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

Anna M. Stewart-Ibarra[1,2]*, Aileen Kenneson[1], Christine A. King[1,3], Mark Abbott[1,3], Arturo Barbachano-Guerrero[3], Efraín Beltrán-Ayala[4], Mercy J. Borbor-Cordova[5], Washington B. Cárdenas[5], Cinthya Cueva[1], Julia L. Finkelstein[6], Christina D. Lupone[1], Richard G. Jarman[7], Irina Maljkovic Berry[7], Saurabh Mehta[6], Mark Polhemus[1,2], Mercy Silva[8], Sadie J. Ryan[1,9,10,11], and Timothy P. Endy[1,2,3]

[1] Center for Global Health & Translational Sciences, SUNY Upstate Medical University, Syracuse, NY, USA
[2] Department of Medicine, SUNY Upstate Medical University, Syracuse, NY, USA
[3] Department of Microbiology & Immunology, SUNY Upstate Medical University, Syracuse, NY, USA
[4] Universidad Técnica de Machala, Machala, El Oro Province, Ecuador
[5] Escuela Superior Politecnica del Litoral (ESPOL), Guayaquil, Ecuador
[6] Division of Nutritional Sciences, Cornell University, Ithaca, NY, USA
[7] Viral Diseases Branch of the Walter Reed Army Institute of Research (WRAIR), Silver Springs, MD, USA
[8] Ministry of Health, Machala, El Oro, Ecuador
[9] Department of Geography, University of Florida, Gainesville, FL, USA
[10] Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA
[11] College of Life Sciences, University of Kwazulu-Natal, Durban, South Africa
*Corresponding author:
Email: stewarta@upstate.edu (AMSI)

Abstract

Background: Dengue (DENV), chikugunya (CHIKV) and zika (ZIKV) viruses are arboviruses transmitted by the *Ae. aegypti* mosquito, that cause febrile illness and present a major public health challenge in tropical low- and middle-income countries such as Ecuador. 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. **Methods:** Individuals whom presented at one of four sentinel clinics or the central hospital of the Ministry of Health with suspected DENV (index cases) were recruited into the study (n = 324). Index cases with confirmed acute DENV infection triggered a cluster investigation (n = 44) of DENV infections in the index household and four neighboring households (associates) within 200 meters, (n = 397). We conducted genomic sequencing and phylogenetic analysis of select DENV positive samples from 2014. **Results:** In 2014, 72.5% of index patients and 35.6% of associates had evidence of acute or recent DENV infections. In 2015, 28.3% and 12.85% of index patients and associates, respectively, had acute or recent infections. The first cases of CHIKV were detected in an associate on epidemiological week 12 in 2015. There were a total of 54 cases of acute CHIKV infections, including seven DENV/CHIKV co-infections. No cases of ZIKV were detected. DENV symptoms varied significantly by age and by primary versus secondary infections. Symptoms that were associated with DENV and CHIKV infections are presented. Phylogenetic analyses of isolates 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. **Discussion:** Findings from this study highlight the importance of (1) implementing rapid active surveillance protocols

and (2) strengthening local molecular diagnostic capacities in resource-limited settings where DENV, CHIKV, and ZIKV co-circulate.

Key words: dengue, chikungunya, zika virus, 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 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]. The burden of apparent DENV cases has increased globally over the last three decades, from 8.3 million cases in 1990, to 58.4 million cases in 2013 [2]. When inapparent DENV cases are considered, studies report upper estimates of 390 million cases per year [3].

DENV re-emerged in Latin America and the Caribbean in the late 1980s. Over the last three decades the distribution, severity, and incidence has increased, from 16.4 cases per 100,000 in the 1980's to 71.5 cases per 100,000 from 2000 to 2007 [4,5]. Current estimates of apparent DENV infection in LAC range from 1.5 million [2] to 13.3 million [3] cases per year. In many countries in the Americas, DENV has replaced malaria as the leading cause of vector-borne febrile illness [6]. 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 [7].

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 [8]. The first cases of ZIKV (family *Flaviviridae*, genus *flavivirus*) were reported in Brazil

in 2015 [9,10]. On February 1, 2016, the World Health Organization declared a Public Health Emergency of International Concern in response to the potential neurological and autoimmune complications associated with ZIKV infections (i.e., Guillain-Barré syndrome and zika congenital syndrome) [11]. To date, 738,783 suspected and confirmed autochthonous cases of ZIKV have been reported from 48 countries and territories (as of January 18, 2017) [12]

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 [13,14]. 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 [15]. 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 [16].

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 [17]. 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 [18,19]. DENV epidemics in the region are associated with El Niño climate events that cause increased rainfall and warmer air temperatures [18]. Local social-ecological risk factors include poor housing conditions and infrequent access to piped water in

the urban periphery, lack of knowledge of DENV transmission, and water storage behavior [19–21].

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 3,585 suspected and confirmed cases of ZIKV have been reported to date (as of January 18, 2017) [12].

In Ecuador, diagnosed cases of DENV, ZIKV, and CHIKV infections require mandatory notification to the Ministry of Health (MoH). The national surveillance system is based on passive surveillance of cases from MoH clinics and hospitals. Most reported cases are only clinically diagnosed. 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 (qPCR) at the national reference laboratory of the National Institute for Public Health Research (INSPI) of the MoH. Positive cases trigger focal vector control interventions by the MoH (i.e., fumigation and larvicide application). Challenges include lack of access to low-cost diagnostics, resource limitations to conduct vector control and to purchase diagnostic reagents, and under-reporting of mild or subclinical infections due to lack of active surveillance and limited healthcare seeking by community members [20,22]. Improved surveillance data are urgently needed to estimate the true burden of disease and to inform targeted disease control interventions.

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 (associate cases) living within 200 meters of the index patient. We focus specifically on:

1. Characterization of infections in index and associate cases (i.e., symptoms, serotypes, serology)
2. Prevalence of DENV infection and expansion factors (EF) from spatiotemporal clusters
3. Phylogenetic analysis of DENV circulating in 2014
4. Detection of the emergence of CHIKV in Machala in 2015
5. Multi-variate models of symptoms associated with DENV and CHIKV infections
6. Surveillance for acute ZIKV infections

This study contributes to an ongoing collaboration with the MoH of Ecuador to strengthen febrile vector-borne disease surveillance in southern coastal Ecuador, providing key epidemiological information for the region [23].

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. 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 Sites.

Machala, Ecuador, (population 245,972, 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 [19,24,25]. In 2014 and 2015, 1,196 and 2,791 dengue cases, respectively, were reported from Machala (mean annual incidence 48.6 and 113.5 cases per 10,000 people) [26]. 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.

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. 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 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. 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 symptoms, symptoms during illness, medications, and aural temperature. Travel history and assessment of contact with animals were also recorded. Nutritional assessments included 24-hour dietary recall, and anthropometric measurements (height, weight, waist and mid-upper arm circumference). 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 NIH 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. A study nurse or technician performed a two-week follow-up visit to collect a convalescent blood sample only from previously hospitalized patients.

Each week, up to four index participants 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 and individuals from four surrounding households located within a 200-meter radius of the index household, the typical flight range of the *Ae. aegypti* mosquito. This resulted in approximately nine to 10 associate cases per index case in each spatiotemporal cluster. The diagnostic tests and

assessments described above for index patients were repeated for all associates. Prokopack backpack aspirators were used to collect adult mosquitoes inside and outside the home [27]. Mosquitoes were maintained on ice until they were taken to the laboratory, where they were counted, sexed, sorted by species, and stored at or below -80°C for future testing for viruses. Household surveys were conducted with heads of households to identify risk factors for illness, such as demographics, housing condition, and access to public services. The location (latitude, longitude) of each home was recorded using handheld Garmin GPS units. Entomology and household risk factor results are not reported here. 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 [28],

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 [28–30].

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 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 [31] (primers and probes in Supplementary 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 [32] 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).

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 PCR were classified as having acute CHIKV or ZIKV infections.

Expansion factors (EF) are a means to estimate true case burdens from reported burdens, by creating a correction factor for underreporting. There are a variety of methods used to do this in the literature (REF EF papers), and here we use a local EF estimate from our cluster study. The DENV expansion factor (EF) is the ratio of the best estimate of DENV cases (often from active surveillance) to the number of reported cases (often from passive surveillance) [33]. An EF = 1 reflects 100% reporting of DENV infections, and EF > 1 indicates underreporting [33]. In this case, we treat the index cases in the cluster (plus any that recently sought hospital care) as 'reported' and the acute and recent dengue associate cases as the 'best estimate of DENV cases'. Data from the spatiotemporal clusters were used to estimate the weekly and cluster-level dengue infection expansion factor (EF), by dividing the total number of acute or recent cases of dengue infection in the associate cases by the number of initiating acute index cases, plus reporting associates. As the purpose of deriving this EF was to correct MoH reported cases, associate cases (n=7) who had sought medical care for DENV infections in the past two weeks were added to the index case, as they would have been captured by the MoH surveillance system. Thus, our cluster level calculated EF is:

EF = #positive in population tested / (Index case + # reporting associates).

