated with Zika virus were detected in Ecuador in early May 2017.

Clinical predictors of DENV and CHIKV.

In general, the frequencies of symptoms that were observed in DENV infections are consistent with other reports [58–64]. Findings from this study indicate that symptoms associated with DENV infections may vary year to year, likely due to both differences in the dominant serotypes in circulation [65,66] and the ratio of primary versus secondary infections [23,63,67]. In the multivariate model, rash, diarrhea and abdominal pain were associated with DENV infections; rhinorrhea and cough were associated with infections that were neither DENV nor CHIKV. Prior studies also reported that gastrointestinal symptoms were predictive of DENV infections in a multivariate model [65]. In our study group, diarrhea was more predictive of DENV in 2014, when DENV2 was prevalent and more secondary infections were found. Abdominal pain was more predictive in 2015, when DENV1 was prevalent and more primary infections were observed. However, other studies did not find differences in rates of diarrhea and abdominal pain between DENV-1 and DENV-2 [68,69]. Therefore, the difference that we observed between the two years is more likely to be due to differences in the ratio of primary to secondary infections. Consistent with prior studies, we found that secondary infections had a higher proportion of severe outcomes including hospitalization, bleeding, and vomiting [23,63,67].

People infected with CHIKV versus DENV were older on average, consistent with the disease being newly introduced into the population. MoH reports indicated that the highest burden of CHIKV in Machala was among adults aged 20 to 49. We found that rash and muscle or joint pain were more commonly reported by people with CHIKV infections than those with DENV, which is consistent with previous reports [60,64].

Associates with acute or recent DENV infections had symptoms similar to those reported by acute or recent DENV index cases, but symptoms were reported less frequently. Prior studies that report asymptomatic illness, defined asymptomatic as afebrile whereas we use a broader definition of asymptomatic to include the absence of any dengue-like symptom [70]. The overall ratio of DENV positive associates with dengue symptoms to those without was 1:0.7. The proportion of subclinical infection is similar to prior studies [23,70], and highlights the

importance of active surveillance protocols to capture subclinical infections not registered in traditional passive surveillance systems.

Phylogenetic analysis

Phylogenetic analyses of DENV1 showed Ecuadorian samples falling into two distinct clusters, sharing a common ancestor with viruses from Colombia in one cluster and a common ancestor with viruses from Venezuela in the other one. These well-separated clusters indicate at least two distinct introductions of DENV1 into Ecuador. Given the early sampling of Venezuelan and Colombian genomes (between 2004 and 2008), and given that recent DENV1 full genome samples from Peru are not available, we cannot exclude with certainty the role that Peru may have played in the DENV1 introductions into Ecuador. However, the results suggest a close genetic relationship of viruses circulating in Venezuela and Colombia and support the notion of commonly occurring DENV1 flow between the countries. Similar to DENV1, DENV2 genomes from Ecuador were most closely related to genomes from Venezuela and Colombia. However, unlike DENV1, DENV2 genomes from Ecuador made up a single monophyletic clade separated from the rest of the South American taxa with high support. This indicates a single introduction and subsequent spread of this virus in Ecuador without further DENV2 introductions and mixing from other regions. Even though older sequences from Peru clustered further away from genomes sampled in Ecuador, Venezuela, and Colombia, suggesting they did not play a role in the current DENV2 epidemic in Ecuador, the lack of recent full genomes from Peru prevent us from determining the involvement of Peru in the observed DENV2 spread in Ecuador. The unavailability of recent full genomes from countries surrounding Ecuador was most evident in DENV4, where the exact placement of the only Ecuadorian genome in the tree could not be determined due to low node support. Nevertheless, the results suggested a close relationship between DENV4 in Ecuador, Venezuela, Colombia and Brazil. It is important to note that samples from Peru were missing here as well, and that there is a possibility this country was also involved in the circulation of DENV4 in this region. Thus, our results suggest frequent flow of DENV between Ecuador and surrounding countries, including introduction and re-introduction of different serotypes and different lineages of the same serotype. In addition, our results show the importance of continuous surveillance, including genetic sequencing efforts. If available, virus full genomes from these countries would allow for more accurate analysis of the patterns of DENV movement and spread in this region.

Public health implications

This study contributes to a long-term collaboration with the MoH and other governmental and academic partners to strengthen infectious disease surveillance in southern coastal Ecuador, a strategic area to monitor endemic and emerging pathogens. The collaboration has been successful due to a shared vision for integrated active surveillance that includes the virus, vector, climate and other social-ecological drivers; ongoing training of physicians, researchers and students; and improvement of local diagnostic and research infrastructure.

Rapid active surveillance studies, such as this, provide high-resolution spatiotemporal data on the distribution of symptomatic and subclinical arboviral infections across the population. This is especially important in places and in subgroups with low-health care seeking behavior, which result in underreporting and continued disease transmission, as reported in Machala [18,71]. Enhanced surveillance systems have been shown to detect an increase in disease cases earlier than passive surveillance systems [24], providing a warning of an escalating

outbreak. These data are currently being used to parameterize and calibrate local epidemic forecast models (Lowe, Stewart-Ibarra et al, *in review*). These data also allow the public health sector to more accurately estimate the social and economic cost of the disease, allowing for informed decision making regarding the allocation of scarce resources for current and future interventions, such as vector control, community mobilization, and vaccines. The age-stratified seroprevalence data generated through this study design also provides important information for the design of vaccine trials and vaccination campaigns.

Disclaimer. Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting the views of the Department of the Army, or the Department of Defense.

