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Zika and Chikungunya virus detection in naturally infected *Aedes aegypti* in Ecuador

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ABSTRACT

The wide and rapid spread of Chikungunya (CHIKV) and Zika (ZIKV) viruses represent a global public health problem, especially for tropical and subtropical environments. The early detection of CHIKV and ZIKV in mosquitoes may help to understand the dynamics of the diseases in high-risk areas, and to design data based epidemiological surveillance to activate the preparedness and response of the public health system and vector control programs. This study was done to detect ZIKV and CHIKV viruses in naturally infected fed female *Aedes aegypti* (L.) mosquitoes from active epidemic urban areas in Ecuador. Pools (n = 193; 22 pools) and individuals (n = 22) of field collected *Ae. aegypti* mosquitoes from high-risk arboviruses infection sites in Ecuador were analyzed for the presence of CHIKV and ZIKV using RT-PCR. Phylogenetic analysis demonstrated that both ZIKV and CHIKV viruses circulating in Ecuador correspond to the Asian lineages. Minimum infection rate (MIR) of CHIKV for Esmeraldas city was 2.3% and the maximum likelihood estimation (MLE) was 3.3%. The minimum infection rate (MIR) of ZIKV for Portoviejo city was 5.3% and for Manta city was 2.1%. Maximum likelihood estimation (MLE) for Portoviejo city was 6.9% and 2.6% for Manta city. Detection of arboviruses and infection rates in the arthropod vectors may help to predict an outbreak and serve as a warning tool in surveillance programs.

1. Introduction

Multiple outbreaks of emerging arboviral diseases have been documented worldwide, predominantly in tropical areas. This emerging group of infectious diseases has drawn attention from the global health community due to the potential for large-scale epidemics and rapid spread across the borders of several countries in the Americas.

Dengue, zika and chikungunya diseases are present in Ecuador and affect mainly the population on the Pacific coastal zones, where dengue is considered endemic. There have been 10,104 confirmed cases of dengue in Ecuador during 2017, until the epidemiological week (EW) 31, with incidences of 22.6–242.6 (cases per 100s,000 population) in the coastal provinces. While in the Amazon basin provinces the incidences of dengue vary from 4.7 to 221.9. Since the introduction of Zika in 2016, there have been 5125 confirmed cases in Ecuador with incidences of 0.8–201.8 (per 100,000 population) in the Pacific coast provinces, and from 0 to 8.4 in the Amazon basin provinces (2016 and up to EW 23, 2017). Chikungunya cases, since the first locally acquired cases in 2014, reached 35,698 confirmed cases with incidences of 68–2,019.7 (per 100,000 population) in the coastal provinces, and 2.9–104.6 in the Amazon basin region (2016 – EW 35, 2017) (Ministerio de Salud Pública, 2017)

The rapid expansion of Chikungunya and Zika viruses demand the identification of the circulating lineages to design effective surveillance programs. Zika virus (ZIKV) belongs to the genus *Flavivirus*, family *Flaviviridae*. The study of the genome of ZIKV is ongoing and so far there are two lineages reported, the Asian and the African lineages (Enfissi et al., 2016). Chikungunya virus (CHIKV), on the other hand, is a member of the genus *Alphavirus*, family *Togaviridae*, and has been responsible for major outbreaks of febrile arthralgia in humans (Gould

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and Higgs, 2009). There are three reported lineages of CHIKV: West African, East central African (ECSA), and Asian (Weaver and Forrester, 2015). These viruses are transmitted in the urban areas by the mosquitoes *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse), although the viruses have also been found in other mosquito species (Vorou, 2016; Lourenço de Oliveira et al., 2003; Chouin-Carneiro et al., 2016; Haddow et al., 2012; Faye et al., 2008).

Symptomatic infections of ZIKV and CHIKV can present in a similar manner and a large number of infected humans are asymptomatic (Gallian et al., 2017; Yactayo et al., 2016). ZIKV is an emerging arbovirus originally detected in Africa and it is closely related to other flaviviruses of public health significance, such as Dengue virus (DENV) (Waggoner et al., 2016).