We calculated cluster-wise estimates of EF, and present average and range values for this. 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.

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.

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 [34]. 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 [35]. Assemblies were further processed using samtools version 0.1 [36] 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 [37].

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 [38]. 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) [39] 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) [40]. Maximum Likelihood (ML) phylogenetic trees for each of the DENV1, DENV2 and DENV4 datasets were inferred using Phyml v 4.9.1 [41,42]. 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 [43] 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 patients 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). Of these, 72 (22.2%) tested positive for acute DENV infection using the NS1 rapid test; 44 of these individuals were randomly selected as initiates of spatiotemporal clusters, from which 397 associates were recruited into the study (310 associates in 2014, 87 in 2015). On average, associates were more likely to be female ($p<0.0001$) and were older ($p<0.0001$) than index cases (Table 1). DENV transmission was highly seasonal in both years of the study. Transmission began in January and February, peaked in May, and tailed off in September and October (Fig 3). CHIKV transmission followed a similar seasonal curve in 2015.

Epidemiology

In 2014, 72.5% of index patients and 35.6% of associates had evidence of acute or recent DENV infections (Table 1). The prevalence of DENV disease decreased in 2015, with 28.3% and 12.85% of index patients and associates, respectively, with acute or recent infections. The

first cases of CHIKV were detected in an associate individual on epidemiological week 12 in 2015. We detected a total of 50 index cases with acute CHIKV infections (50/122; prevalence of 41%), including six index cases with DENV/CHIKV co-infections. There were four associates with CHIKV infections (4/87, prevalence 4.6%), including one associate with a DENV/CHIKV co-infection. No cases of ZIKV were detected.

In 2014 index cases were hospitalized in 24.1% of DENV and 8.82% of non-DENV cases (excluding CHIKV) (p=0.06) (Supplementary Table 2). In 2015 index cases were hospitalized in 31.0% of DENV and 13.2% of non-DENV cases (p=0.07). Of associates with acute or recent DENV infections, 6.5% and 0% sought medical care in 2014 and 2015, respectively (Table 2).

The prevalence of DENV was greatest in children and young adults under the age of 20, whom accounted for 51% of all acute or recent DENV infections (Fig 4a). Acute and recent dengue infections in index patients and associates peaked in children 11-20 years. The prevalence of primary DENV infections in associates peaked at 11-20 years and in index cases peaked at 21 to 30 years (4c). The prevalence of secondary index infections was greatest in adults 31-40 years, and there was no clear peak age class in secondary associate cases, likely due to the small sample size. Accordingly, the mean age of individuals presenting with primary DENV infections was significantly lower than of secondary infections (18 vs. 24 years) (p=0.02).

Patients with CHIKV infections were significantly older (34 years, SD=18.0) than those with DENV infections (21 years, SD = 14.0) (p<0.0001) (Fig 4b). Adults (>20 years) accounted for 72% of acute CHIKV infections. Significantly more index patients with CHIKV infections were female (63.9%) compared to index patients with DENV infections (45.3%) (p = 0.03) (Supplemental Table 3).

The proportion of primary and secondary DENV infections varied from year to year, as did the dominant DENV serotypes in circulation (Table 2). In 2014, there were a greater proportion of secondary DENV infections in index patients and associates, whereas in 2015 there were a greater proportion of primary DENV infections. In 2014, all four DENV serotypes were detected, and most cases were DENV2 (84.3% of serotyped index patients) (Table 2). In 2015, DENV1 and DENV2 were detected, and most cases were DENV1 (59.1% of serotyped index patients). The mean age of individuals with DENV2 infections (25 years) was significantly higher than that of individuals with DENV1 infections (15 years) (p=0.02) (Supplementary Table 4), consistent with the fact that the DENV2 infections observed in our study were predominantly secondary infections (60.0%), while most of the DENV1 infections were primary (76.9%).

Clinical presentation

The prevalence of index and associate cases with febrile acute or recent DENV infections decreased with increasing age (Table 3). In associates, children 0 to 10 years of age had the highest prevalence of febrile acute or recent DENV infections (13% of associates 0-10 years of age). In index cases, children 11 to 20 years of age had the highest prevalence of febrile acute or recent DENV infections (64.8% of index cases 11-20 years of age). In contrast, the prevalence of febrile acute CHIKV infections increased with age. Index subjects aged 51 to 60 years had the highest prevalence of febrile acute CHIKV infections (75% of index subjects 51-60 years).

In a comparison of index individuals with secondary versus primary DENV infections, we found more severe illness in secondary infections (Supplemental Table 5). Vomiting (p=0.04) and hospitalization (p=0.001) were significantly more common among individuals with secondary infections. Bleeding and diarrhea were also more common in individuals with

secondary infections, although the differences were not statistically significant ($p>0.05$). Fever (temperature measured >38°C) was significantly less common among secondary infections ($p = 0.02$).

There were significant differences in DENV symptoms by age group and by primary versus secondary infections (Table 4). Diarrhea and muscle/joint pain were less common in the 0 to 10 year age group ($p<0.05$). Among secondary infections, retro-orbital pain and drowsiness/lethargy were significantly less more among children age 0 to 10 years than among older children (11 to 20 years) and adults (21+ years) ($p<0.05$).

Of the 116 associate cases with acute or recent DENV infections (excluding one DENV/CHIKV co-infection), 49 (43.8%) reported no dengue symptoms, i.e., no fever within the last 7 days, rash, muscle or joint pain, abdominal pain or tenderness, bleeding, drowsiness or lethargy. One quarter (26.8%; 30/116) reported no symptoms of any kind (Supplementary Table 6). Associates with primary infections were significantly less likely to report symptoms (37.5% with no symptoms, 15/40) than associates with secondary infections (15.6% with no symptoms, 7/45) ($p=0.02$). There were 16 associate cases with a febrile illness that was neither DENV nor CHIK. The frequencies of their symptoms are presented in Supplemental Table 6.

Multivariate analysis of DENV and CHIKV symptoms

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

The best model to explain DENV vs. non-DENV cases (excluding CHIKV cases) indicated that the presence of rhinorrhea was associated with decreased odds of DENV infection (Adj OR=0.279, 95% CI: 0.141-0.554, 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 2). Possible reasons for different clinical features between 2014 and 2015 include differences between serotypes and between primary versus secondary infections (Supplementary Tables 4 and 5).

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 3).

The best model to explain CHIKV versus non-CHIKV infections (excluding DENV cases) 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).

Spatiotemporal clusters

The distance between the households of associate cases 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.

Within clusters, the percent of associate cases with evidence of an acute or recent DENV infection ranged from 0% to 87.5%. The index case had a DENV1 infection in 10 of 44 clusters. 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 cases in the clusters to the number of index and reported associate cases, was 3.22. Cluster estimates of expansion factors ranged from 1 to 10, with a median of 3 and a mean of 3.23 (SD=2.15). The mean EFs were 3.83 for 2014 and 1.77 for 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).

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).

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.

Discussion

DENV, CHIKV, and ZIKV, febrile illnesses transmitted by the *Ae. aegypti* mosquito, present a major public health challenge in low- and middle-income countries, such as Ecuador. In our study, children had the greatest burden of symptomatic DENV infections. Differential

diagnosis between the diseases is difficult due to similar clinical presentation and limited access to laboratory diagnostics. In the absence of effective vaccines and therapeutics, people without severe symptoms do not seek care in the public health system, leading to significant underreporting of disease and continued transmission. Active and passive surveillance studies, such as this, provide important information regarding the burden of disease, which would not be captured through traditional surveillance methods.

Burden of disease and EF estimates.

This study provides one of the first estimates of the community-based prevalence of symptomatic and subclinical DENV infections in the region. On average over the two years of the study, 32.1% 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 [1]. In Thailand, cluster DENV prevalence ranged from 10.1% to 14.3% using PCR or serology [2,3]. 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 cluster prevalence rate varied by dengue 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 [4].

The expansion factor (EF) for DENV in Machala was estimated using the ratio of all cases to the number of medically-attended cases among the 44 clusters. Our overall estimate was 3.22 (3.83 in 2014, 1.77 in 2015), indicating that estimates of dengue incidence based on reporting from clinics and hospitals miss approximately 69% of cases. This EF is comparable to the low end of a range of previously reported EFs for the PAHO region [5]. 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 48.6 cases per 10,000 population in 2014 and 113.5 cases per 10,000 population in 2015, the estimated actual annual incidences are 186.1 cases per 10,000, and 200.9 cases per 10,000 in 2014 and 2015, respectively. Interestingly, we found that the EF was higher in 2014 (3.83) than 2015 (1.77), 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). The rapid surveillance methods developed in this study 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.

