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Genetic Characterization of Northwestern Colombian Chikungunya Virus Strains from the 2014–2015 Epidemic

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Abstract. Chikungunya fever, an acute and often chronic arthralgic disease caused by the mosquito-borne alphavirus, chikungunya virus (CHIKV), spread into the Americas in late 2013. Since then it has caused epidemics in nearly all New World countries, the second largest being Colombia with over 450,000 suspected cases beginning in September, 2014, and focused in Bolivar Department in the north. We examined 32 human sera from suspected cases, including diverse age groups and both genders, and sequenced the CHIKV envelope glycoprotein genes, known determinants of vector host range. As expected for Asian lineage CHIKV strains, these isolates lacked known *Aedes albopictus*–adaptive mutations. All the Colombian strains were closely related to those from the Virgin Islands, Saint Lucia, Mexico, Puerto Rico, and Brazil, consistent with a single, point-source introduction from the southeast Asia/Pacific region. Two substitutions in the E2 and E1 envelope glycoprotein genes were found in the Colombian strains, especially E1-K211E involving a residue shown previously to affect epistatically the penetrance of the E1-A226V *A. albopictus*–adaptive substitution. We also identified two amino acid substitutions unique to all American CHIKV sequences: E2-V368A and 6K-L20M. Only one codon, 6K-47, had a high nonsynonymous substitution rate suggesting positive selection.

INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus that belongs to the *Togaviridae* family, genus *Alphavirus*. The viral genome has a positive-sense, single-stranded RNA of approximately 11.5 kb, and encodes four nonstructural proteins (nsP1–4) and three main structural proteins (capsid, E2 and E1 envelope glycoproteins) in the genomic and a subgenomic RNA, respectively.[1] The envelope glycoproteins include a fusion peptide (E1) and the ligand for receptor-mediated endocytic viral entry (E2); these proteins are known to be major determinants of evolutionary adaption and dissemination of this arbovirus.[2–4] The alphavirus E2 protein consists of three distinct domains, A, B, and C, and E2 has been previously implicated as the major target of the protective host immune response.[5,6] Domain B is believed to code for the tip of alphavirus spikes and to interact with cellular receptors. It includes neutralizing epitopes.[7]

In humans, CHIKV causes an acute undifferentiated febrile syndrome, called chikungunya fever (CHIKF), characterized by high fever, headache, back pain, myalgia, and polyarthralgia among other signs and symptoms.[8] In its original, sylvatic cycle in the African continent, mosquitoes from the *Aedes* genus transmit CHIKV among nonhuman primates.[9] However, in urban environments of Africa, Asia, and Europe, and now in the New World, CHIKV circulates efficiently between anthropophilic mosquitoes, principally *Aedes aegypti*, and humans to infect a large number of persons and cause explosive outbreaks. However, in tropical and subtropical countries, CHIKV can be difficult to diagnose based only on signs and symptoms mainly due to the presence of dengue virus (DENV), yellow fever virus (YFV), and other viruses associated with acute undifferentiated febrile syndromes, such as St. Louis encephalitis virus (SLEV), Venezuelan equine encephalitis virus (VEEV), West Nile virus (WNV), Mayaro virus (MAYV), Oropouche virus (OROV), and more recently Zika virus (ZIKV).[10–14]

CHIKV was first isolated in the 1950s in Tanzania (east Africa), and since has spread to the Indian Ocean basin, Asia (India, Sri Lanka, and southeast Asia in 2005[15,16]), Europe (Italy, 2007[17] and France, 2010[18] and 2014[19]), and the Americas in 2013.[20,21] Since the original discovery of CHIKV in Africa, there have been large outbreaks in the Asian and American continents involving millions of human cases, resulting in major interest in the news media and scientific journals.