The first local transmission of CHIKV and ZIKV occurred in the Western Hemisphere in 2013 and 2015, respectively (Patterson et al., 2016). Since then, these viruses have spread rapidly in the Americas. The first cases of chikungunya in Ecuador were introduced from Colombia and the first autochthonous case was reported in Manabí province in December 2014 (Ministerio de Salud Pública, 2017). Ecuador reported an outbreak of chikungunya mainly in the coastal provinces of Esmeraldas, Guayas, and Manabí in October 2015 (Ministerio de Salud Pública, 2017).

In January 2016, Ecuador confirmed the first two ZIKV cases in travelers returning from Colombia. Two months later, the epidemiological bulletin of the Ecuadorian Ministry of Public Health reported 66 cases of Zika nationwide, 23 were identified in the province of Manabí (Ministerio de Salud Pública, 2017).

The purpose of this study was to detect ZIKV and CHIKV viruses in naturally infected *Aedes aegypti* mosquitoes from active epidemic urban areas in three coastal cities of Ecuador. This information, in turn, may help to design better strategies to prevent and control arbovirus outbreaks.

2. Materials and methods

2.1. Collection sites and mosquitoes sampling

Collections were conducted in the provinces of Esmeraldas and Manabí on the Pacific coast of Ecuador at sea level (Fig. 1). The sampling took place in urban households in areas considered as high-risk sites for arbovirus transmission, due to the high number of clinical cases in those areas and high abundance of *Aedes aegypti* along several previous years (Ministerio de Salud Pública, 2017). One collection in the port city of Esmeraldas (0°57′00″N 79°40′00″O) took place during the rainy season (April 2015), while mosquitoes were collected in two sites in the city of Portoviejo (1°03′22″S 80°27′19″W), and in four sites in Manta city (00°57′00.08″S 80°42′58.32″W) during the dry season (August and October 2016).

Adult *Ae. aegypti* mosquitoes were collected inside homes using a battery powered Improved CDC Backpack Aspirator (Model 2846), from 9 am to 3 pm. The sampled homes were located about 500 m from each other. A total of 80 homes were surveyed, 60 in Manabí and 20 in Esmeraldas. Mosquitoes were taken to the laboratory and identified using a stereomicroscope and following taxonomic keys for *Aedes* species (Rueda, 2004) and blood feed engorgement was noted. All collected engorged female mosquitoes in a house were grouped in the same pool or tested individually if a single engorged female was collected in a home.

Aedes aegypti female mosquitoes were stored in RNA later Invitrogen® and transported to the Entomology Laboratory at the National Reference Center for Vector Research (CIREV) of the National Institute of Public Health (INSPI), and preserved at 4 °C.

2.2. Nucleic acid extraction and RT-PCR

RNA was extracted from the whole mosquito body using the QIAamp Viral RNA Mini kit following manufacturer's recommendations (Qiagen, Hilden, Germany). The RNA was stored at −80 °C until processing. Samples of Aedes aegypti from the three cities were processed individually or in pools, depending on the number of individuals collected in each inspected house, and all mosquitoes collected in a house were processed as a pool. Samples from Esmeraldas were processed in pools of 3–10 fed females, while samples from Manta and Portoviejo in pools of 2–6 fed females. Individual mosquitoes from Portoviejo (10) and Manta (12) were also tested for CHIKV and ZIKV. Extraction of genetic material from Esmeraldas samples was done in 2015, previous to ZIKV introduction, and for the rest of the locations in 2016.

Mosquitoes from Esmeraldas, Manta and Portoviejo cities were analyzed for ZIKV and CHIKV using a multiplex real-time RT-PCR, performed on a LightCycler96 (Roche) instrument. The ZIKV, CHIKV and DENV assay (ZCD) has been described previously and consists of a single multiplex reaction for the three viruses (Waggoner et al., 2016). Each run of the ZCD assay included a positive control consisting of the specific viral RNA (DENV2, ZIKV or CHIKV) and water as the negative control. All the runs using water resulted negative. Confirmatory runs of positive and negative controls were done twice using the ZIKV-CHIKV assay following the same protocols.