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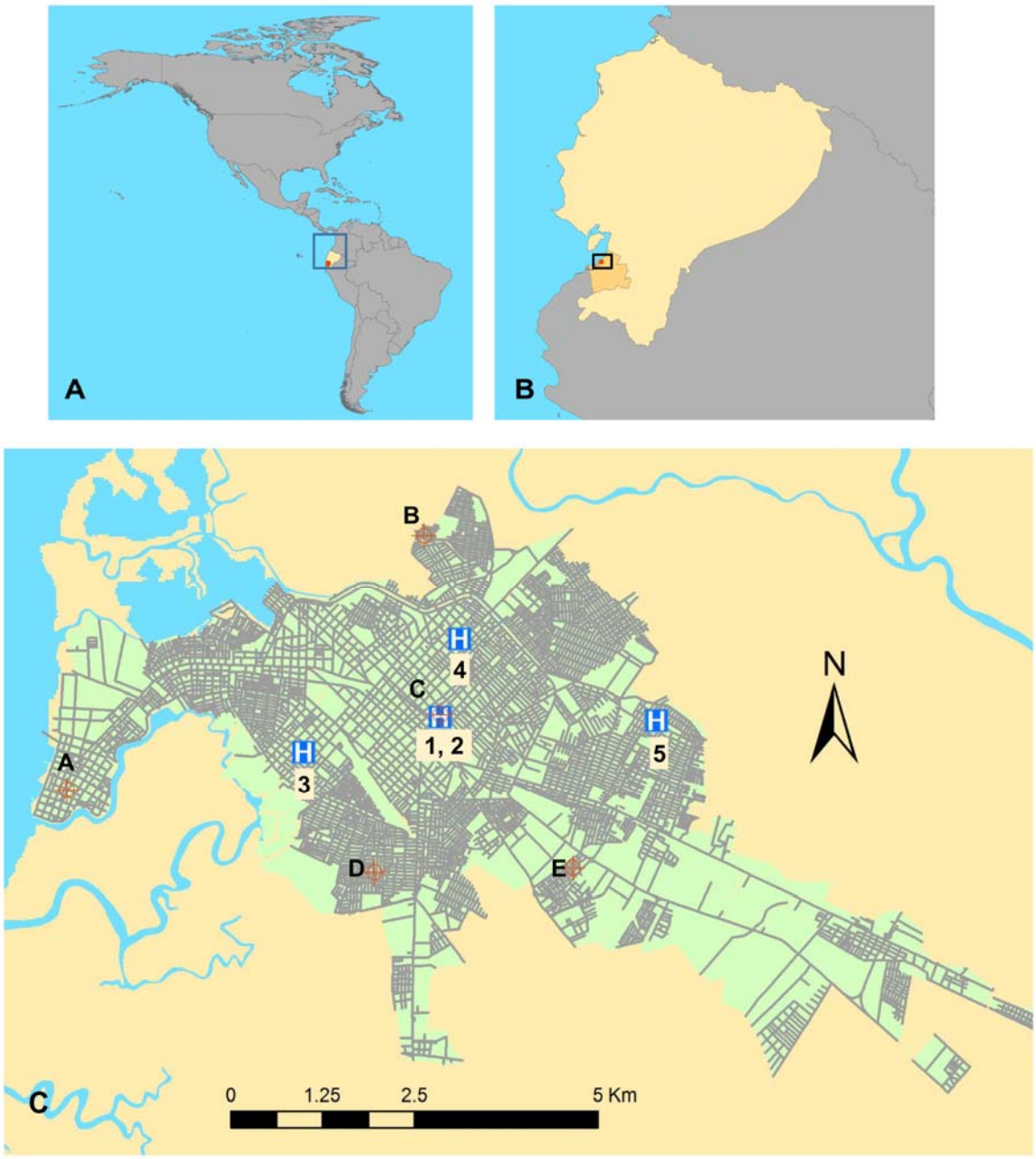

Fig 1: Map of the study site: A. Location of Ecuador in the Americas. B. Location of El Oro Province in Ecuador, the city of Machala indicated as a red dot. C. The city of Machala, showing the five Ministry of Health clinical sites/hospital: 1. Mabel Estupiñan Clinic, 2. Teofilo Davila Hospital, 3. Brisas del Mar Clinic, 4. El Paraiso Clinic, 5. Rayito de Luz Clinic. The location of meteorological stations are indicated by A-E as follows: A. Puerto Bolivar, B. Los Esteros, C. Mabel Estupiñan; D. Florida; E. Crucitas.

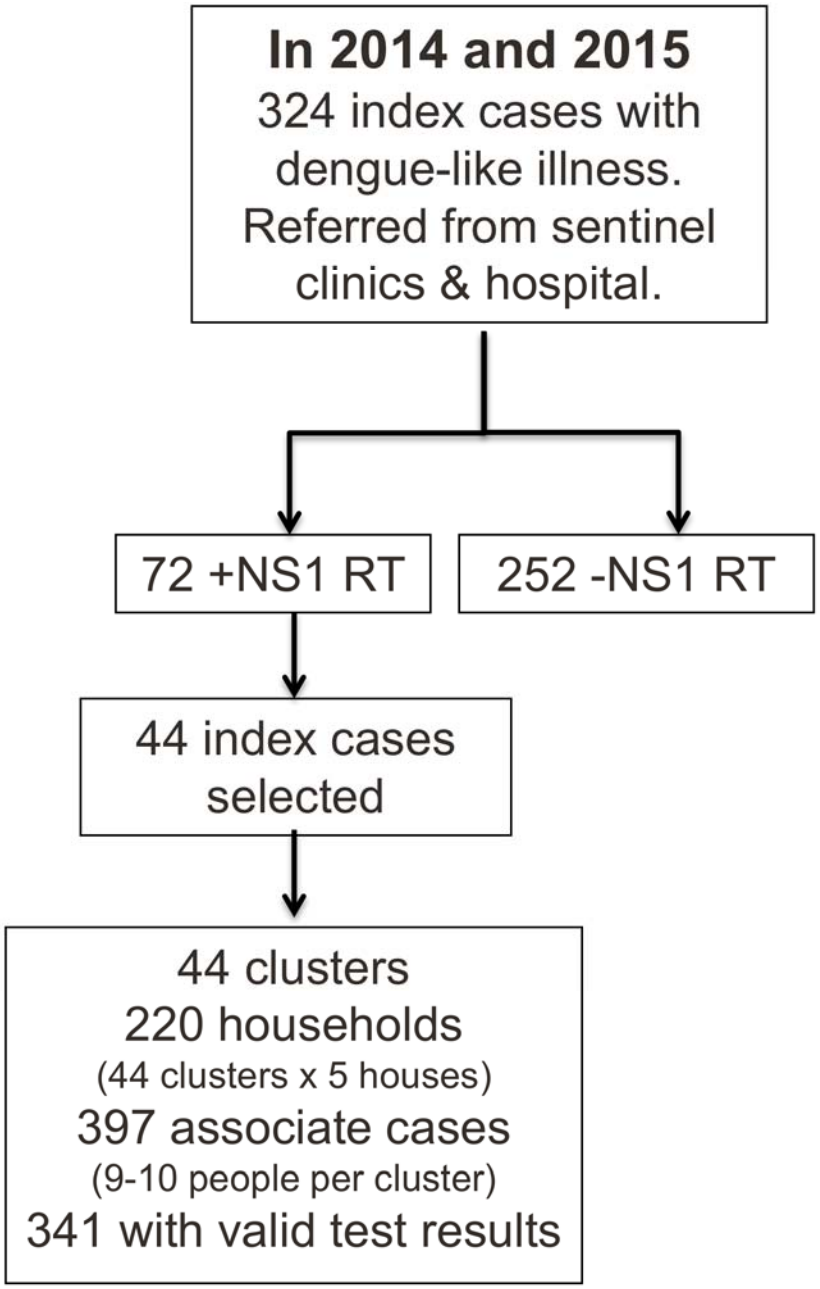

Fig 2. Study design. DENV surveillance study design in Machala, Ecuador, in 2014 and 2015.