Colombia has been the second most affected nation during the current CHIKV outbreak in the Americas (second only to the Dominican Republic, which had over 500,000 suspected cases between 2014 and 2015),[22,23] with more than 450,000 suspected cases,[24] most only diagnosed based on signs and symptoms. Thus, these two countries represent more than half of the case burden CHIKV has imposed in the Americans.[25] According to the national health agency (Instituto Nacional de Salud [INS]), there have been 73 CHIKV-related deaths in Colombia, seven of them involving coinfection with DENV.[24]

Initial phylogenetic analyses of CHIKV revealed three main lineages or clades: the west African and east/central/South Africa (ECSA) enzootic lineages, and the Asian endemic/epidemic lineage.[26] However, beginning in 2005, an ECSA lineage strain spread into the Indian Ocean basin from Kenya and also to India, giving rise to the new epidemic Indian Ocean lineage (IOL) characterized by human-mosquito transmission. This emergence was quickly accompanied by an E1-A226V substitution that dramatically enhanced transmission by *Aedes albopictus*.[4] Subsequently, this IOL evolved into at least four sublineages, each accompanied by further adaptive evolution for this new vector involving E2 substitutions.[27] However, epistatic interactions within the E1 protein restrict the expression of the E1-A226V mutation in the Asian lineage, suggesting that the

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strains of this lineage now circulating in the Americas will be principally transmitted by *A. aegypti*.[28] The CHIKV landscape in the Americas was further complicated by the arrival of an ECSA strain directly from Africa in 2014.[29] The potential for efficient *A. albopictus* transmission by this strain remains unknown.

The first CHIKF cases in Colombia were detected between June and August 2014. By September 2014, the INS reported 10 autochthonous cases diagnosed clinically in three municipalities of Bolivar Department (on the Atlantic coast), including the major tourist destination of Cartagena (Figure 1).[30] The presence of dengue fever (DENF) in many areas of Colombia probably masked the first CHIKF cases, although polyarthralgia and rash raised suspicion of CHIKF.[31] Known CHIKV vectors are present nearly throughout Colombia; *A. aegypti* is abundant in most of regions, and *A. albopictus* also occurs in the Amazon, Andean, and Pacific regions, but not in the Atlantic, where the samples of this study came from (Figure 1).[32]

To determine the source and patterns of initial spread of CHIKV in Colombia, we attempted to isolate, amplify via reverse transcription polymerase chain reaction (RT-PCR), and genetically analyze CHIKV from over 30 sera obtained from febrile patients from the northwestern area of the country as part of dengue surveillance. To determine the main CHIKV lineages circulating and to identify vector-adaptive mutations described previously, we sequenced the E1 and E2 glycoprotein genes from all strains detected to help determine the public health risks to predict the future spread of the outbreak. Viral sequences of 14 CHIKV isolates analyzed from Sincelejo (Sucre Department, Colombia) were members of the Asian lineage and, consistent with previous evidence, none