Zika virus positive samples were amplified again with a one-step rRT-PCR protocol that amplifies a region of the E gene that codifies for the envelope protein (Faye et al., 2008). Chikungunya positive samples were amplified again with a one-step rRT-PCR protocol that amplifies a 767 base-pair fragment, including a portion of a nonstructural protein 1 (nsP1) (Stapleford et al., 2016). Amplicons from conventional assays were visualized on agarose gel stained with 2% sybr safe.

PCR products were detected by agarose gel electrophoresis in TAE 1X buffer, stained with SYBR® Safe 10000X in agarose gel, and visualized under UV light. All positive PCR products were purified and sequenced using the Sanger methodology at Macrogen sequencing service, Seoul, South Korea.

2.3. Phylogenetic analysis

Amplicons from conventional RT-PCRs were sequenced using Sanger sequencing (Macrogen), and a multiple sequence alignment (Clustal W) phylogenetic analysis was performed with Geneious V.9. The Maximum Likelihood method, based on the Tamura-Nei model MEGA 7, was used for determining the genotype of each sequence. ZIKV and CHIKV sequences isolated in the Ecuador localities were compared with sequences from South America, Asia and Africa deposited in GenBank.

2.4. Accessions of sequences deposited in GenBank

Nucleotide sequences obtained in this study were deposited in GenBank: CHIKV (KT876070) and ZIKV (KY005879, KY465607).

2.5. Statistical analysis

The proportion of infected mosquitoes with ZIKV grouped in pools was calculated for each location using the MIR (minimum infection rate) and MLE (maximum likelihood estimation) (Condotta et al., 2004). The pool size used for MLE was an average of all pools sizes, and it is assumed that at least one mosquito carried the virus in the positive pools (Gu et al., 2008)

Minimum infection rate (MIR) = (no. positive pools/total no. mosquitoes tested) × 1000