Burden of CHIKV and other febrile illness:

In 2015 we found that 41% of clinically diagnosed DENV cases were positive for CHIKV, higher than the proportion of laboratory-confirmed dengue cases (28.3%). We also found evidence of six co-infections in index cases (4.6% prevalence in 2015), one co-infection among associate cases (1.1% prevalence in 2015), and 96 cases with undiagnosed febrile illness (non-dengue, non-chik, non-zika) among the index cases. 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 [6]) 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, the study would have detected some ZIKV cases if they were present in Machala due to the overlapping clinical presentations of DENV and ZIKV infections.

Clinical predictors of DENV and CHIKV.

In general, the frequencies of symptoms that we observed in DENV cases are consistent with other reports [7–13]. 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 [14] and the ratio of primary versus secondary infections [12,15,16]. The relative contribution of human leukocyte antigen (HLA) genotype in the population was not assessed in this study but likely also have a significant impact on host response and thus symptoms in the patient. In our 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 [14]. 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 [17,18]. 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 [12,15,16].

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 also found that DENV cases were more likely to be male than CHIKV cases. On average, index cases of DENV or CHIKV were more likely to be male than associate cases. These differences may reflect variation in exposure to infectious mosquito bites, a greater propensity for severe symptoms in men, or gender differences in health-seeking behaviors. This could be a spurious result, although prior studies have reported a higher prevalence of DENV in men than in women [19,20]. 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 [9,13].

Symptoms in acute or recent DENV associate cases were 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 [21]. 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 [15,21], 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 (1) a shared vision for integrated active surveillance that includes the virus, vector, climate and other social-ecological drivers; (2) ongoing training of physicians, researchers and students; and (3) improvement of local diagnostic and research infrastructure.

The results of this study highlight the importance of (1) rapid active surveillance protocols to detect subclinical infections and to compare infections rates to different regions, (2) strengthening of local molecular diagnostic capacities in resource-limited settings where DENV, CHIKV, and ZIKV are endemic and co-circulate, and (3) sharing of full virus genomes from neighboring countries to better understand the spread of arboviruses in the region.

The use of expansion factors (EFs) is important for these diseases, and in this study, we highlight the utility of cluster-based estimates over a longer period of cohort time than is usually available. However, we also revealed the vulnerability of these estimates to low sample sizes that are often encountered in the local cohort setting. Our EF estimate in 2014 is roughly double that for 2015, indicating that depending on the year and setting, we could have underreporting off by

a factor of 2. This adds further impetus to the need for improved surveillance and updating of both infection and reporting trends.

Availability of data and materials

The de-identified datasets in the current study are available from the corresponding author on reasonable request. Genetic sequences have been deposited in GenBank under accession numbers KY474303-KY474335.

Competing Interests

The authors declare no competing interests, financial or non-financial.

Funding

This study was supported in part by the Department of Defense Global Emerging Infection Surveillance (GEIS) grant (P0220_13_OT) and the Department of Medicine of SUNY Upstate Medical University. AMS and SJR were additionally supported by NSF DEB EEID 1518681 and NSF DEB RAPID 1641145. Additional support was provided to AMSI through the Prometeo program of the National Secretary of Higher Education, Science, Technology, and Innovation (SENESCYT) of Ecuador.

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.

Authors' Contributions

AMSI and TE had overall responsibility for the study, which included the study conception, collaboration with co-authors on the study protocol, and oversight of data collection. AMSI, CAK, EBA, MS, MJBC, and CC were responsible for data collection; SM, JLF, and SJR supported database development; CAK, WBC, MP, ABG, MA, and CDL supported development of diagnostic SOPs. MA conducted PCR analysis. IMB and RJ conducted phylogenetic analyses. AMSI, AK, and IMB analyzed the data and drafted the manuscript. All co-authors provided critical revision of the article and approved the final manuscript.

Acknowledgements

This project was possible thanks to support from colleagues from the Ministry of Health, the National Institute of Meteorology and Hydrology, the National Secretary of Higher Education, Science, Technology, and Innovation (SENESCYT) of Ecuador and community members from Machala, Ecuador. We thank our local field team and coordinators for their dedication and perseverance: Jefferson Adrian, Victor Arteaga, Jose Cueva, Reagan Deming, Carlos Enriquez, Prissila Fernandez, Froilan Heras, Naveed Heydari, Jesse Krisher, Lyndsay Krisher, Elizabeth McMahon, Eunice Ordoñez, and Tania Ordoñez. Many thanks to Rosemary Rochford, Lisa Ware, Holly Chanatry, David Amberg and Marti Benedict for supporting the development of the research platform with partners in Ecuador. We also thank Danielle Safaty and Laura Sorenson in the Center for Global Health and Translational Science at SUNY Upstate Medical University

for technical support in sample preparation, RT-PCR analysis, and data compilation. We also thank Dr. Renato Leon for supporting the development of the entomology protocol, and Ing. Raul Mejia and Dr. Angel Muñoz for supporting climate surveillance. Thank you to Dr. Butsaya Thaisomboonsuk PhD and Dr. Louis Macareo MD, JD from AFRIMS for sharing surveillance and diagnostic protocols. Thank you to Clinical Research Management (CRM) for supporting continued surveillance activities.

References

1. WHO. Dengue: guidelines for diagnosis, treatment, prevention and control. World Health Organization; 2009.

2. Stanaway JD, Shepard DS, Undurraga EA, Halasa YA, Coffeng LE, Brady OJ, et al. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. Lancet Infect Dis. 2016; doi:10.1016/S1473-3099(16)00026-8

3. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013; Available: http://www.nature.com/nature/journal/vaop/ncurrent/full/nature12060.html?WT.ec_id=NATURE-20130411

4. Dick OB, Martín JLS, Montoya RH, Diego J del, Zambrano B, Dayan GH. The History of Dengue Outbreaks in the Americas. Am J Trop Med Hyg. 2012;87: 584–593. doi:10.4269/ajtmh.2012.11-0770

5. San Martín JL, Brathwaite O, Zambrano B, Solórzano JO, Bouckenooghe A, Dayan GH, et al. The Epidemiology of Dengue in the Americas Over the Last Three Decades: A Worrisome Reality. Am J Trop Med Hyg. 2010;82: 128 –135. doi:10.4269/ajtmh.2010.09-0346

6. Cifuentes SG, Trostle J, Trueba G, Milbrath M, Baldeón ME, Coloma J, et al. Transition in the Cause of Fever from Malaria to Dengue, Northwestern Ecuador, 1990–2011. Emerg Infect Dis. 2013;19: 1642.

7. WHO | Dengue and severe dengue. In: WHO [Internet]. [cited 11 Jun 2014]. Available: http://www.who.int/mediacentre/factsheets/fs117/en/

8. PAHO. Number of Reported Cases of Chikungunya Fever in the Americas, by Country or Territory. [Internet]. Available: http://www.paho.org/hq/index.php?option=com_topics&view=readall&cid=5927&Itemid=40931&lang=en

9. Zanluca C, Melo VCA de, Mosimann ALP, Santos GIV dos, Santos CND dos, Luz K, et al. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015;110: 569–572. doi:10.1590/0074-02760150192

10. Campos GS, Bandeira AC, Sardi SI. Zika Virus Outbreak, Bahia, Brazil. Emerg Infect Dis. 2015;21: 1885–1886. doi:10.3201/eid2110.150847

11. WHO | Zika virus. In: WHO [Internet]. [cited 25 Feb 2016]. Available: http://www.who.int/mediacentre/factsheets/zika/en/

12. Zika cases and congenital syndrome associated with Zika virus reported by countries and territories in the Americas, 2015 - 2016. Cumulative cases [Internet]. PAHO/WHO; 2016. Available: http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&Itemid=270&gid=36012&lang=en

13. Camargo S. History of Aedes aegypti eradication in the Americas. Bull World Health Organ. 1967;36: 602.

14. Gonzalez V, Jurado H. Guayaquil: Aedes aegypti, 1740 - 2007. Guayaquil, Ecuador: Servicio Nacional para La Eradicaccion de Malaria (SNEM); 2007.

15. Dengue Epidemic - Ecuador 1988. Mortal Morb Wkly Rep. 38: 419–421.

16. Alava, A., Mosquera, C., Vargas, W., Real, J. Dengue en el Ecuador 1989-2002. Rev Ecuat Hig Med Trop. 2005;42: 11–34.