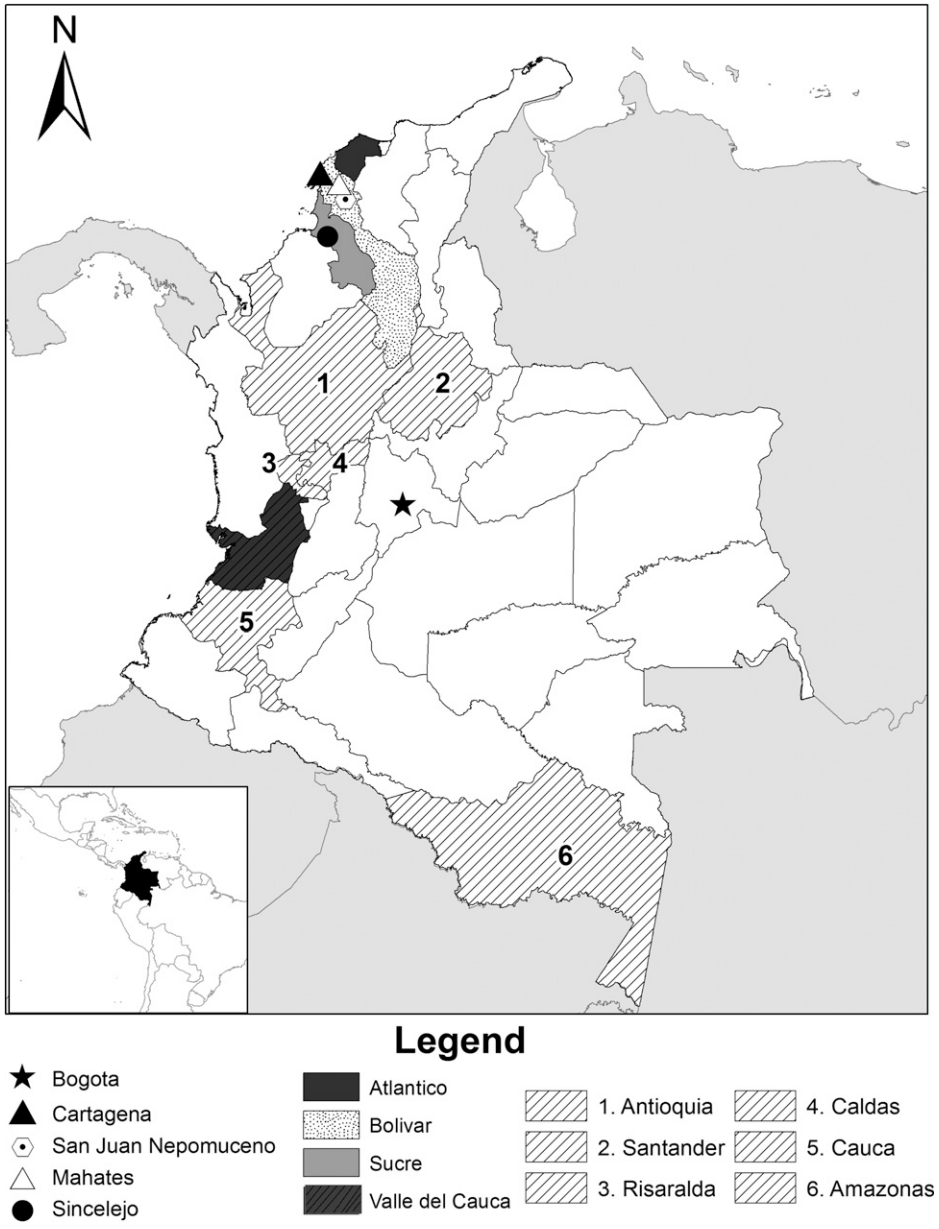

FIGURE 1. Geographic location of Sincelejo city (Sucre Province, Colombia). This map shows the origins of the human samples used in this study, the location of first chikungunya fever cases in Colombia, and provinces where *Aedes albopictus* is present in Colombia.

had known *A. albopictus*–adaptive mutations,[9] consistent with prior predictions for the Asian lineage.[28]

MATERIALS AND METHODS

Study subjects. Thirty-two acute sera from patients with an undifferentiated febrile syndrome compatible with DENF were collected between October 18, 2014, and January 15, 2015, from Sincelejo (Sucre Department, Figure 1). Sucre ranks third or fourth among Colombian provinces in the number of suspected CHIKV cases, with over 20,000 between 2014 and 2015, about a 10th of the national total.[33]

Ethics statement. The study was funded by a grant from Colciencias (code 112954531519, "Eco-epidemiological surveillance system of Dengue Virus in the department of Sucre"). In compliance with the international regulations, the study protocol was reviewed and approved by the University of Sucre Bioethics Committee. Written consent was obtained from patients 18 years of age and older; for patients younger than 18 years, written consent was obtained from a parent or legal guardian.