Maximum likelihood estimation (MLE) = $[1 - (n - \chi/n)^{1/m}] \times 1000$

n = number of pools tested
χ = number of positive pools

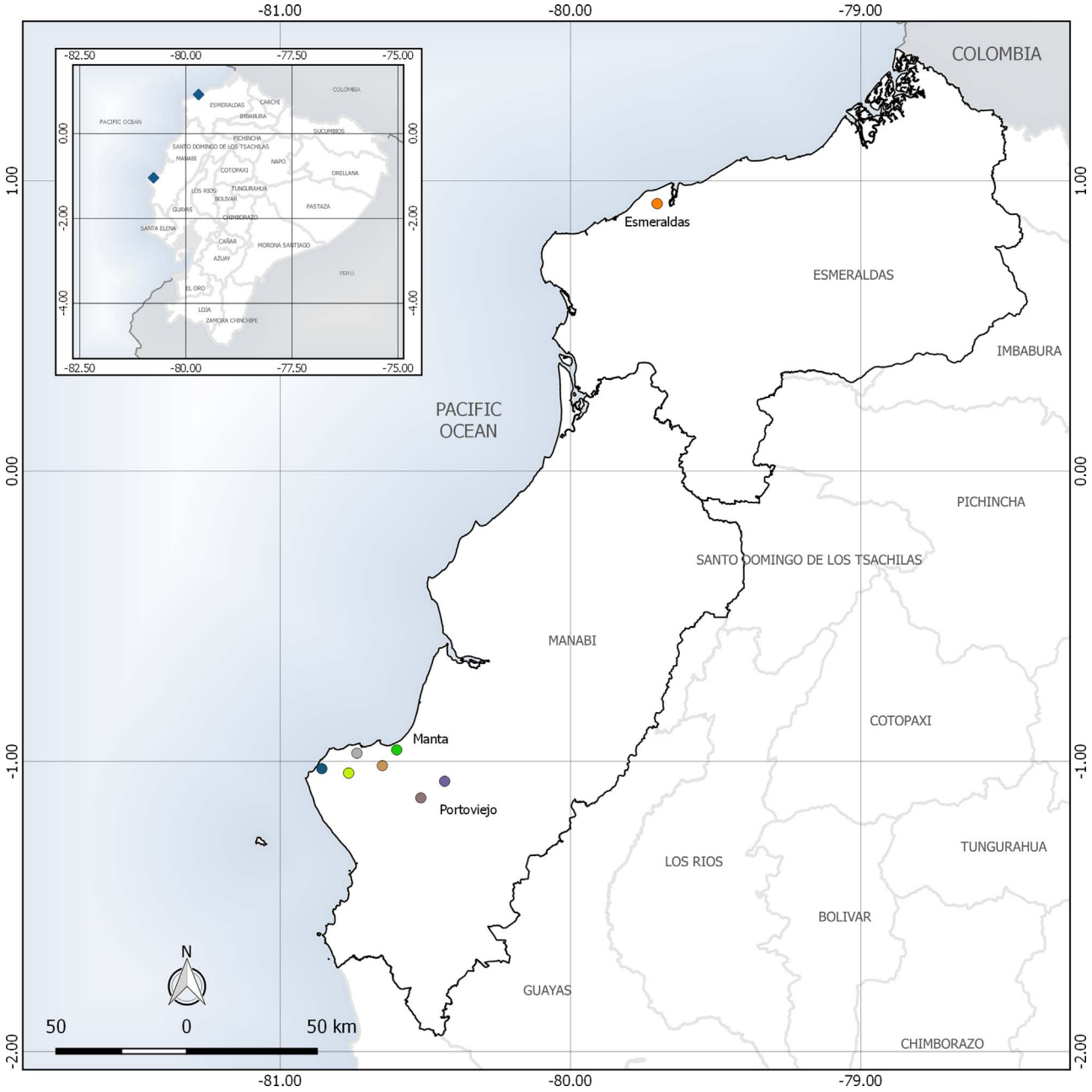

Fig. 1. Collecting sites of mosquitoes in Esmeraldas, Manta and Portoviejo. A) Orange circle represents the specific collecting site in Esmeraldas, 2015. B) Blue, grey, pink, yellow and dark green circles represent the sampling locations of Manta city. Violet and brown circles show the localities in Portoviejo. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

m = pool size

3. Results

A total of 282 mosquitoes were collected, 193 were identified as *Aedes aegypti* blood-fed female mosquitoes from the cities of Esmeraldas (43), Portoviejo (50) and Manta (100). Fed female mosquitoes were grouped in ten pools from Manta, four from Portoviejo and 8 pools from Esmeraldas. One out of eight (12.5%) mosquito pools were found positive for CHIKV, while two (14.3%) out of fourteen mosquito pools were positive for ZIKV. The positive pool for CHIKV was from Esmeraldas and consisted of 10 blood-fed females. Samples from Portoviejo and Manta cities (Manabí province) were negative for CHIKV. The two positive pools for ZIKV consisted of six blood-fed females each from Portoviejo and Manta cities. Pools size from Portoviejo and Manta varied from 2 to 6 fed females collected in August and October 2016, respectively that corresponds to the dry season. Table 1 shows the cycle threshold (C_t) results for each run using the ZCD assay. All samples were negative for DENV 1–4 serotypes.

Mosquitoes analyzed during 2016 were part of a surveillance system of the health system of Ecuador. As part of our standardization trials, we included singleplex assays with no positive results. Detection of ZIKV and CHIKV viruses were consistent after the implementation of multiplex assays developed by Waggoner and co-workers (Waggoner et al., 2016).

Infection rates were calculated using the total number of tested engorged females. Minimum infection rate (MIR) of CHIKV for Esmeraldas city was 2.3% and the maximum likelihood estimation (MLE) was 3.3%. The minimum infection rate (MIR) of ZIKV for the city of Portoviejo was 5.3% and for the city of Manta was 2.1%. Maximum

likelihood estimation (MLE) for Portoviejo city was 6.9% and for Manta city was 2.6%.

To further confirm virus identity amplicons were sequenced. The CHIKV amplicon showed 100% nucleotide identity with CHIKV isolate TR206/H804187, complete genome (KP164572).