17. PAHO. Number of reported cases of dengue and severe dengue (DS) in the Americas by country (1995-2015) [Internet]. Washington D.C.: Pan American Health Organization; 2011. Available: www.who.int/denguenet

18. Stewart Ibarra AM, Lowe R. Climate and non-climate drivers of dengue epidemics in southern coastal Ecuador. Am J Trop Med Hyg. 2013;88: 971–981. doi:10.4269/ajtmh.12-0478

19. Stewart Ibarra AM, Ryan SJ, Beltrán E, Mejía R, Silva M, Muñoz Á. Dengue Vector Dynamics (Aedes aegypti) Influenced by Climate and Social Factors in Ecuador: Implications for Targeted Control. PLOS ONE. 2013;8: e78263.

20. Stewart Ibarra AM, Luzadis VA, Borbor Cordova MJ, Silva M, Ordoñez T, Beltrán Ayala E, et al. A social-ecological analysis of community perceptions of dengue fever and Aedes aegypti in Machala, Ecuador. BMC Public Health. 2014;14: 1135. doi:10.1186/1471-2458-14-1135

21. Stewart Ibarra AM, Munoz AG, Ryan SJ, Borbor MJ, Ayala EB, Finkelstein JL, et al. Spatiotemporal clustering, climate periodicity, and social-ecological risk factors for dengue during an outbreak in Machala, Ecuador, in 2010. BMC Infect Dis. 2014;14: 610. doi:10.1186/s12879-014-0610-4

22. Handel AS, Ayala EB, Borbor-Cordova MJ, Fessler AG, Finkelstein JL, Espinoza RXR, et al. Knowledge, attitudes, and practices regarding dengue infection among public sector healthcare providers in Machala, Ecuador. Trop Dis Travel Med Vaccines. 2016;2: 8. doi:10.1186/s40794-016-0024-y

23. Borbor-Cordova M, Beltran Ayala E, Cardenas W, Endy TP, Finkelstein JL, King CA, et al. Case study 5.C Vector-virus microclimate surveillance system for dengue control in Machala, Ecuador. Climate Services for Health: Improving public health decision-making in a new climate. Geneva, Switzerland: World Meteorological Association and World Health Organization; 2016. Available: http://public.wmo.int/en/resources/library/climate-services-health-case-studies

24. Sommerfeld J, Kroeger A. Eco-bio-social research on dengue in Asia: a multicountry study on ecosystem and community-based approaches for the control of dengue vectors in urban and peri-urban Asia. Pathog Glob Health. 2012;106: 428–435. doi:10.1179/2047773212Y.0000000055

25. Quintero J, Brochero H, Manrique-Saide P, Barrera-Pérez M, Basso C, Romero S, et al. Ecological, biological and social dimensions of dengue vector breeding in five urban settings of Latin America: a multi-country study. BMC Infect Dis. 2014;14: 38. doi:10.1186/1471-2334-14-38

26. Casos de Dengue Reportados en el Epi Local por Semanas Epidemiologicas. Machala, Ecuador: Departamento de Epidemiologia, Direccion Provincial de Salud de El Oro, Ministerio de Salud Publica; 2010.

27. Vazquez-Prokopec GM, Galvin WA, Kelly R, Kitron U. A New, Cost-Effective, Battery-Powered Aspirator for Adult Mosquito Collections. J Med Entomol. 2009;46: 1256–1259.

28. Thomas SJ, Aldstadt J, Jarman RG, Buddhari D, Yoon I-K, Richardson JH, et al. Improving dengue virus capture rates in humans and vectors in Kamphaeng Phet Province, Thailand, using an enhanced spatiotemporal surveillance strategy. Am J Trop Med Hyg. 2015;93: 24–32.

29. Pan-ngum W, Blacksell SD, Lubell Y, Pukrittayakamee S, Bailey MS, de Silva HJ, et al. Estimating the True Accuracy of Diagnostic Tests for Dengue Infection Using Bayesian Latent Class Models. PLoS ONE. 2013;8. doi:10.1371/journal.pone.0050765

30. Pal S, Dauner AL, Valks A, Forshey BM, Long KC, Thaisomboonsuk B, et al. Multicountry Prospective Clinical Evaluation of Two Enzyme-Linked Immunosorbent Assays and Two Rapid Diagnostic Tests for Diagnosing Dengue Fever. J Clin Microbiol. 2015;53: 1092–1102. doi:10.1128/JCM.03042-14

31. Santiago GA, Vergne E, Quiles Y, Cosme J, Vazquez J, Medina JF, et al. Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus. PLoS Negl Trop Dis. 2013;7: e2311.

32. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14: 1232–9.

33. Toan NT, Rossi S, Prisco G, Nante N, Viviani S. Dengue epidemiology in selected endemic countries: factors influencing expansion factors as estimates of underreporting. Trop Med Int Health. 2015;20: 840–863.

34. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; btu170.

35. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. ArXiv Prepr ArXiv13033997. 2013; Available: http://arxiv.org/abs/1303.3997

36. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;25: 2078–2079.

37. Hunter JD, others. Matplotlib: A 2D graphics environment. Comput Sci Eng. 2007;9: 90–95.

38. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30: 2725–2729.

39. Myers WGWME, Altschul SF, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215: 403–10.

40. Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol. 2008;25: 1253–1256.

41. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 2003;52: 696–704.

42. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010;59: 307–321.

43. Pyke AT, Moore PR, Taylor CT, Hall-Mendelin S, Cameron JN, Hewitson GR, et al. Highly divergent dengue virus type 1 genotype sets a new distance record. Sci Rep. 2016;6. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4770315/

44. Krisher LK, Krisher J, Ambuludi M, Arichabala A, Beltrán-Ayala E, Navarrete P, et al. Successful malaria elimination in the Ecuador–Peru border region: epidemiology and lessons learned. Malar J. 2016;15: 573. doi:10.1186/s12936-016-1630-x

45. Anders KL, Van Thuy NT, Van Ngoc T, Tam CT, Tai LTH, Truong NT, et al. Households as foci for dengue transmission in highly urban Vietnam. PLoS Negl Trop Dis. 2015;9: e0003528.

46. Yoon I-K, Getis A, Aldstadt J, Rothman AL, Tannitisupawong D, Koenraadt CJM, et al. Fine Scale Spatiotemporal Clustering of Dengue Virus Transmission in Children and Aedes aegypti in Rural Thai Villages. PLoS Negl Trop Dis. 2012;6: e1730. doi:10.1371/journal.pntd.0001730

47. Mammen Jr MP, Pimgate C, Koenraadt CJ, Rothman AL, Aldstadt J, Nisalak A, et al. Spatial and temporal clustering of dengue virus transmission in Thai villages. PLoS Med. 2008;5: e205.

48. Beckett CG, Kosasih H, Faisal I, Tan R, Widjaja S, Listiyaningsih E, et al. Early detection of dengue infections using cluster sampling around index cases. Am J Trop Med Hyg. 2005;72: 777–782.

49. Ali A, ur Rehman H, Nisar M, Rafique S, Ali S, Hussain A, et al. Seroepidemiology of dengue fever in Khyber Pakhtunkhawa, Pakistan. Int J Infect Dis. 2013;17: e518–e523.

50. Fernández E, Smieja M, Walter SD, Loeb M. A predictive model to differentiate dengue from other febrile illness. BMC Infect Dis. 2016;16: 694.

51. Zim MM, Sam I-C, Omar SS, Chan YF, AbuBakar S, Kamarulzaman A. Chikungunya infection in Malaysia: comparison with dengue infection in adults and predictors of persistent arthralgia. J Clin Virol. 2013;56: 141–145.

52. Murray KO, Rodriguez LF, Herrington E, Kharat V, Vasilakis N, Walker C, et al. Identification of dengue fever cases in Houston, Texas, with evidence of autochthonous transmission between 2003 and 2005. Vector-Borne Zoonotic Dis. 2013;13: 835–845.

53. Parreira R, Conceição C, Centeno-Lima S, Marques N, da Cunha JS, Abreu C, et al. Angola's 2013 dengue outbreak: clinical, laboratory and molecular analyses of cases from four Portuguese institutions. J Infect Dev Ctries. 2014;8: 1210–1215.

54. Thai KT, Phuong HL, Nga TTT, Giao PT, Hung LQ, Van Nam N, et al. Clinical, epidemiological and virological features of Dengue virus infections in Vietnamese patients presenting to primary care facilities with acute undifferentiated fever. J Infect. 2010;60: 229–237.

55. Waggoner JJ, Gresh L, Vargas MJ, Ballesteros G, Tellez Y, Soda KJ, et al. Viremia and Clinical Presentation in Nicaraguan Patients Infected with Zika Virus, Chikungunya Virus, and Dengue Virus. Clin Infect Dis. 2016; ciw589. doi:10.1093/cid/ciw589

56. Le Gonidec E, Maquart M, Duron S, Savini H, Cazajous G, Vidal P-O, et al. Clinical Survey of Dengue Virus Circulation in the Republic of Djibouti between 2011 and 2014 Identifies Serotype 3 Epidemic and Recommends Clinical Diagnosis Guidelines for Resource Limited Settings. PLoS Negl Trop Dis. 2016;10: e0004755.