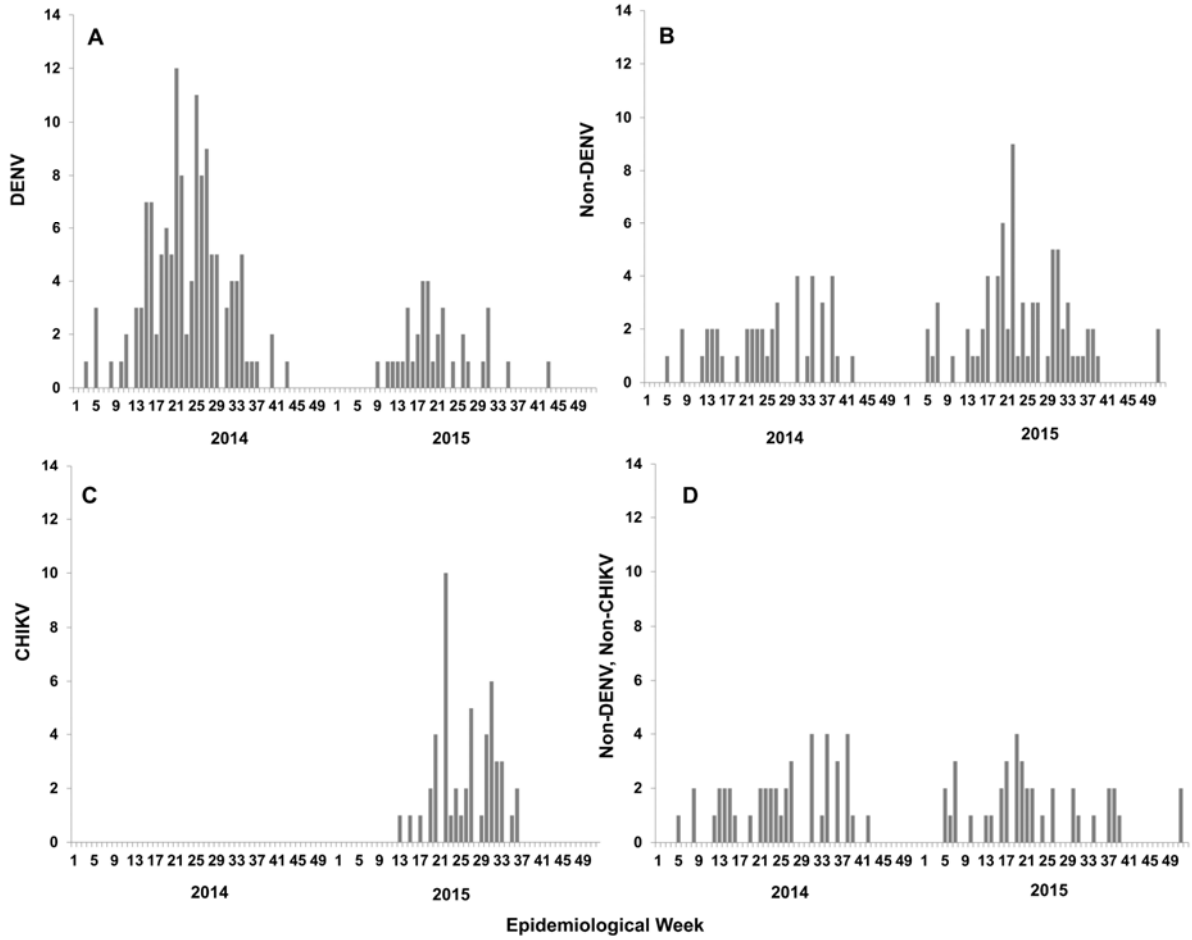

Fig 3. Weekly DENV and CHIKV infections in 2014 and 2015. (A) Acute or recent DENV infection, (B) Non-DENV infections, (C) CHIKV infections, and (D) non-DENV, non-CHIKV infections. Note: no surveillance was conducted in week 30 of 2014.

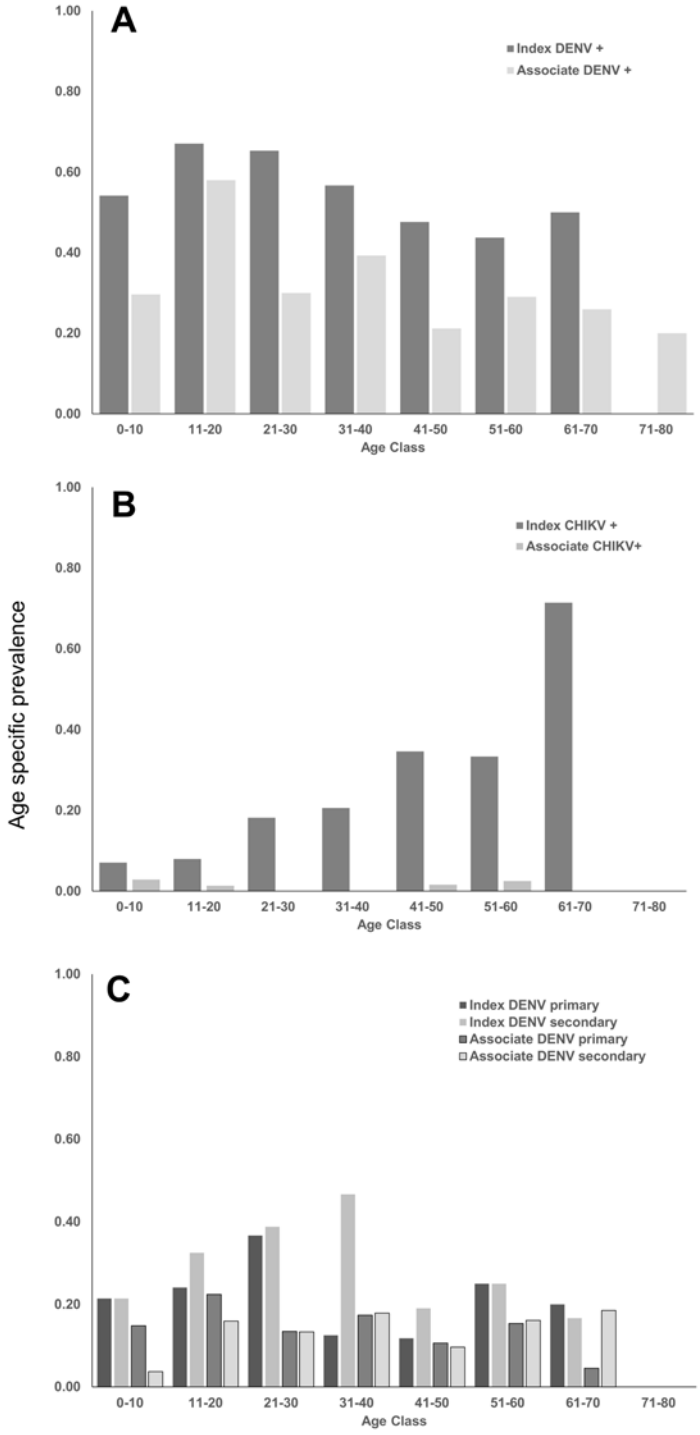

Fig 4: Age specific prevalence of index and associate cases. (A) Acute or recent DENV infection, (B) acute CHIKV infection, (C) primary versus secondary DENV infections.