ZIKV P1 amplicon of sequence with accession number KY005879 showed 99.7% nucleotide identity with Zika virus isolate BR/UFBA/LabViro/23 envelope protein gene, partial cds, (KR816333). While MA10 ECU16184 amplicon of sequence with accession number KY465607 showed 99.6% nucleotide identity with isolate Zika virus GD01 polyprotein gene, complete cds (KU740184) using BLAST analysis. The ZIKV lineages found in mosquito samples from the Portoviejo and Manta cities corresponded to the Asian lineage and grouped with the sequence (KY879604) reported from human blood of a patient in Esmeraldas (Márquez et al., 2017) (Fig. 3). The sequences from this study (KY465607, KY005879) grouped with isolates from Haiti 2014, Brazil 2015, Honduras 2015, Venezuela 2016, Mexico 2016, Ecuador 2016, China 2016, Colombia 2015, Martinique 2015, French Polynesia 2013 and Philippines 2016. This group is clearly different from the sequences from Senegal 1984 and Uganda 1947 that belong to the African lineage (Fig. 3).

CHIKV sequence with accession number KT876070 from Esmeraldas mosquitoes showed 100% homology with all sequences reported in GenBank and corresponded to the Asian lineage.

Nucleotide sequences obtained in this study were deposited in GenBank under accession numbers KT876070 (CHIKV) and KY005879, KY465607 (ZIKV).

4. Discussion

Direct detection of arboviruses in their arthropod vectors may help to predict an outbreak and serve as a warning tool in surveillance programs. Furthermore, information on vector viral load can be used in transmission models as well as for vector control interventions. This is of significant importance in diseases such as zika due to the serious effects on the central nervous system of newborns born to women infected during pregnancy, including severe zika syndrome that includes microcephaly (Rasmussen et al., 2016) However, only a few studies report naturally infected *Ae. aegypti* mosquitoes with ZIKV and CHIKV (Costa-da-Silva et al., 2017; Ferreira-De-Brito et al., 2016; Guerbois et al., 2016; Marchette et al., 1969; Mourya et al., 2001). The number of infected engorged females detected in our samples may include mosquitoes that already have the ability to transmit the viruses (infectious), and infected mosquitoes that are going through the extrinsic incubation period (EIP) denominated as latent (Bustamante and Lord, 2010).

The infection rates (IR) in the mosquitoes are determined by testing each individual vector; however, this approach has limitations of time and costs. A feasible alternative is to calculate MIR as the ratio of the positive pools for ZIKV and CHIKV to the total of tested mosquitoes and assumes that there is only one infected individual per pool. Other indicator to measure the proportion of infected mosquitoes is the MLE that can be used with samples of less than 1000 mosquitoes (Bernard et al., 2001). The values obtained in this study shows that MIR for CHIKV is 2.3%, which by some authors is a rather high value and it may indicate a potential outbreak (Day, 2001). This high value of MIR could have predicted the upcoming outbreak of CHIKV in the city of Esmeraldas during 2015, which by the end of epidemiological week 40 had caused a reported 5572 cases of chikungunya (Ministerio de Salud Pública, 2017). By contrast, the MLE for the same location was 3.3%, which is potentially more accurate as it estimates the proportion of infected mosquitoes in the sample (Gu et al., 2008) since the MIR estimates the lower bound of the infection rates and MLE the infection rate itself. Under the assumption that there was one infected mosquito in the positive pool, the infection rates (IR) may be inferred for CHIKV and ZIKV and in this case, it will be equal to the MIR values. Infection rates higher than 0.1% have been adopted as an indicator of an outbreak for arboviruses (Gu et al., 2008).