57. Thomas L, Verlaeten O, Cabié A, Kaidomar S, Moravie V, Martial J, et al. Influence of the dengue serotype, previous dengue infection, and plasma viral load on clinical presentation and outcome during a dengue-2 and dengue-4 co-epidemic. Am J Trop Med Hyg. 2008;78: 990–998.

58. Burattini MN, Lopez LF, Coutinho FA, Siqueira-Jr JB, Homsani S, Sarti E, et al. Age and regional differences in clinical presentation and risk of hospitalization for dengue in Brazil, 2000-2014. Clinics. 2016;71: 455–463.

59. Halsey ES, Marks MA, Gotuzzo E, Fiestas V, Suarez L, Vargas J, et al. Correlation of serotype-specific dengue virus infection with clinical manifestations. PLoS Negl Trop Dis. 2012;6: e1638.

60. Gill BS. Epidemiology of dengue in Malaysia from 2005 - 2010 and factors contributing to its emergence [Internet]. University of Western Australia. 2012. Available: http://research-repository.uwa.edu.au/en/publications/epidemiology-of-dengue-in-malaysia-from-2005--2010-and-factors-contributing-to-its-emergence(52431773-d415-4b6f-8573-8d38e2fcb64a)/export.html?uwaCustom=thesis

61. Prasith N, Keosavanh O, Phengxay M, Stone S, Lewis HC, Tsuyuoka R, et al. Assessment of gender distribution in dengue surveillance data, the Lao People's Democratic Republic. Methods. 2011; Available: http://www.wpro.who.int/entity/wpsar/volumes/04/2/2012.3.4.020_OR_Prasith.EN.pdf

Figure Legends

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 and associate group demographic data and infection status.

	2014		2015	
	Index cases (n = 194)	**Associate cases (n = 310)**	**Index cases (n = 130)**	**Associate cases (n = 87)**
Demographics				
Age in years, mean (SD)	20.4 (15.7)	34.9 (19.8)	27.0 (18.8)	38.4 (20.2)
Gender, % female	47.4%	65.5%	53.8%	67.8%
Fever				
Acute fever (>38°C)	17.3%	0.66%	20.2%	0%
History of fever (self-report)	96.9%	11.0%	96.2%	3.6%
Fever by either measure	97.4%	11.0%	96.2%	3.6%
DENV infection				
Acute infection (NS1 RT, NS1 ELISA or PCR pos)	41.2%	15.1%	19.4%	5.8%
Recent infection (NS1 RT/NS1 ELISA/PCR neg, IgM pos)	31.3%	20.5%	8.87%	7.0%
IgG only	2.75%	12.8%	12.1%	14.0%
Negative by all tests (NS1 RT/ELISA/PCR, IgG, IgM)	24.7%	51.7%	59.7%	73.3%
Missing/incomplete	12	15	6	1
Health care utilization				
Sought medical care	100%	2.36%	100%	1.15%
Hospitalized	19.4%	0%	16.2%	0%
Other infections				
Chikungunya	0%	0%	41.0%	4.6%
Zika	0%	0%	0%	0%

The characteristics of index and associate cases 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)**	**Index cases (n = 35)**	**Associate cases (n = 11)**
Demographics				
Age in years, mean (SD)	20.9 (14.4)	29.8 (18.3)	22.7 (14.8)	27.6 (14.4)
Gender, % female	43.2%	69.8%	60.0%	54.5%
Fever				
Acute fever (>38°C)	13.6	1.92	30.3	0%
History of fever (self-report)	95.4	17.6	97.1	9.09%
Fever by either measure	96.2	17.6	97.1	9.09%
Health care utilization				
Sought medical care	100%	6.48%	100%	0%
Hospitalized	24.1%	0%	28.6%	0%
Serology				
Primary infection	21.3%	35.8%	60.0%	36.4%
Secondary infection	59.8%	40.6%	28.6%	18.2%
None	18.8%	23.6%	11.4%	45.4%
DENV serotype				
1	7.84%	16.7%	59.1%	0%
1 & 2	1.96%	0%	0%	0%
2	84.3%	55.6%	40.9%	100%
3	3.92%	27.8%	0%	0%
4	1.96%	0%	0%	0%
PCR negative	81	88	13	10

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 and associate cases 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	Associate cases	Index Cases	Associate cases
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 cases			Primary DENV cases			Secondary DENV cases		
	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 = 14)	11-20 years (n = 26)	21+ years (n = 42)
Demographics									
Gender (% female)	41.0%*	32.2%*	62.3%*	33.3%	46.2%	63.2%	46.7%*	26.9%*	66.7%*
Symptoms									
Fever	100%	96.6%	92.8%	100%	100%	89.5%	100%	92.3%	95.2%
Headache	73.7%	79.7%	82.6%	86.7%	92.3%	73.7%	60.0%	73.1%	83.3%
Anorexia and nausea	61.5%	69.5%	55.1%	60.0%	76.9%	42.1%	60.0%	65.4%	64.3%
Muscle/joint pain	65.8%*	86.3%*	87.0%*	66.7%	76.9%	89.5%	53.3%	76.9%	83.3%
Rash	15.4%	22.4%	20.3%	26.7%	41.7%	15.8%	6.67%	23.1%	21.4%
Bleeding	7.7%	11.9%	8.8%	6.7%	15.4%	0%	13.3%	15.4%	14.3%
Rhinorrhea	18.0%	17.0%	13.0%	26.7%	0%	15.8%	13.3%	15.4%	11.9%
Vomiting	56.4%	47.5%	33.3%	53.3%	30.8%	15.8%	80.0%	53.8%	45.2%
Drowsiness/ lethargy	76.9%	81.4%	89.9%	80.0%	84.6%	73.7%	73.3%*	88.5%*	97.6%*
Cough	33.3%	25.4%	26.1%	26.7%	30.8%	10.5%	46.7%	19.2%	31.0%
Abdominal pain	71.0%	50.8%	60.3%	66.7%	46.2%	61.1%	86.7%	57.7%	59.5%
Diarrhea	12.8%*	35.6%*	27.5%*	13.3%	30.8%	31.6%	6.67%	42.3%	31.0%
Retro-orbital pain	55.2%	72.9%	68.1%	73.3%	84.6%	63.2%	28.6%*	65.4%*	66.7%*

*Significant differences between 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 co-infections), (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