Fig 5. Maximum likelihood phylogenetic tree of DENV1 genotypes from Ecuador in 2014. Samples from Ecuador are colored magenta (dark and light). The two clades containing the genomes from Ecuador are marked in the tree (A and B). aLRT confidence values are shown next to the respective node. The tree is rooted on the sylvatic genotype *VI* sample. Some clades were collapsed in the tree to increase clarity. All collapsed clades were supported with high (>0.75) aLRT values and contained only genomes from a single country, indicated in the name of the clade. Colored taxa represent known genotype references.

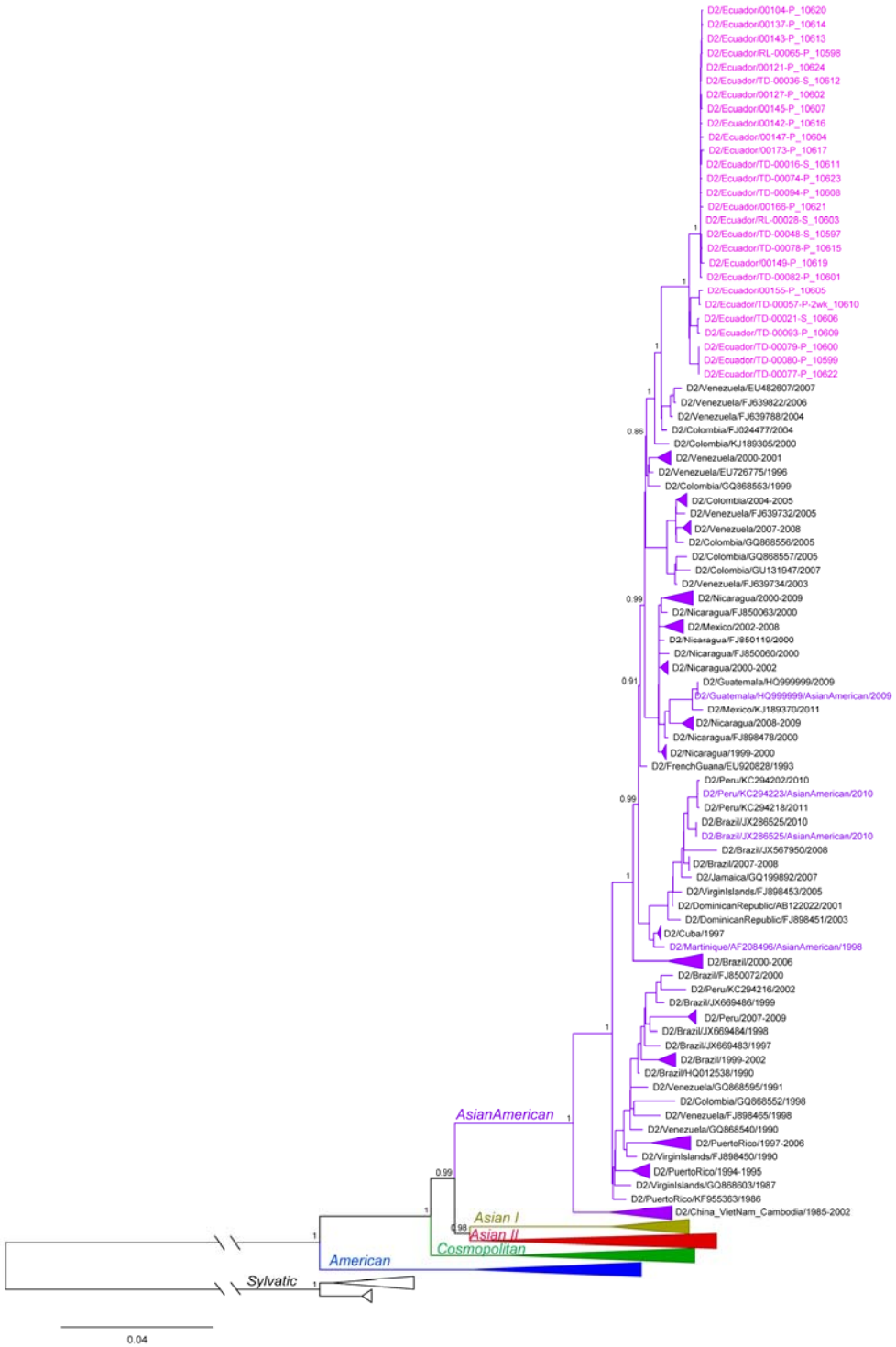

Fig 6. Maximum likelihood phylogenetic tree of DENV2 genotypes from Ecuador in 2014. Samples from Ecuador are colored magenta in a monophyletic clade A. aLRT confidence values are shown next to the respective node. The tree is rooted on the sylvatic genotype outgroup. Some clades were collapsed in the tree to increase clarity. All collapsed clades were supported with high (>0.75) aLRT values and contained only genomes from a single country, indicated in the name of the clade. Colored taxa represent known genotype references.