Cell culture and virus isolation. Initially, each serum (100 μL) from Sincelejo was inoculated into two 12.5-cm^2 plastic flasks containing cultures of Vero E6 (African Green Monkey, ATCC CCL-81, Manassas, VA) and C6/36 (*A. albopictus* mosquito, ATCC CRL-1660) cells. Cell lines were propagated at 37°C (Vero) or 28°C (C6/36) in 5% CO_2 environment. Dulbecco's minimal essential medium containing 2% (v/v) fetal bovine serum (FBS), sodium pyruvate (1 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL) was the maintenance medium. Cultures that developed cytopathic effects (CPEs) were tested using an indirect immunofluorescence antibody test (IFAT). On observation of CPE, or 6 days after inoculation if none was observed, cells were removed and placed on 12-well glass spot-slides for microscopic examination after drying and fixation in cold acetone. A standard indirect immunofluorescence assay (IFA) was performed using hyperimmune mouse ascitic fluid (HMAF) against a panel of local arbovirus (DENV-1–4, SLEV, CHIKV, YFV, MAYV, VEEV, and WNV), followed by the addition of fluorescein-conjugated goat antimouse IgG, as previously described.[34,35] The IFA tests were performed by using HMAF at dilutions of 1:10 and 1:20 and a commercial fluorescein isothiocyanate–conjugated goat antimouse IgG (Sigma, St. Louis, MO). Uninfected cells tested with the HMAF and the IgG antimouse antibody were used as negative controls.

RNA extraction and RT-PCR amplification and sequencing. RNA was extracted from the supernatants of infected C6/36 cells using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocols, and was used to synthesize complementary DNA using the Super Script III First Strand kit (Invitrogen, Waltham, MA), and random hexamers following the manufacturer's protocol. The Phusion® High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA) was then used to generate three overlapping PCR amplicons from E1/E2 regions, which were then purified using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA). Primer sequences are available from the authors on request.

The three sets of primers were used to amplify three overlapping fragments of approximately 600–700 nucleotides (nt). This integrated amplicon spanned 1,679 nt and allowed for the analysis of approximately 57% of E2, 100% of 6K, and 59% of E1. These segments were carefully chosen to analyze the most significant adaptive mutations that have impacted CHIKV evolution during historic outbreaks.[27,28] We used a CHIKV RNA obtained from Vero cells infected with a genetically distinct viral isolate from Puerto Rico as a positive control. As negative controls, we used RNase-free water for the RT and an aliquot of this RT as a negative control for the PCR.

Sequences were generated using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The products of the sequencing reactions were purified using a 96-well plate format (Performa DTR v3 96-well short plates; EdgeBio, Gaithersburg, MD), and then analyzed on a 3500 Genetic Analyzer (Applied Biosystems). Sequence products were analyzed and edited using Sequencher 5.3 for Sanger Sequencing Analysis (Gene codes Corporation, Ann Arbor, MI). The sequences were deposited in the GenBank database under accession nos. KU518330–KU518343.

Phylogenetic analysis. We used MEGA 6.06 (Molecular Evolutionary Genetics Analysis, Arizona State University, Tempe, AZ) for most sequence analyses, and the MrBayes 3.2.2 (available at http://mrbayes.sourceforge.net/) for Bayesian studies.[36] Partial nucleotide sequences of E2/6K/E1 genes (genome positions 9,064–10,742) of 14 Colombian isolates and 49 homologous sequences from GenBank were aligned using the Muscle algorithm (available at http://www.drive5.com/muscle/) implemented in MEGA. Strains belonging to all previously described CHIKV lineages (west African as outgroup, ECSA, IOL, and Asian) were included to maximize context.

The maximum likelihood (ML) method used with the Kimura-2 parameter model[37] and a discrete Gamma distribution was selected to model evolutionary rate differences among sites (3 categories [+ G, parameter = 0.30]). The E2/E1 sequences were analyzed using 63 CHIKV strains (49 from GenBank and 14 unique Colombian strains chosen from the 32 we amplified and sequenced, excluding identical sequences). We used 1,000 bootstrap replicates to determine the robustness of groupings.

The Bayesian analysis for the E2/E1 gene segments was run with the same 63 sequences, 3 million generations and using the K2 + G model of nucleotide substitution and gamma distributed rate variation in the MrBayes 3.2.2.

To test for adaptive evolution in the sequences included in the dataset, we used the Fast Unconstrained Bayesian Approximation (FUBAR),[38] a program that uses a Markov chain Monte Carlo routine to estimate the synonymous (dS) and nonsynonymous (dN) substitutions rates and the posterior probabilities of selection pressures at individual codons in a Bayesian context.