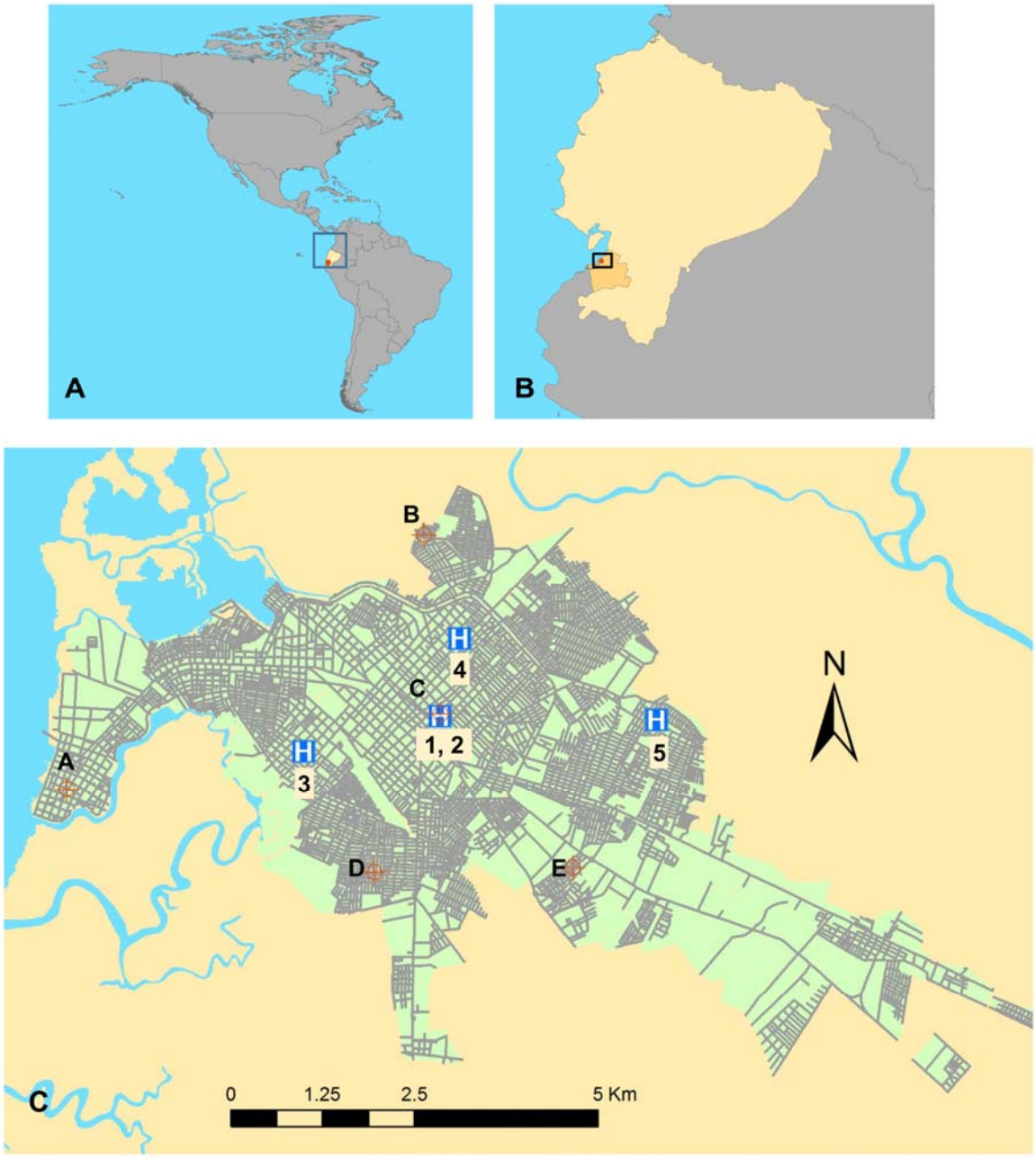

Fig 1: Map of the study site: A. Location of Ecuador in the Americas; study site in red. B. Location of El Oro Province in southern coastal Ecuador, study site in red. C. The city of Machala, showing the Ministry of Health sentinel clinics and hospital: 1. Mabel Estupiñan Clinic, 2. Teofilo Davila Hospital, 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.

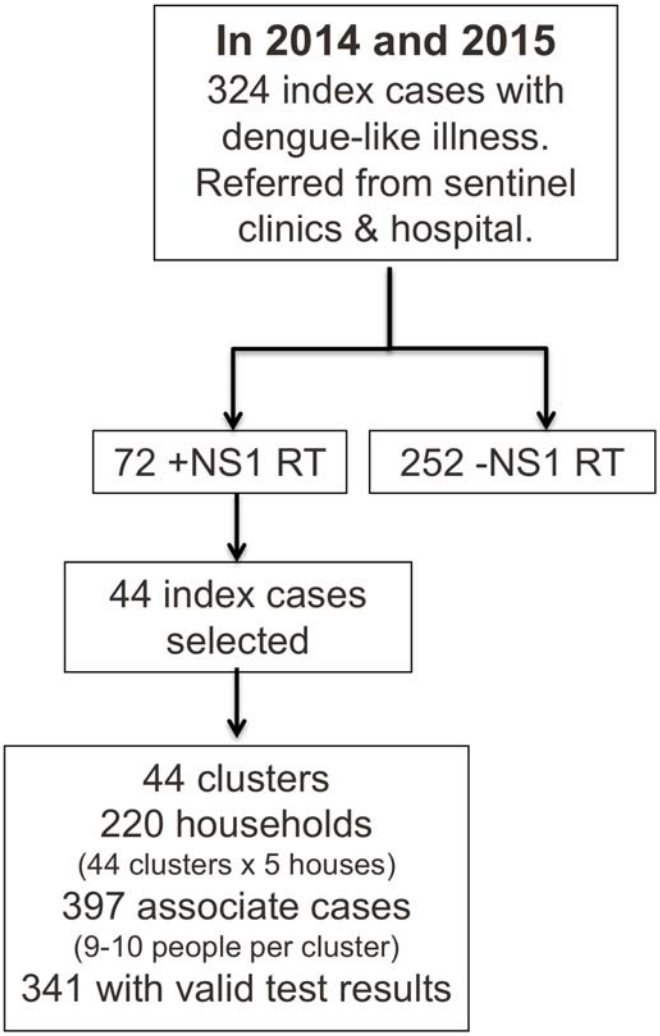

Fig 2. Study design. DENV surveillance study design in Machala, Ecuador, in 2014 and 2015.

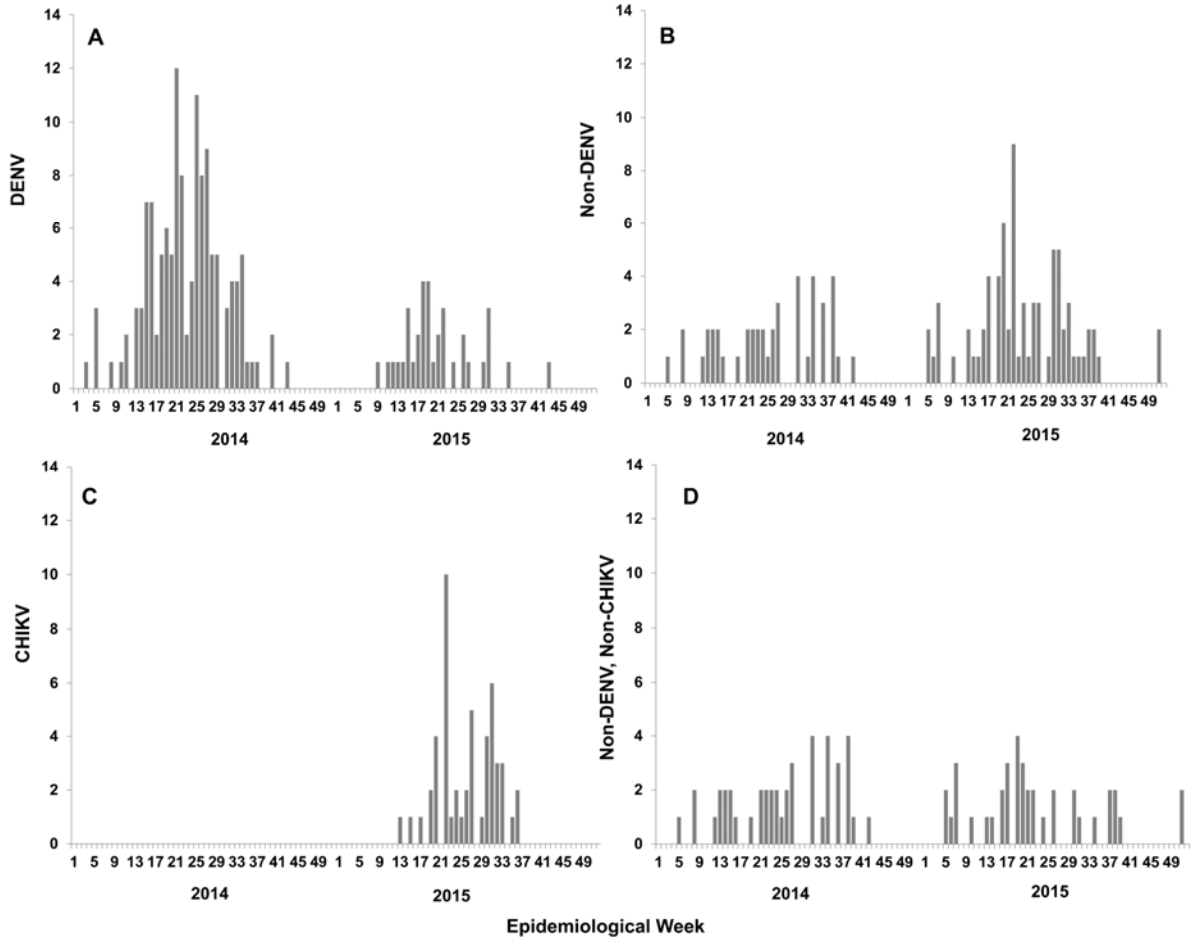

Fig 3. Weekly DENV and CHIKV infections in 2014 and 2015. (A) Acute or recent DENV infections, (B) Non-DENV infections, (C) CHIKV infections, and (D) non-DENV, non-CHIKV infections. Note: no surveillance was conducted in week 30 of 2014.