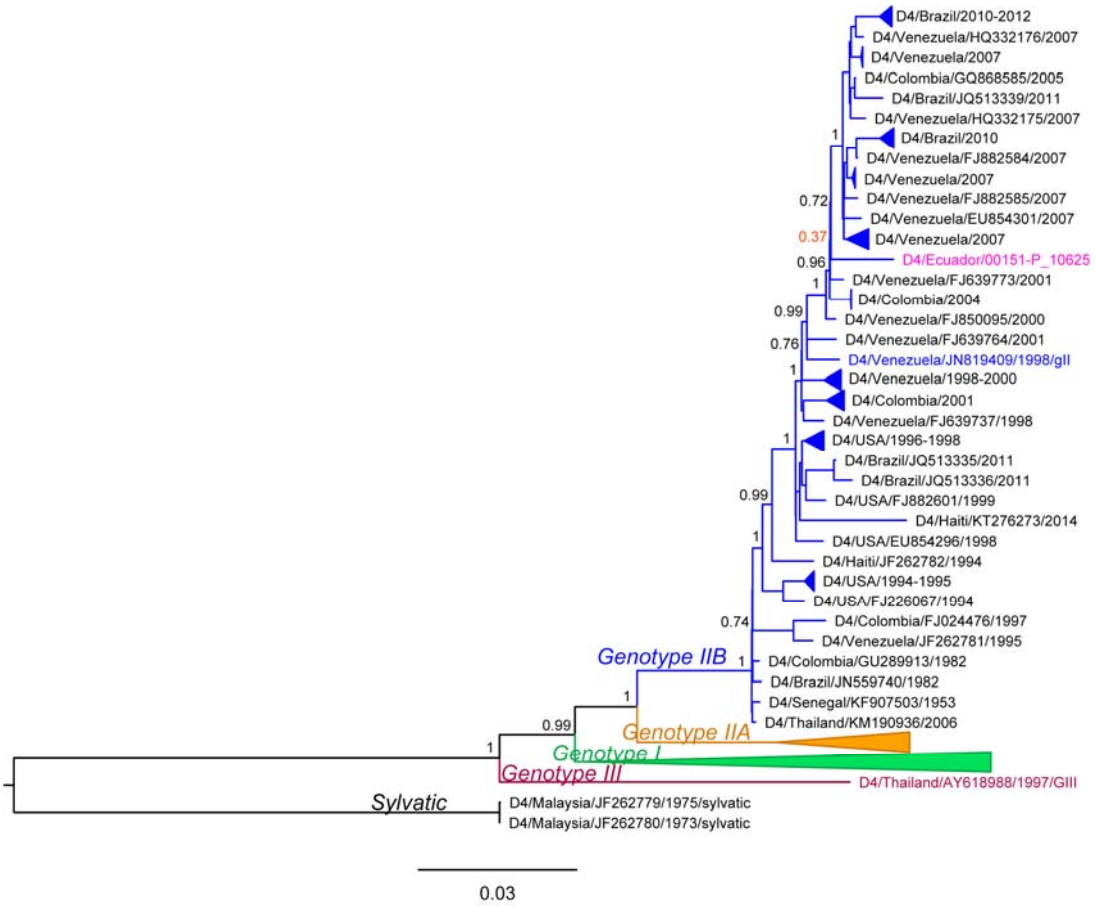

Fig 7. Maximum likelihood phylogenetic tree of DENV4 genotypes from Ecuador in 2014. Sample from Ecuador is colored in magenta. aLRT confidence values are shown next to the respective node. Low aLRT values are highlighted in red. The tree is rooted on the sylvatic genotype outgroup. Some clades were collapsed in the tree to increase clarity. All collapsed clades were supported with high (>0.75) aLRT values and contained only genomes from a single country, indicated in the name of the clade. Colored taxa represent known genotype references.

Table 1. Index cases and associate demographic data and infection status.

	2014		2015	
	Index cases (N = 194)	**Associates (N = 310)**	**Index cases (N = 130)**	**Associates (N = 87)**
Demographics				
Age in years, mean (SD)	20.4 (15.7) N=194	34.9 (19.8) N=310	27.0 (18.8) N=130	38.4 (20.2) N=87
Gender, % female	92/194 (47.4%)	203/310 (65.5%)	70/130 (53.8%)	59/87 (67.8%)
Fever				
Acute fever (>38°C)	153/185 (17.3%)	2/304 (0.66%)	25/124 (20.2%)	0/87 (0%)
History of fever (self-report)	187/193 (96.9%)	33/300 (11.0%)	125/130 (96.2%)	3/84 (3.6%)
Fever by either measure	188/193 (97.4%)	33/300 (11.0%)	125/130 (96.2%)	3/84 (3.6%)
DENV infection				
Acute infection (NS1 RT, NS1 ELISA or PCR pos)	75/182 (41.2%)	45/298 (15.1%)	24/124 (19.4%)	5/86 (5.8%)
Recent infection (NS1 RT/NS1 ELISA/PCR neg, IgM pos)	57/182 (31.3%)	61/298 (20.5%)	11/124 (8.87%)	6/86 (7.0%)
IgG only	5/182 (2.75%)	38/298 (12.8%)	15/124 (12.1%)	12/86 (14.0%)
Negative by all tests (NS1 RT/ELISA/PCR, IgG, IgM)	45/182 (24.7%)	154/298 (51.7%)	74/124 (59.7%)	63/86 (73.3%)
Health care utilization				
Sought medical care	194/194 (100%)	8/310 (2.36%)	130/130 (100%)	1/87 (1.15%)
Hospitalized	32/165 (19.4%)	0/310 (0%)	21/130 (16.2%)	(0/87) 0%
Other infections				
Chikungunya virus	0/194 (0%)	0/194 (0%)	50/122 (41.0%)	4/87 (4.6%)
Zika virus	Not tested	Not tested	0/122 (0%)	0/87 (0%)

The characteristics of index cases and associates in 2014 and 2015: mean age (standard deviation = SD) and gender, febrile status, health care seeking behavior, and arbovirus infection status (DENV, CHIKV, and ZIKV).

Table 2. Fever status and infection characterization of individuals with acute and recent DENV infections.

	2014			2015		
	Index cases (N = 132)	**Associate cases (N = 106)**	**p-value**	**Index cases (N = 35)**	**Associate cases (N = 11)**	**p-value**
Demographics						
Age in years, mean (SD)	20.9 (14.4) N=132	29.8 (18.3) N=106		22.7 (14.8) N=35	27.6 (14.4) N=11	
Gender, % female	57/132 (43.2%)	74/106 (69.8%)	<0.0001	21/35 (60.0%)	6/11 (54.5%)	0.7486
Fever						
Acute fever (>38°C)	17/125 (13.6%)	2/104 (1.92%)	0.0013	10/33 (30.3%)	0/11 (0%)	0.0457
History of fever (self-report)	125/131 (95.4%)	18/102 (17.6%)	<0.0001	34/35 (97.1%)	1/11 (9.09%)	<0.0001
Fever by either measure	126/131 (96.2%)	18/102 (17.6%)	<0.0001	34/35 (97.1%)	1/11 (9.09%)	<0.0001
Health care utilization						
Sought medical care	132/132 (100%)	7/106 (6.60%)		35/35 (100%)	0/11 (0%)	
Hospitalized	28/116 (24.1%)	0/106 (0%)		10/35 (28.6%)	0/11 (0%)	
Serology						
Primary infection	26/122 (21.3%)	38/106 (35.8%)	0.0116	21/35 (60.0%)	4/11 (36.4%)	0.0460
Secondary infection	73/122 (59.8%)	43/106 (40.6%)		10/35 (28.6%)	2/11 (18.2%)	
None	23/122 (18.8%)	25/106 (23.6%)		4/35 (11.4%)	5/11 (45.4%)	
DENV serotype						
1	4/51 (7.84%)	3/18 (16.7%)	0.0311	13/22 (59.1%)	0/1 (0%)	0.4348
1 & 2	1/51 (1.96%)	0/18 (0%)		0/22 (0%)	0/1 (0%)	
2	43/51 (84.3%)	10/18 (55.6%)		9/22 (40.9%)	1/1 (100%)	
3	2/51 (3.92%)	5/18 (27.8%)		0/22 (0%)	0/1 (0%)	
4	1/51 (1.96%)	0/18 (0%)		0/22 (0%)	0 (0%)	
PCR negative	81/132 (61.4%)	88/106 (83.0%)	0.0004	13/35 (37.1%)	10/11 (90.9%)	0.0057

Cases with acute or recent DENV infections in 2014 and 2015: mean age (standard deviation = SD) and gender, febrile status, percent hospitalized, serology (primary versus secondary infections), and DENV serotype (DENV1-4, one person positive for DENV1 and DENV2).