RESULTS

Human subjects. The 32 patients from Sincelejo (Sucre Department) represented a very wide range of ages from 5 days to over 40 years old. Most patients showed the same common features described in previous CHIKF outbreaks including the Colombian epidemic.[39] Of the 32 patients, 26 experienced fever (81%), 23 rash (72%), 21 arthralgia (65%), 19 myalgia (59%), and 11 headache (34%). These results were similar to others previously described in the literature.[4,18,21]

Virus isolation and identification by IFA. All 32 sera produced CPE on Vero and C6/36 cells, and CHIKV was

identified by IFA using a set of antibodies for arboviruses from different families (*Bunyaviridae*, *Flaviviridae*, *Togaviridae*, *Orthomyxoviridae*, *Reoviridae*, and *Rhabdoviridae*; data not shown). The VectorTest®, a rapid assay for CHIKV antigen detection (Vector Test Systems, Thousand Oaks, CA), was also performed on culture fluid from the IFAT-positive cultures to confirm these results (data not shown).

RT-PCR products and sequence analysis. The 32 samples were confirmed as CHIKV positive by RT-PCR using three sets of primers covering the E2/6K/E1 genes (not shown). Sanger sequences of the three overlapping amplicons were concatenated to yield 1,679 nt located at genome positions 9,064–10,742. The Colombian sequences varied only by 0–2 nt (0.00–0.12%) in this genome region, and differed from other American strains by up to 3 nt (0.18%).

Phylogenies were estimated with maximum likelihood Bayesian and neighbor-joining analyses. Only minor differences were observed in terminal groupings with different methods. ML phylogenies are provided in Figure 2 (other analyses are provided as Supplemental Figures 1 and 2).

FIGURE 2. Phylogenetic analysis of 63 chikungunya strains, including 14 Colombian strains and 49 homologous sequences obtained from the GenBank library and representing the major lineages: east/central/South Africa (ECSA) (gave rise in 2004 to the Indian Ocean lineage) and Asian. This analysis was rooted with west African lineage as an outgroup. The evolutionary history was inferred by the maximum likelihood method, using 1,000 replications of bootstrap and based on the Kimura-2 parameter with gamma distributed rate variation among sites (K2 + G). The analysis was based on 1,679 nucleotides representing genome positions 9,064–10,742 that includes 57% of E2 and 59% of the E1 gene as well as all of 6K/TF. Colombian isolates sequenced in this study are indicated by black triangles. The recently described Brazilian ECSA lineage is labeled with a black dot. Isolates from Kenya and Reunion Island that gave rise to the Indian Ocean lineage are labeled with black diamonds. All main lineages had a branch support > 70%. Virus strain information is found in Supplemental Table 1.

According to the ML analysis, the Sincelejo Colombia samples were closely related to each other in a paraphyletic group with other Caribbean, Central and South American sequences within the Asian lineage (Figure 2). It was not surprising to find such genetic similarity in the Sincelejo strains because all were obtained within the same municipality during a short period of time (October 2014–January 2015).

The closest viral strains to our Colombian isolates were from the 2014 outbreak in the Virgin Islands, Saint Lucia, Mexico, Puerto Rico, and Brazil. The clade corresponding to the American outbreak was well supported (100% bootstrap), and closely related to other sequences from previous isolates from Asia (KJ689453 Micronesia 2013, AB860301 Philippines 2013, KC488650 Philippines 2012, and KF318729 China 2012), consistent with a point-source introduction from one of the later regions.