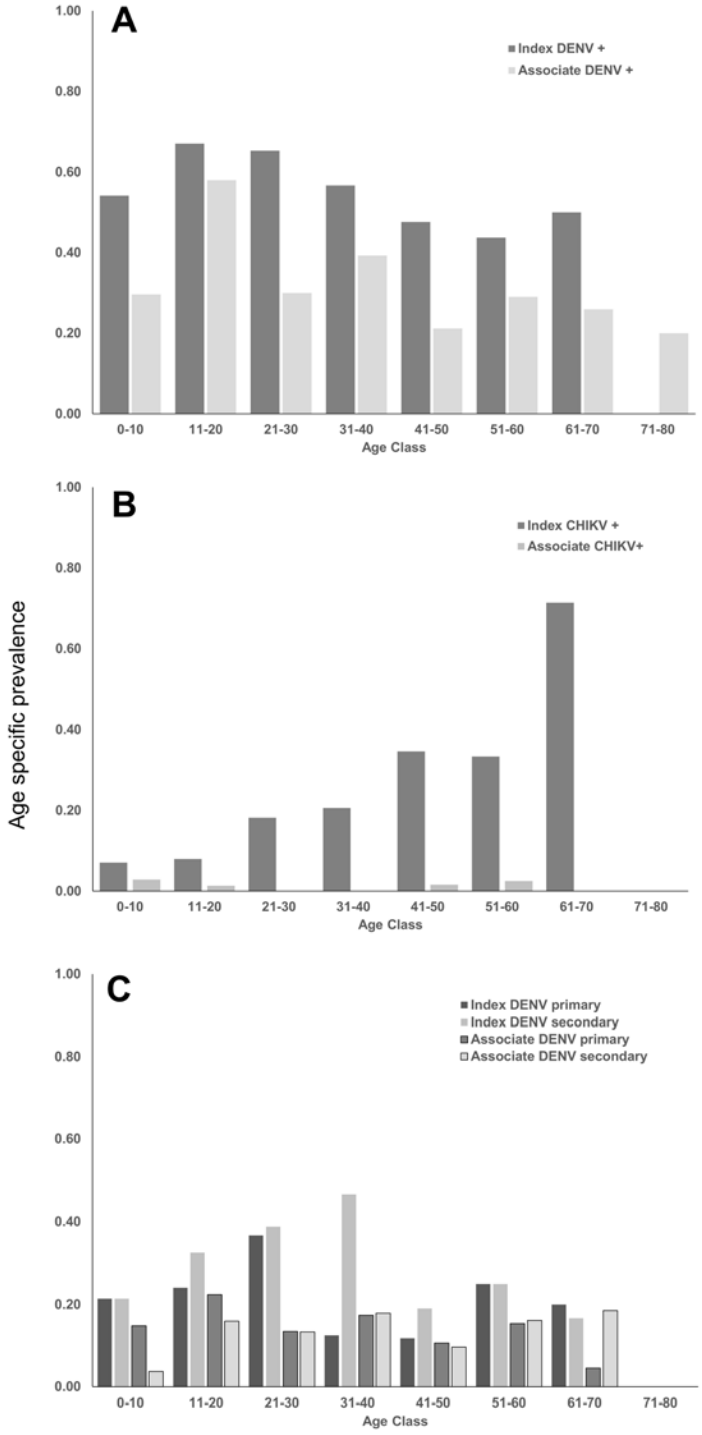

Fig 4: Age specific prevalence of index and associate cases. (A) Acute or recent DENV infection, (B) acute CHIKV infection, (C) primary versus secondary DENV infections.

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..

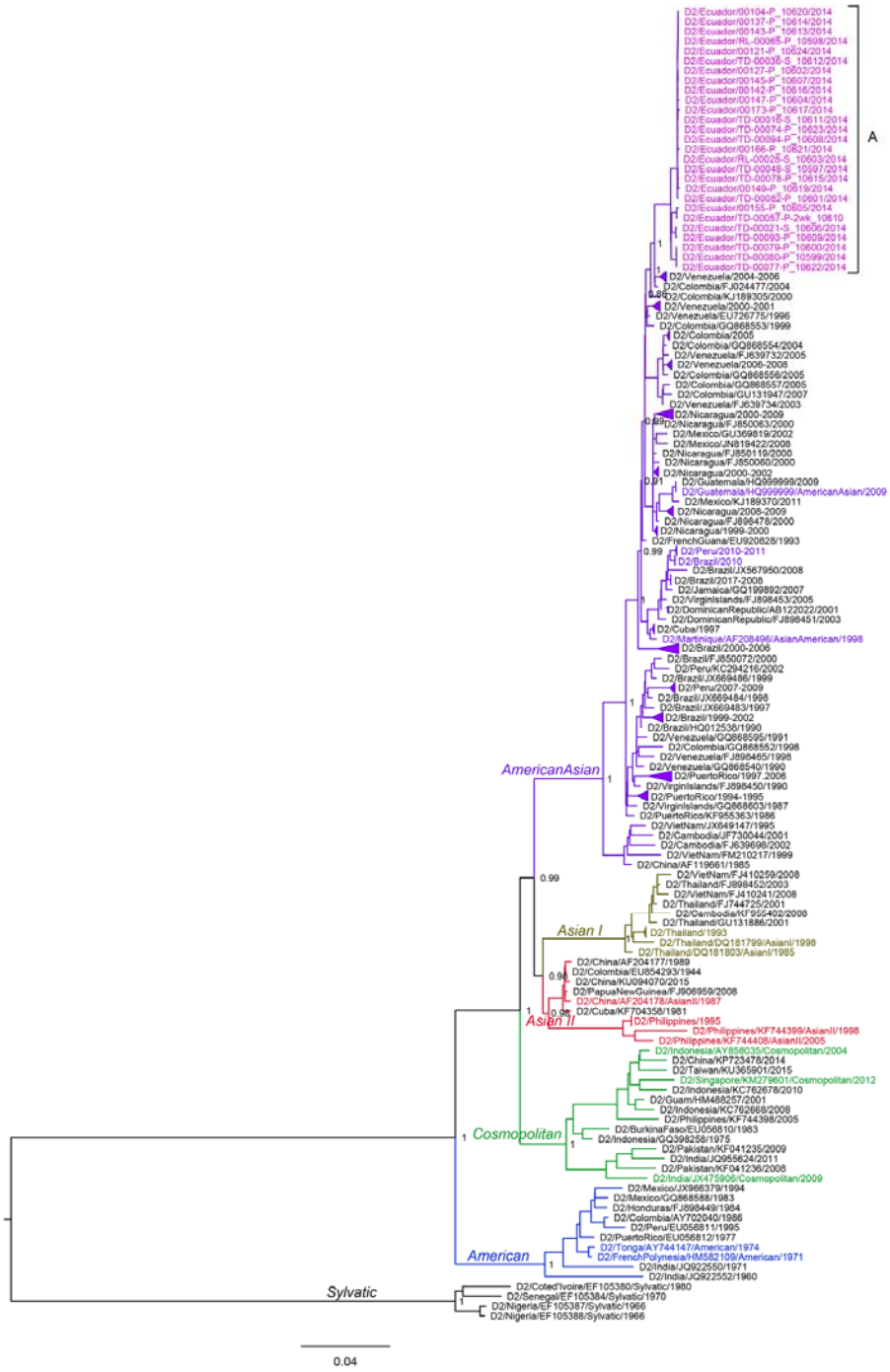

Fig 6. Maximum likelihood phylogenetic tree of DENV2 genotypes from Ecuador in 2014. Samples from Ecuador are colored magenta in a monophyletic clade A. aLRT confidence values are shown next to the respective node. The tree is rooted on the sylvatic genotype outgroup. Some clades were collapsed in the 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.