Table 3: Percentage of index cases and associates that were febrile and had recent or acute DENV infections or acute CHIKV by age group.

	Acute or recent DENV and febrile*		Acute CHIKV and febrile**	
	Index Cases	Associates	Index Cases	Associates
0-10 years	38/71 = 53.5%	3/23= 13.0%	6/25 = 24.0%	0/1 = 0%
11-20 years	57/88 = 64.8%	7/63= 11.1%	7/27 = 25.9%	0/17 = 0%
21-30 years	30/49 = 61.2%	4/59 = 6.78%	9/27 = 33.3%	0/15 = 0%
31-40 years	17/30 = 56.7%	3/56 = 5.36%	6/14 = 42.9%	0/15 = 0%
41-50 years	9/21 = 42.9%	1/52 = 1.92%	9/14 = 64.3%	0/11 = 0%
51-60 years	7/16 = 43.8%	1/31 = 3.23%	6/8 = 75.0%	0/8 = 0%
61-70 years	2/6 = 33.3%	0/26 = 0%	5/5 = 100%	0/9 = 0%
71-80 years	4/4 = 100%	0/9 = 0%	0/2 = 0%	0/5 = 0%
81-90 years	NA	0/2 = 0%	NA	NA

*Data shown for 2014 and 2015. Febrile defined as temperature > 38°C or self-reported fever.

**Data shown only for 2015

Table 4: Symptoms by age class and by serology for index cases with acute or recent DENV.

	All DENV infections			Primary DENV infections			Secondary DENV infections		
	0-10 years (n = 39)	11-20 years (n = 59)	21+ years (n = 69)	0-10 years (n = 15)	11-20 years (n = 13)	21+ years (n = 19)	0-10 years (n = 15)	11-20 years (n = 26)	21+ years (n = 42)
Demographics									
Gender (% female)	16/39[b] (41.0%)	19/59[c] (32.2%)	43/69[b,c] (62.3%)	5/15 (33.3%)	6/13 (46.2%)	12/19 (63.2%)	7/15 (46.7%)	7/26[c] (26.9%)	28/42[c] (66.7%)
Symptoms									
Fever	38/38 (100%)	57/59 (96.6%)	64/69 (92.8%)	15/15 (100%)	13/13 (100%)	17/19 (89.5%)	14/14 (100%)	24/26 (92.3%)	40/42 (95.2%)
Headache	28/38 (73.7%)	47/59 (79.7%)	57/69 (82.6%)	13/15 (86.7%)	12/13 (92.3%)	14/19 (73.7%)	9/15 (60.0%)	19/26 (73.1%)	35/42 (83.3%)
Anorexia and nausea	24/39 (61.5%)	41/59 (69.5%)	38/69 (55.1%)	9/15 (60.0%)	10/13 (76.9%)	8/19 (42.1%)	9/15 (60.0%)	17/26 (65.4%)	27/42 (64.3%)
Muscle/joint pain	25/38[b] (65.8%)	45/59 (86.3%)	60/69[b] (87.0%)	10/15 (66.7%)	10/13 (76.9%)	17/19 (89.5%)	8/15 (53.3%)	20/26 (76.9%)	35/42 (83.3%)
Rash	6/39 (15.4%)	13/58 (22.4%)	14/69 (20.3%)	4/15 (26.7%)	5/12 (41.7%)	3/19 (15.8%)	1/15 (6.67%)	6/26 (23.1%)	9/42 (21.4%)
Bleeding	3/39 (7.7%)	7/59 (11.9%)	6/68 (8.8%)	1/15 (6.7%)	2/13 (15.4%)	0/18 (0%)	2/15 (13.3%)	4/26 (15.4%)	6/42 (14.3%)
Rhinorrhea	7/39 (18.0%)	10/59 (17.0%)	9/69 (13.0%)	4/15 (26.7%)	0/13 (0%)	3/19 (15.8%)	2/15 (13.3%)	4/26 (15.4%)	5/42 (11.9%)
Vomiting	22/39 (56.4%)	28/59 (47.5%)	23/69 (33.3%)	8/15 (53.3%)	4/13 (30.8%)	3/19 (15.8%)	12/15 (80.0%)	14/26 (53.8%)	19/42 (45.2%)
Drowsiness/ lethargy	30/39 (76.9%)	48/59 (81.4%)	62/69 (89.9%)	12/15 (80.0%)	11/13 (84.6%)	14/19 (73.7%)	11/15[b] (73.3%)	23/26 (88.5%)	41/42[b] (97.6%)
Cough	13/39 (33.3%)	15/59 (25.4%)	18/69 (26.1%)	4/15 (26.7%)	4/13 (30.8%)	2/19 (10.5%)	7/15 (46.7%)	5/26 (19.2%)	13/42 (31.0%)
Abdominal pain	27/38 (71.0%)	30/59 (50.8%)	41/68 (60.3%)	10/15 (66.7%)	6/13 (46.2%)	11/18 (61.1%)	13/15 (86.7%)	15/26 (57.7%)	25/42 (59.5%)
Diarrhea	5/39[a] (12.8%)	21/59[a] (35.6%)	19/69 (27.5%)	2/15 (13.3%)	4/13 (30.8%)	6/19 (31.6%)	1/15 (6.67%)	11/26 (42.3%)	13/42 (31.0%)
Retro-orbital pain	21/38 (55.2%)	43/59 (72.9%)	47/69 (68.1%)	11/15 (73.3%)	11/13 (84.6%)	12/19 (63.2%)	4/14[a,b] (28.6%)	17/26[a] (65.4%)	28/42[b] (66.7%)

a Significant differences between 0-10 and 11-20 age groups, $p < 0.05$

b Significant differences between 0-10 and 21+ age groups, $p < 0.05$

c Significant differences between 11-20 age groups, $p < 0.05$

Table 5: Analysis of maximum likelihood estimates of symptom correlates for (A) DENV infection versus non-DENV infection (excluding CHIKV), (B) CHIKV versus DENV infections (excluding individuals with both acute CHIKV and acute or recent DENV), (C) CHIKV versus non-CHIKV infections (excluding DENV).