Cocirculation of two different CHIKV lineages has been reported in Brazil since 2014 (Figure 2, accession nos. KP164567 and KP164572, the latter labeled with a black circle).[29] However, we only detected the Asian lineage in our samples. When we examined our sequences for *A. albopictus*–adaptive mutations identified previously (Table 1),[2,27,41,48,51] none was found. However, when we examined the E1 and E2 acid-sensitive domains involved in *A. albopictus*–adaptive evolution, two substitutions were found: E2-L248F and E1-K211E, present in all Colombian strains. E2-L248F was described in 2012 CHIKV strains from the Philippines that are part of the B3 cosmopolitan Asian CHIKV clade derived from a common ancestor dated to 2003.[45] All Philippines CHIKV strains isolated from the nationwide 2012 outbreaks within this clade, as well as other CHIKV isolates from China (2012), Micronesia (2013), and the Caribbean (2013–2014) have E2-248F. On the basis of its proximity to known vector-adaptive substitutions, E2-L248F deserves further study.

E2 L248F and E1-K211E were already present in some Asian strains before CHIKV arrival to the Americas, so it is not likely to be associated with adaptive evolution in the Americas. However, we did identify two amino acid substitutions that are unique to all American CHIKV sequences in our dataset: E2-V368A and 6K-L20M (Table 1). In addition, we also found 4 dN substitutions in one Colombian sequence each: E2-H351R, 6K-A47S, 6K- F48L, and E1-L133F, which were present in strains SI190, SI246, SI256, and SI258, respectively. The E2-V371L substitution, shared by recent Asian lineage CHIKV strains but not mapped to *A. albopictus*–adaptive envelope protein domains was also identified in all of our Colombian strains (Table 1).

The results of the analysis of adaptive evolution run in FUBAR showed that in most codons the estimated dS substitution rate was higher than the dN rate (dN < dS) with 285 of 559 codons exhibiting evidence of negative (purifying) selection with posterior probabilities > 0.9. Only one site, codon 6K-47, had a significantly higher dN rate (dN/dS = 5.74; Table 1), suggesting positive selection with a posterior probability of 0.92 and a Bayes factor of 44.8 (not shown). This substitution therefore deserves further study.

DISCUSSION

According to our results, the Colombian CHIKV strains, like others found in the Caribbean and South America,[3] belong to the Asian lineage that lacks *A. albopictus*–adaptive mutations due to the epistatic constraint imposed by E1-T98 (Table 1).[28] Nevertheless, tourism and other aspects of globalization may still intervene to bring the IOL to the Americas where *A. albopictus* is widespread and abundant.[52] In the meantime, possible implications of the unique E2-L248F substitutions found in Colombia, as well as the E1-K211E we observed (Table 1), the latter which corresponds to the IOL sublineage 2,[27] should be studied using reverse

TABLE 1
CHIKV amino acid substitutions of interest and/or found in Colombian strains*

Amino acid substitution	CHIKV lineage where detected	Phenotype	dN/dS† (Pr [dN > dS])
E2-R198Q	IOL-SL3[27,40]	Enhanced infection of *Aedes albopictus* (synergistic with E3-18F in background E1-226V)	0.09 (0.03)
E2-L210Q	IOL-SL4[27,41,42]	Enhanced infection of *A. albopictus*	0.11 (0.03)
E2-V222I	IOL-SL2[27,40]	No increase in ability to infect *A. albopictus*	0.56 (0.26)
E2-K233E/Q	IOL-SL2/artificial[27]	Enhanced infection of *A. albopictus*	0.11 (0.04)
E2-K234E	Artificial[27]	Enhanced infection of *A. albopictus*	0.08 (0.01)
E2-L248Q/F	Artificial (Q)[27] Asian genotype (F)	None described	0.64 (0.34)
E2-K252Q	IOL-SL1[27,43]	Enhanced infection of *A. albopictus* (second-step adaptive mutation)	0.42 (0.23)
E2-H351R	Colombia SI190	None described	0.88 (0.46)
E2-V368A	Asian genotype (American branch)[44]	None described	0.40 (0.19)*
E2-V371L	Asian[45]	None described	0.64 (0.35)
6K-L20M	Asian genotype (American branch)[44]	None described	0.47 (0.19)*
6K-A47T/S	Asian genotype (T) Colombia SI246 (S)	None described	5.74 (0.92)
6K-F48L	Colombia SI256	None described	0.91 (0.45)
6K-A56V	Brazil AMA2798	None described	0.88 (0.46)
E1-A98T	Asian genotype[28]	Epistatic constraint on E1-A226V	0.38 (0.15)
E1-L133F	Colombia SI258	None described	0.40 (0.19)
E1-K211N/E	IOL-SL2 (N)[27,46] Asian (E)[47]	No increased ability to infect *A. albopictus*	1.69 (0.66)
E1-A226V	IOL[48–50]	Initial mutation for enhanced infection of *A. albopictus*	0.45 (0.22)