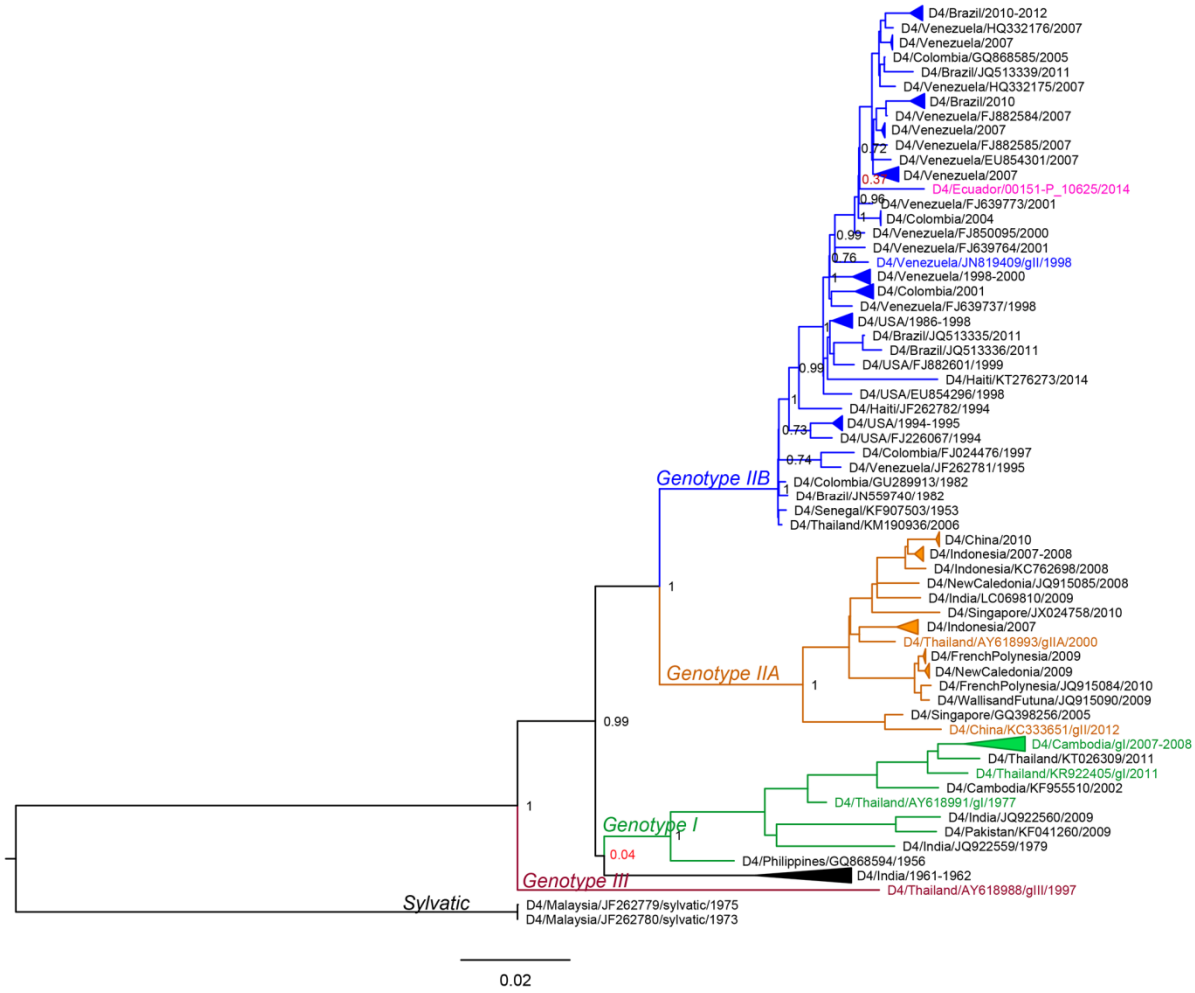

Fig 7. Maximum likelihood phylogenetic tree of DENV4 genotypes from Ecuador in 2014. Sample from Ecuador is colored in magenta. aLRT confidence values are shown next to the respective node. Low aLRT values are highlighted in red. The tree is rooted on the sylvatic genotype outgroup. Some clades were 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.

Supplemental Tables

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. 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)**	**Acute or recent DENV (n = 29)**	**Non-DENV and non-CHIKV (n = 38)**
Demographics				
Age in years, mean (SD)	20.9 (14.4)	20.0 (18.8)	19.0 (11.7)	24.0 (19.7)
Gender, % female	43.2%*	62.2%*	55.2%	39.5%
Acute Febrile				
Temperature > 38°C	13.6%	25.6%	37.0%	18.9%
Symptoms in prior 7 days				
Fever	95.4%	100%	100%	97.4%
Headache	78.6%	83.7%	86.2%	81.6%
Anorexia and nausea	62.9%	57.8%	69.0%	50.0%
Muscle/joint pain	77.9%	69.8%	75.9%	75.7%
Rash	21.1%*	6.82%*	7.14%	18.4%
Bleeding	9.09%	2.22%	14.3%	5.26%
Rhinorrhea	16.7%*	33.3%*	13.8%*	42.1%*
Vomiting	47.0%	31.1%	37.9%	21.0%
Drowsiness/lethargy	84.8%	84.4%	86.2%	92.1%
Cough	30.3%*	51.1%*	17.2%	29.0%
Abdominal pain	57.2%	54.6%	75.0%*	50.0%*
Diarrhea	28.0%*	6.67%*	20.7%	26.3%
Retro-orbital pain	64.9%	59.1%	75.9%	67.6%
Health care utilization				
Sought medical care	100%	100%	100%	100%
Hospitalized	24.1%	8.82%	31.0%	13.2%

* $p < 0.05$.

Supplemental Table 3. 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)
Demographics		
Age in years, mean (SD)*	20.6 (14.0)	33.6 (18.0)
Gender, % female	45.3%*	63.9%*
Acute Febrile		
Temperature > 38°C	17.8%	11.8%
Symptoms in prior 7 Days		
Fever	96.2%	97.2%
Headache	80.0%	80.6%
Anorexia and nausea	64.0%	47.2%
Muscle/joint pain	77.5%*	97.2%*
Rash	18.8%	33.3%
Bleeding	10.0%	5.56%
Rhinorrhea	16.2%	13.9%
Vomiting	45.3%	30.6%
Drowsiness/lethargy	85.1%	94.4%
Cough	28.0%	13.9%
Abdominal pain	60.4%*	41.7%*
Diarrhea	26.7%	33.3%
Retro-orbital pain	66.9%	71.4%
Health care utilization		
Sought medical care	100%	100%
Hospitalized	25.5%	11.1%

* $p < 0.05$.

Supplemental Table 4. Characteristics of DENV infections in index cases by serotype, excluding CHIKV co-infections.

	DENV-1 (n = 17)	DENV-2 (n = 51)
Demographics		
Age in years, mean (SD)	14.9 (10.8)*	25.2 (16.2)*
Gender, % female	47.1%	41.2%
Acute Febrile		
Temperature > 38°C	50%	31.2%
Symptoms in prior 7 days		
Fever	100%	96.1%
Headache	100%	84.3%
Anorexia and nausea	76.5%	62.8%
Muscle/joint pain	70.6%	84.3%
Rash	12.5%	15.7%
Bleeding	11.8%	3.92%
Rhinorrhea	17.6%	15.7%
Vomiting	52.9%	51.0%
Drowsiness/lethargy	94.1%	86.3%
Cough	17.6%	29.4%
Abdominal pain	70.6%	60.8%
Diarrhea	23.5%	23.5%
Retro-orbital pain	76.5%	70.6%
Hospitalization		
Hospitalized	23.5%	15.9%
Serology		
Primary	62.5%	27.7%
Secondary	18.8%	40.4%
None	18.8%	31.9%
Missing/incomplete	1	4

*$p < 0.05$

Supplemental Table 5 Characteristics of DENV infections in index cases by serology (excludes those with CHIKV co-infections)

	Primary DENV infections (n = 43)	Secondary DENV infections (n = 82)
Demographics		
Age in years, mean (SD)	18.0 (13.1)*	23.2 (13.8)*
Gender, % female	44.2%	50.0%
Acute Febrile		
Temperature > 38°C	25.6%*	8.86%*
Symptoms in Prior 7 Days		
Fever	97.7%	95.1%
Headache	86.0%	75.6%
Anorexia and nausea	62.8%	64.6%
Muscle/joint pain	76.7%	75.6%
Rash	21.4%	19.5%
Bleeding	7.14%	14.6%
Rhinorrhea	16.3%	13.4%
Vomiting	34.9%*	54.9%*
Drowsiness/lethargy	83.7%	90.2%
Cough	20.9%	30.5%
Abdominal pain	59.5%	64.6%
Diarrhea	23.3%	30.5%
Retro-orbital pain	74.4%	59.3%
Hospitalization		
Hospitalized	10.8%*	44.0%*
Serotype		
1	10 (43.5%)	3 (12.5%)
1&2	0	1 (4.17%)
2	13 (56.5%)	19 (79.2%)
3	0	1 (4.17%)
4	0	0

* $p<0.05$

Supplemental Table 6. Symptoms of associate cases with acute or recent DENV infections 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
Demographics		
Age in years, mean (SD)	29.4 (17.9)	32.4 (20.0)
Gender, % female	68.1%	68.8%
Acute Febrile		
Temperature > 38°C	1.75%	100%
Symptoms in Prior 7 Days		
No symptoms	26.8%	0%
No dengue-like symptoms	41.4%	0%
Fever**	17.0%	100%
Headache**	29.8%	68.8%
Anorexia and nausea**	8.77%	43.8%
Muscle/joint pain**	27.2%	62.5%
Rash	12.3%	12.5%
Bleeding	0.88%	0%
Rhinorrhea**	12.3%	31.2%
Vomiting**	1.75%	12.5%
Drowsiness/lethargy**	20.2%	43.8%
Cough**	17.5%	62.5%
Abdominal pain	20.2%	31.2%
Diarrhea	8.77%	12.5%
Retro-orbital pain	22.8%	25.0%
Health care utilization		
Sought medical care	6.03%	0%
Hospitalized	0%	0%

*Excludes one case of CHIKV co-infection

**$p < 0.05$