Parameter	Odds Ratio	95% Wald CI	Estimate	Std Error	Wald Chi-Square	PR>ChiSq
A. DENV vs. non-DENV						
Intercept			3954.7	1126.0	12.34	0.0004
Year			-1.9630	0.56	12.33	0.0004
Rhinorrhea	0.28	0.14 – 0.56	-1.2755	0.35	13.33	0.0003
Diarrhea			5423.5	1885.7	8.27	0.0040
Abdominal Pain			-3217.6	1440.2	4.99	0.0255
Year*Diarrhea			-2.6921	0.94	8.27	0.0040
Year*Abdominal Pain			1.5976	0.72	4.99	0.0254
B. CHIKV vs. DENV						
Intercept			-2.83	0.44	40.75	<0.0001
Age	1.05	1.03-1.08	0.05	0.01	16.45	<0.0001
Rash	2.66	1.08-6.52	0.98	0.46	4.53	0.032
Cough	0.33	0.12-0.99	-1.12	0.57	3.89	0.0486
C. CHIKV vs. non-CHIKV						
Intercept			-3.29	1.07	9.40	0.0022
Muscle or joint pain	18.41	2.20-154.19	2.91	1.08	7.22	0.0072
Rash	4.48	1.41-14.28	1.50	0.59	6.43	0.0112
Rhinorrhea	0.19	0.06-0.611	-1.64	0.59	7.83	0.0051

Supplemental Table 1. (A) Primers and (b) probes used for qPCR diagnostics of DENV, CHIKV, and ZIKV.

A. Primers		
Viral Target	**Primer Name**	**Primer Sequence 5' to 3'**
DENV1	D1F	CAAAAGGAAGTCGYGCAATA
DENV1	D1R	CTGAGTGAATTCTCTCTGCTRAAC
DENV2	D2F	CAGGCTATGGCACYGTCACGAT
DENV2	D2R	CCATYTGCAGCARCACCATCTC
DENV3	D3F	GGACTRGACACACGCACCCA
DENV3	D3R	CATGTCTCTACCTTCTCGACTTGYCT
DENV4	D4F	TTGTCCTAATGATGCTRGTCG
DENV4	D4R	TCCACCYGAGACTCCTTCCA
CHIKV	CHIKF_856	ACCATCGGTGTTCCATCTAAAG
CHIKV	CHIKR_962c	GCCTGGGCTCATCGTTATT
ZIKA	ZIKAF_1086	CCGCTGCCCAACACAAG
ZIKA	ZIKAR_1162c	CCACTAACGTTC TTTTGCAGACAT

B. Probes				
Viral Target	**Probe Name**	**Probe Sequence 5' to 3'**	**5' Label**	**3' Quench**
DENV1	D1P	CATGTGGYTGGGAGCRCGC	FAM	BHQ1
DENV2	D2P	CTCYCCRAGAACGGGCCTCGACTTCAA	HEX	BHQ1
DENV3	D3P	ACCTGGATGTCGGCTGAAGGAGCTTG	TexRed	BHQ2
DENV4	D4P	TYCCTACYCCTACGCATCGCATTCCG	Cy5	BHQ3
CHIKV	CHIKP_908	ACAGTGGTT/ZEN/TCGTGTGAGGGCTAC	HEX	IBFQ
ZIKA	ZIKAP_1107	AGCCTACCT/ZEN/TGACAAGCAGTCAGACACTCAA	FAM	IBFQ

Supplemental Table 2. Characteristics of DENV infections in index cases by serotype, excluding CHIKV infections.

	DENV-1 (n = 17)	DENV-2 (n = 51)	p-value
Demographics			
Age in years, mean (SD)	14.9 (10.8)	25.2 (16.2)	0.0173
Gender, % female	8/17 (47.1%)	21/51 (41.2%)	0.6711
Acute Febrile			
Temperature > 38°C	8/16 (50%)	15/48 (31.2%)	0.1758
Symptoms in prior 7 days			
Fever	17/17 (100%)	49/51 (96.1%)	1.000
Headache	17/17 (100%)	43/51 (84.3%)	0.1863
Anorexia and nausea	13/17 (76.5%)	32/51 (62.8%)	0.3828
Muscle/joint pain	12/17 (70.6%)	43/51 (84.3%)	0.2126
Rash	2/16 (12.5%)	8/51 (15.7%)	1.000
Bleeding	2/17 (11.8%)	2/51 (3.92%)	0.2584
Rhinorrhea	3/17 (17.6%)	8/51 (15.7%)	1.000
Vomiting	9/17 (52.9%)	26/51 (51.0%)	0.8886
Drowsiness/lethargy	16/17 (94.1%)	44/51 (86.3%)	0.6687
Cough	3/17 (17.6%)	15/51 29.4%	0.5270
Abdominal pain	12/17 (70.6%)	31/51 (60.8%)	0.4678
Diarrhea	4/17 (23.5%)	12/51 (23.5%)	1.000
Retro-orbital pain	13/17 (76.5%)	36/51 (70.6%)	0.7613
Hospitalization			
Hospitalized	4/17 (23.5%)	7/44 (15.9%)	0.4811
Serology			
Primary	10/16 (62.5%)	13/47 (27.7%)	0.0429
Secondary	3/16 (18.8%)	19/47 (40.4%)	
None	3/16 (18.8%)	15/47 (31.9%)	
Missing/incomplete	1	4	

*p < 0.05

Supplemental Table 3 Characteristics of DENV infections in index cases by serology (excludes those with CHIKV infections)

	Primary DENV infections (n = 43)	Secondary DENV infections (n = 82)	p-value
Demographics			
Age in years, mean (SD)	18.0 (13.1)	23.2 (13.8)	0.0460
Gender, % female	19/43 (44.2%)	41/82 (50.0%)	0.5365
Acute Febrile			
Temperature > 38°C	10/39 (25.6%)	7/79 (8.86%)	0.0146
Symptoms in Prior 7 Days			
Fever	42/43 (97.7%)	77/81 (95.1%)	0.6578
Headache	37/43 (86.0%)	62/82 (75.6%)	0.1720
Anorexia and nausea	27/143 (62.8%)	53/82 (64.6%)	0.8384
Muscle/joint pain	33/43 (76.7%)	62/82 (75.6%)	0.8878
Rash	9/42 (21.4%)	16/82 (19.5%)	0.8012
Bleeding	3/42 (7.14%)	12/82 (14.6%)	0.2621
Rhinorrhea	7/43 (16.3%)	11/82 (13.4%)	0.6648
Vomiting	15/43 (34.9%)	45/82 (54.9%)	0.0335
Drowsiness/lethargy	36/43 (83.7%)	74/82 (90.2%)	0.2864
Cough	9/43 (20.9%)	25/82 (30.5%)	0.2540
Abdominal pain	25/42 (59.5%)	53/82 (64.6%)	0.5772
Diarrhea	10/43 (23.3%)	25/82 (30.5%)	0.3923
Retro-orbital pain	32/43 (74.4%)	48/81 (59.3%)	0.0931
Hospitalization			
Hospitalized	4/37 (10.8%)	33/75 (44.0%)	0.0005
Serotype			
1	10/23 (43.5%)	3/24 (12.5%)	0.0760
1&2	0/23 (0%)	1/24 (4.17%)	
2	13/23 (56.5%)	19/24 (79.2%)	
3	0/23 (0%)	1/24 (4.17%)	
4	0/23 (0%)	0/24 (0%)	

Supplemental Table 4. Symptoms of index patients with acute or recent DENV infection versus non-DENV and non-CHIKV cases.