Values of MIR for infected mosquitoes with ZIKV in Portoviejo and Manta cities were 5.3% and 2.1%, respectively. The MLE value for Portoviejo city was 6.9% and for Manta city was 2.6%. These values of MIR and MLE are high considering that the sampling in Portoviejo and Manta cities was done during the dry season (August and October 2016, respectively), when transmission of arboviruses declines annually and corroborated by the decrease of reported human zika infections, with an estimated 20 cases in the country (Guerbois et al., 2016; Ministerio de Salud Pública, 2017) found MLE values of 5.2% and 17.3% for ZIKV in mosquitoes from southern Mexico. According to Gu et al. (2008), the MIR and MLE are similar under low transmission conditions, but differ significantly when the infection rates are high. Under high infection levels the MLE is larger than MIR and should be coupled with variable pool size. In the case of ZIKV infected mosquitoes from Portoviejo and Manta the values of MIR and MLE are high, but failed to predict an outbreak likely due to low density of *Aedes aegypti* populations due to control activities, low rainfall or a combination of factors. Minimum infection rate (MIR) values for dengue in Brazil are between 23 and 70%, while in Malaysia are 3.8% and 0.6% in Delhi, India (Costa et al., 2009; Lau et al., 2015).

Genomic sequence for CHIKV confirmed that the circulating virus in Esmeraldas belongs to the Asian lineage, which is primarily transmitted by *Aedes aegypti* (Rúa-Uribe et al., 2012). The other competent vector, *Aedes albopictus,* has recently been reported in Ecuador (Ponce et al., 2017).

The CHIKV Asian lineage has been reported in several South Pacific islands (2011–2015) and introduced in Brazil in early 2015 (Musso et al., 2015; Nunes et al., 2015; Patterson et al., 2016; Roth et al., 2014) when an outbreak of CHIKV was reported in the north Caribbean area of Colombia. The first autochthonous cases of CHIKV in Ecuador were reported in the province of Manabí in December 2014 and spread mostly in the coastal provinces, and by the 40th epidemiological week reached 33,245 cases, mainly in three adjacent coastal provinces (Ministerio de Salud Pública, 2017). However, the dynamics of the spread of the disease in the lowlands in the coastal areas and in Eastern Amazonian Ecuador is unclear, in part due to the arrival of ZIKV and the limited number of laboratory confirmed cases (Ministerio de Salud Pública, 2017).

The CHIKV sequence obtained in our study is a 100% match pairwise with sequences reported in Brazil, México, New Caledonia, Yap Island, Philippines, Thailand, and Malaysia (Fig. 2). The East, Central, South Africa (ECSA) and the West African genotype have also been reported in Brazil and the Caribbean, although the Asian lineage has been predominant in the Americas (Souza et al., 2017). Since the first imported cases of CHIKV in Ecuador matched to isolates from Colombia (Ministerio de Salud Pública, 2017) it is plausible that it was introduced from that country. The lineage of the CHIKV in Ecuador does not show any described mutations that render adaptive traits for *Aedes albopictus*, which coincides with reports in neighboring countries (Rodas et al., 2016).

However, it may be just a matter of time before the ECSA genotype of CHIKV arrives in Ecuador, considering the mobility of humans and commerce (Rodas et al., 2016). On the 26th epidemiological week there were 857 confirmed CHIKV cases (Ministerio de Salud Pública, 2017). By epidemiological week 29th there were 29,969 cases, mainly in Esmeraldas, Manabí, and Guayas provinces in the coastal region (Ministerio de Salud Pública, 2017) an increase of 3497%. The spread of the CHIKV infection in the human population in the western and eastern lowlands of Ecuador may follow the pattern of DENV, which is endemic. However, transmission pattern is not easy to elucidate due to the similar clinical symptoms between DENV infections and the newly arrived ZIKV at the beginning of 2016 (Ministerio de Salud Pública, 2017), and the limited number of cases confirmed by laboratory tests. It is known that the rainy season (January-June) is the time when *Aedes aegypti* populations increase (Cevallos unpublished) and the risk of