CHIKV = chikungunya virus; IOL = Indian Ocean lineage.
*Amino acid substitutions unique to all American CHIKV sequences.
†dN/dS = nonsynonymous to synonymous difference ratio; Pr (dN/dS) = posterior mean of the site-level probability of positive selection.

genetic approaches to determine whether they represent vector-adaptive evolution of CHIKV in the Americas. Complete genomic sequences from different regions and times in the Colombian territory are also needed to further define the origin of the index case and the port of entry for the Colombian outbreak.

Regarding the origin of the current CHIKF Colombian outbreak, our results support a recent analysis of the transcontinental movement of the Asian genotype.[53] The Colombian strains showed a very close identity between two isolates from Micronesia (accession nos. KJ451622 and KJ451623) and a CHIKV isolate from Virgin Islands (accession no. KJ451624, the same strain used in our analysis); these strains differ by only 18–19 nt.[53] Lanciotti and Valadere also showed that two CHIKV isolates from Philippines, 2012 (accession no. KC488650) and China-Zhejiang, 2012 (accession no. KF318729), both strains included in our analysis, are strongly supported sisters in this Asian subclade, and concluded that the CHIKV strain from the Caribbean could have originated from strains recently circulating in China, the Philippines, or Micronesia.[53]

Concerning the two amino acid substitutions that are unique to all American CHIKV sequences in our dataset, E2-V368A and 6K-L20M (Table 1), they had been recently described by a Mexican group in all the American strains that arrived to this country and belonged to the Asian genotype that was also detected in Virgin Islands.[44]

On the other hand, the search for positive selection identified amino acid position 6K-47 as a likely candidate. In this polymorphic site, most sequences of west African and ECSA genotypes exhibit an A (alanine) that changes to T (threonine) in the branch leading to the Asian genotype (Figure 2); however, it is valine in Ivory Coast 1993, isoleucine in India 1973 and serine in Si246 Colombia 2014 (not shown). Whether this variability represents adaptation to different host environments or it is just the result of random genetic drift should be tested in larger datasets or in a reverse genetic system.

During 2014, 106,763 suspected CHIKF cases occurred in Colombia, the vast majority unconfirmed by laboratory diagnosis.[54] There are no specific treatments available for CHIKF; accurate diagnosis is important to improve understanding of CHIKF epidemiology and to rule out DENV infection, which is more often life threatening. Colombia has reported 73 deaths attributed to CHIKV infection since the beginning of the outbreak in 2014, for a case-fatality rate of 0.016%. This rate is lower than those from previous outbreaks in Reunion Island and India, where rates as high as 0.1% and 4.9%, respectively, were reported.[55,56] Nevertheless, the Colombian burden of CHIKF in terms of disability-adjusted life years and the cost of treatment should be estimated along with studies throughout the Americas to determine if the Asian CHIKV lineage differs from the IOL in virulence.[57–60]

Infections in Colombia with CHIKV could also be influenced by the presence of other pathogens. The alphaviruses MAYV and CHIKV, closely related in the Semliki Forest complex, could cross-protect to some degree.[13,29] The former virus circulates enzootically in forested regions throughout the Amazon Basin, probably including Colombia. Coinfection of CHIKV with DENV, MAYV, and ZIKV in Colombia also deserves surveillance and further study.[24] Other unrecognized viral and bacterial diseases that typically remain undiagnosed (OROV, and *Leptospira* and *Rickettsia* bacteria, among others), could also cause coinfections during major CHIKF epidemics with altered pathogenesis.

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