	2014			2015		
	Acute or recent DENV (n = 132)	**Non-DENV and non-CHIVK (n = 45)**	**p-value**	**Acute or recent DENV (n = 29)**	**Non-DENV and non-CHIKV (n = 38)**	**p-value**
Demographics						
Age in years, mean (SD)	20.9 (14.4)	20.0 (18.8)	0.7479	19.0 (11.7)	24.0 (19.7)	0.1970
Gender, % female	57/132 (43.2%)	28/45 (62.2%)	0.0273	16/29 (55.2%)	15/38 (39.5%)	0.2016
Acute Febrile						
Temperature > 38°C	17/125 (13.6%)	11/43 (25.6%)	0.0690	10/27 (37.0%)	7/37 (18.9%)	0.1051
Symptoms in prior 7 days						
Fever	125/131 (95.4%)	45/45 (100%)	0.3401	29/29 (100%)	37/38 (97.4%)	1.000
Headache	103/131 (78.6%)	36/43 (83.7%)	0.4696	25/29 (86.2%)	31/38 (81.6%)	0.7449
Anorexia and nausea	83/132 (62.9%)	26/45 (57.8%)	0.5435	20/29 (69.0%)	19/38 (50.0%)	0.1189
Muscle/joint pain	102/131 (77.9%)	30/43 (69.8%)	0.2818	22/29 (75.9%)	28/37 (75.7%)	0.9860
Rash	28/132 (21.1%)	3/44 (6.82%)	0.0381	2/28 (7.14%)	7/38 (18.4%)	0.2820
Bleeding	12/132 (9.09%)	1/45 (2.22%)	0.1886	4/28 (14.3%)	2/38 (5.26%)	0.3887
Rhinorrhea	22/132 (16.7%)	15/45 (33.3%)	0.0176	4/29 (13.8%)	16/38 (42.1%)	0.0156
Vomiting	62/132 (47.0%)	14/45 (31.1%)	0.0635	11/29 (37.9%)	8/38 (21.0%)	0.1228
Drowsiness/ lethargy	112/132 (84.8%)	38/45 (84.4%)	0.9481	25/29 (86.2%)	35/38 (92.1%)	0.4555
Cough	40/32 (30.3%)	23/45 (51.1%)	0.0118	5/29 (17.2%)	11/38 (29.0%)	0.2655
Abdominal pain	75/131 (57.2%)	24/44 (54.6%)	0.7540	21/28 (75.0%)	19/38 (50.0%)	0.0399
Diarrhea	37/132 (28.0%)	3/45 (6.67%)	0.0033	23/29 (20.7%)	10/38 (26.3%)	0.5925
Retro-orbital pain	85/131 (64.9%)	26/44 (59.1%)	0.4899	22/29 (75.9%)	25/37 (67.6%)	0.4601
Health care utilization						
Sought medical care	132/132 (100%)	45/45 (100%)	1.000	29/29 (100%)	38/38 (100%)	1.000
Hospitalized	28/116 (24.1%)	3/34 (8.82%)	0.0570	9/29 (31.0%)	5/38 (13.2%)	0.0745

Supplemental Table 5. Symptoms of index cases with acute or recent DENV infections versus CHIKV infections (excluding co-infections).

	Acute or recent DENV infections (n = 161)	Acute CHIKV infections (n = 36)	p-value
Demographics			
Age in years, mean (SD)	20.6 (14.0)	33.6 (18.0)	0.0002
Gender, % female	73/161 (45.3%)	23/36 (63.9%)	0.0441
Acute Febrile			
Temperature > 38°C	27/152 (17.8%)	4/34 (11.8%)	0.6103
Symptoms in prior 7 Days			
Fever	154/160 (96.2%)	35/36 (97.2%)	1.000
Headache	128/160 (80.0%)	29/36 (80.6%)	0.9399
Anorexia and nausea	103/161 (64.0%)	17/36 (47.2%)	0.0626
Muscle/joint pain	125/160 (77.5%)	35/36 (97.2%)	0.0041
Rash	30/160 (18.8%)	12/36 (33.3%)	0.0540
Bleeding	16/160 (10.0%)	2/36 (5.56%)	0.5356
Rhinorrhea	26/161 (16.2%)	5/36 (13.9%)	0.7364
Vomiting	73/161 (45.3%)	11/36 (30.6%)	0.1049
Drowsiness/lethargy	137/1616 (85.1%)	34/36 (94.4%)	0.1767
Cough	45/161 (28.0%)	5/36 (13.9%)	0.0797
Abdominal pain	96/159 (60.4%)	15/36 (41.7%)	0.0406
Diarrhea	43/161 (26.7%)	12/36 (33.3%)	0.4231
Retro-orbital pain	107/160 (66.9%)	25/35 (71.4%)	0.6018
Health care utilization			
Sought medical care	161/161 (100%)	36/36 (100%)	1.000
Hospitalized	37/145 (25.5%)	4/36 (11.1%)	0.0762

Supplemental Table 6. Symptoms of associate cases with acute or recent DENV infections (excluding CHIKV infection) versus associates who were febrile and negative for DENV and CHIKV.

	Acute or recent DENV infections N=116	Febrile, but DENV and CHIKV negative N=16	p-value
Demographics			
Age in years, mean (SD)	29.4 (17.9)	32.4 (20.0)	0.5348
Gender, % female	79/119 (68.1%)	11/16 (68.8%)	0.9585
Acute Febrile			
Temperature > 38°C	2/112 (1.75%)	0/16 (0%)	1.000
Symptoms in Prior 7 Days			
No symptoms	30/112 (26.8%)	0/16 (0%)	0.0432
No dengue-like symptoms	49/112 (43.8%)	2/16 (12.5%)	0.1027
Fever	19/112 (17.0%)	16/16 (100%)	<0.0001
Headache	34/114 (29.8%)	11/16 (68.8%)	0.0022
Anorexia and nausea	10/114 (8.77%)	7/16 (43.8%)	0.0001
Muscle/joint pain	31/114 (27.2%)	10/16 (62.5%)	0.0044
Rash	14/114 (12.3%)	2/16 (12.5%)	1.000
Bleeding	1/113 (0.88%)	0/16 (0%)	1.000
Rhinorrhea	14/114 (12.3%)	5/16 (31.2%)	0.0443
Vomiting	2/114 (1.75%)	2/16 (12.5%)	0.0738
Drowsiness/lethargy	23/114 (20.2%)	7/16 (43.8%)	0.0361
Cough	20/114 (17.5%)	10/16 (62.5%)	<0.0001
Abdominal pain	23/114 (20.2%)	5/16 (31.2%)	0.3129
Diarrhea	10/114 (8.77%)	2/16 (12.5%)	0.6428
Retro-orbital pain	26/114 (22.8%)	4/16 (25.0%)	0.7624
Health care utilization			
Sought medical care	7/116 (6.03%)	0/16 (0%)	0.5972
Hospitalized	0/116 (0%)	0/16 (0%)	1.000