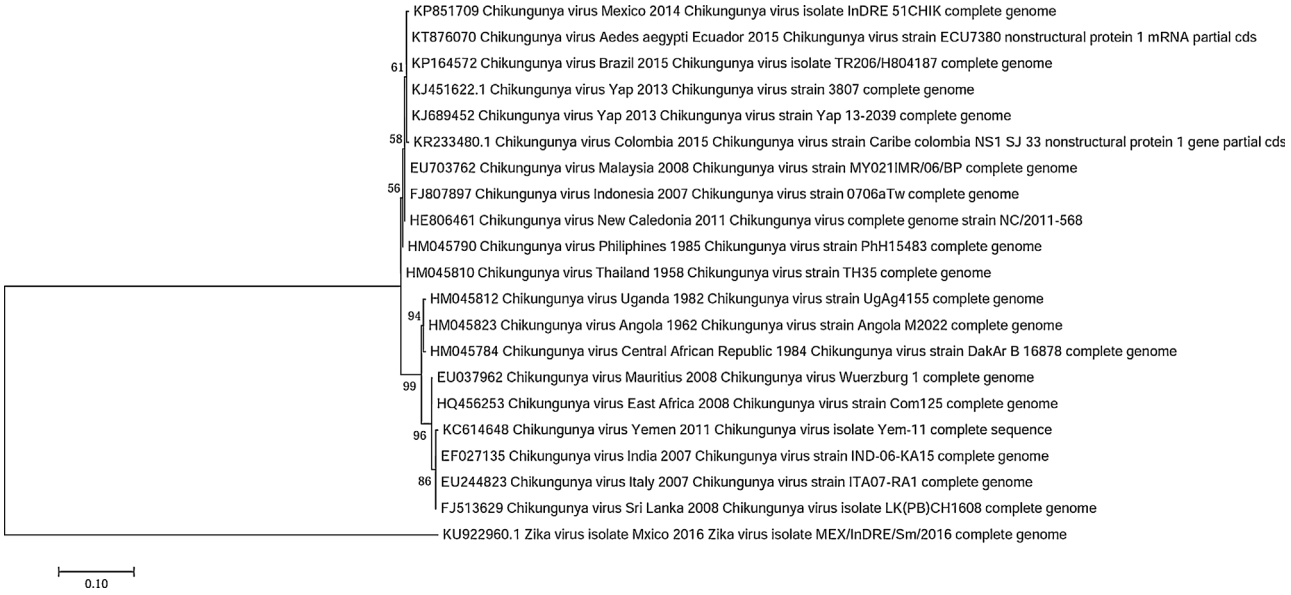

Fig. 2. Phylogenetic analysis of CHIKV. 20 sequences of nonstructural polyprotein 1 (NS1) were analyzed using the Maximum Likelihood method based on the Tamura-Nei model with MEGA 7. CHIKV lineage isolated from *Aedes aegypti* pool in Ecuador is shown. Scale bar 0.01.

transmission of the viruses transmitted by this mosquito is high, especially in the coastal areas.

We report ZIKV from naturally infected *Aedes aegypti* mosquitoes and its genotype corresponds to the Asian lineage. The sequence obtained from febrile patients in Esmeraldas city (Márquez et al., 2017) showed 64% (bootstrap analysis) of similarity with one of the sequences obtained from mosquitoes collected in Portoviejo (Fig. 3). This may be due to the fact that our sequence is only a region of the E gene that codifies for the envelope protein. The analysis showed that ZIKV isolates found in Ecuador clustered with the Asian genotype reported in other countries in the Americas (Enfissi et al., 2016; Haddow et al., 2012; Shen et al., 2016; Zanluca et al., 2015) (Fig. 3). The ZIKV strain found in Ecuador shows high similarity (99.7%) to strains identified in Brazil (KR816333.1) and Colombia (KX548902), raising the possibility that this resulted from a spillover from the epidemic in Colombia, where 65,726 cases were reported by April 2016 (Pacheco et al., 2016) The high human mobility between Ecuador and Colombia may suggest that ZIKV spread from Colombia to Ecuador.

The dispersal of the ZIKV in 2016 was somewhat interrupted due to the end of the rainy season in the lowlands as reflected by the few number of cases observed. However, it is necessary to monitor the advance of the virus in mosquito populations and clinical cases to better predict outbreaks and prepare timely responses. In addition, the percentage of infected field mosquitoes is a sensitive parameter needed for mathematical modeling and understanding of arboviral disease transmission (Manore et al., 2014). Zika virus detection in mosquitoes may be an important tool for arbovirus surveillance and disease outbreak prediction, considering the rapid expansion of ZIKV in the region and the potential link with birth defects and Guillian-Barré syndrome.

The epidemiological scenario of the viruses spread by *Aedes aegypti* in the Eastern lowlands and in the subtropical areas of Ecuador poses a high risk, since the mosquito is widely distributed and on the eastern mountain slopes reaches up to 1650 m (Cevallos unpublished). Most of the Zika virus infections during 2016 through 2017 (EW 31) have occurred in the Pacific coastal provinces, only in the Manabí province there have been 3260 confirmed cases (63.6%) out of the 5125 reported for Ecuador, and only 16 cases (0,3%) have been reported from all the provinces in the Amazon basin (Ministerio de Salud Pública, 2017).

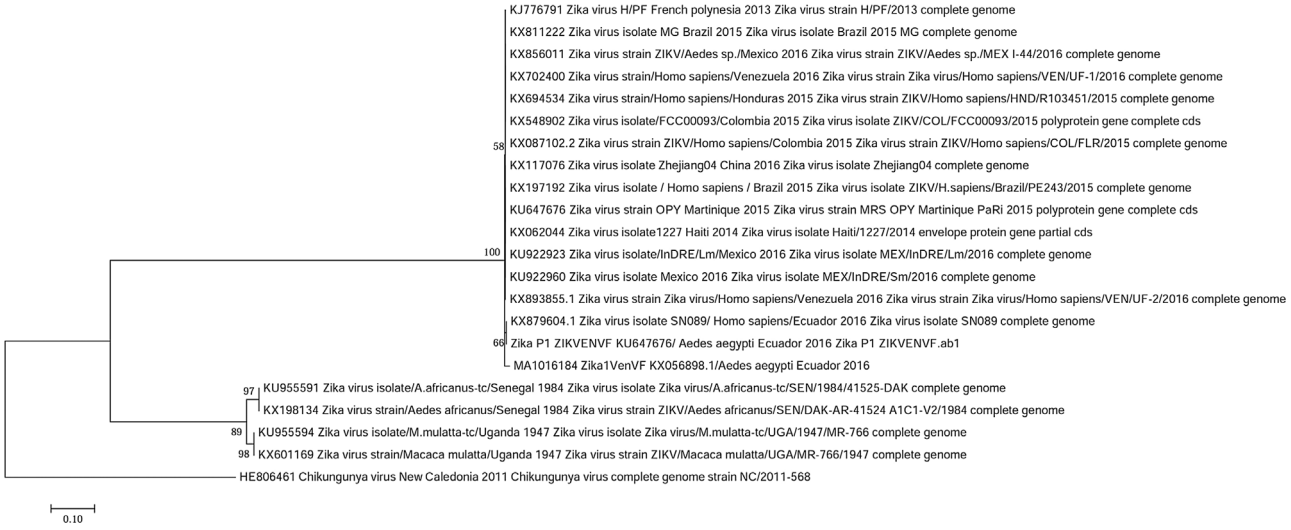

Fig. 3. Phylogenetic analysis of ZIKV. 17 partial sequences of the envelope protein gene were analyzed using the Maximum Likelihood method based on the Tamura-Nei model with MEGA 7. ZIKV lineages isolated from *Aedes aegypti* pools in Ecuador are shown. Scale bar 0.01.

Chikungunya confirmed cases during 2015 through EW 31 of 2017 have reached 35,698, and only from Esmeraldas, Manabí and Guayas provinces there were 30,186 cases (84.6%), while in the Amazon basin region there have been only 248 confirmed cases (0.7%). Consequently, the sampled areas may be considered as reference to understand the spread of arboviral diseases in the western lowland areas of Ecuador. However, the dynamics of vector disease transmission in the Ecuadorian Amazon basin may be different due to ecological differences and relatively low human population.

The detection of arboviruses in mosquitoes may help to understand the dynamics of the diseases for epidemiological surveillance and the design of effective interventions to reduce the risk of transmission, and preparation of the public health system. Further studies may be done adjusting some parameters including the pool size to establish improved early detection programs in areas of high risk of transmission and may include Mayaro, Oropuche and other emerging arboviruses in the region.

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