Novel Molecular Signatures of Chikungunya Virus in Puerto Rico

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Objective: The chikungunya virus (CHIKV) is an arthropod-borne *Alphavirus* transmitted to humans, primarily via *Aedes* mosquitoes. In Puerto Rico, the first locally transmitted infections were reported in May 2014. Although the virus strain in Puerto Rico is related to the Asian/American lineage, many autochthonous cases have emerged recently in the Caribbean region (including Puerto Rico), raising the question of how CHIKV will evolve and adapt in PR. Taking the role of the envelope glycoprotein (E1) in viral evolution and transmission as a given, we analyzed the genetic diversity of the Puerto Rican (PR) E1 gene sequences and the phylogenetic relationships between those sequences and sequences from other parts of the world.

Materials and Methods: To analyze the overall genetic variation, 772 nucleotide sequences of the E1 gene were obtained from the Virus Pathogen Resource (ViPR). A maximum-likelihood analysis was performed to determine the phylogenetic relationships between the PR sequences and sequences from 48 countries around the world.

Results: The analysis of the E1 gene identified variations at 4 nucleotide positions, which included synonymous and nonsynonymous mutations. In addition, 2 nonsynonymous amino acid changes, T207M and S120L, were unique to the PR CHIKV sequences, and T155I was found to be shared by the PR (n = 3) and Colombia (n = 1) strains.

Conclusion: Our analysis of the E1 gene revealed new molecular signatures in PR CHIKV sequences, 1 of which was also found in Colombia. While studies have shown possible relationships between T98A and A226V with viral adaptation and spread, no other PR sequence contained these vector-adaptive mutations. Thus, constant monitoring of the virus remains an essential factor in the establishment of control strategies to track viral spread. [*P R Health Sci J 2019;38:27-32*]

Key words: Chikungunya virus, Evolution, Envelope glycoprotein 1

The chikungunya virus (CHIKV) is an arthropod-borne virus (arbovirus) that is transmitted to humans primarily via the bite of an infected *Aedes* species mosquito (1, 2). The virus is a member of the genus *Alphavirus*; many alphaviruses are important human pathogens (3, 4). The CHIKV infection is characterized by acute febrile illness and severe polyarthralgia that can persist for a long time (5, 6). The first reported case was in the early 1950s, on the border of Tanzania and Mozambique (the Makonde Plateau) (7). In the Caribbean, the first reported case was in Saint Martin (2013), which is a part of the French Antilles (8). Immediately, further cases were identified in other countries in that area (9, 10). The principal vector in the region is *Aedes aegypti*. However, *Aedes albopictus* is also present in some Caribbean countries (11, 12). In May 2014, the first laboratory-positive chikungunya case was reported in Puerto Rico (13). The CHIKV fever expanded rapidly, and 4,465 confirmed and 30,247 suspected cases were reported during that year (14, 15). The expansion of the virus that was observed during 2014 and 2015 was typical of a new pathogen in an immunologically naïve population (16). Currently, the number of new cases has dropped in the last 2 years, due, in part, to increased efforts by the government to eradicate the vector (*Aedes aegypti*), the use of repellent, and the implementation of educational strategies (13, 17).

The CHIKV genome is composed of 11.8 kb of single-stranded, positive-sense RNA. It codes for 4 non-structural and 5 structural proteins and includes the E1 envelope glycoprotein (18). The gene that encodes this protein is composed of approximately 1,300 nucleotides. The E1 glycoprotein is a significant participant in the virion attachment to the host cell and in the fusion of

the virus membrane with the host endosome membrane (19). CHIKV has 3 main genotypes— Asian, East/Central/South African, and West African—which can be distinguished by differences in the E1 glycoprotein gene (20). The CHIKV in Puerto Rico is related to the Asian/Pacific and American strains; however, many autochthonous cases have emerged recently in the Caribbean and South America (21, 22). Single mutations in the E1 gene can potentiate the capacity of the vector to transmit the CHIKV (5, 23). The A226V mutation promotes increased infectivity of the midgut, dissemination to the salivary glands, and transmission of the virus by *Aedes albopictus* mosquitoes (23–25). The CHIKV with the A226V mutation was responsible for several outbreaks, worldwide (26–29).

Although the CHIKV in Puerto Rico is related to the Asian/ American lineage, many autochthonous cases have emerged recently in the Caribbean region, raising the question of how CHIKV will evolve and adapt in Puerto Rico. Our current study wanted to evaluate the hypothesis that during the CHIKV epidemic in Puerto Rico, unique sequences emerged. Taking the role of the envelope glycoprotein (E1) in viral evolution and transmission as a given, we analyzed the E1 envelope glycoprotein partial nucleotide sequences from Puerto Rico, which were available in the Virus Pathogen Resource (ViPR). This analysis can help us to identify the possible autochthonous cases, which could be the result of a mutation that enhances viral fitness and replication capacity in the mosquito and human cells.

Materials and Methods

Ethics Approval

The protocol underwent review at the Institutional Review Board of the Ponce Medical School Foundation and was determined to be exempt from the federal policy for the protection of human subjects, as the data are publicly available from the ViPR, a National Institute of Allergy and Infectious Diseases (NIAID)–sponsored database and analysis resource that supports the research of viral pathogens in categories A, B, and C.

Sequence data and Analysis

At the time of the study, a total of 2,055 CHIKV E1 gene nucleotide sequences were obtained from the ViPR database. Of these sequences, a total of 772 CHIKV E1 gene sequences from 50 countries, including 42 sequences from Puerto Rico, were included in the analysis (30). In choosing non-PR sequences, only those that covered the same region and 696-nucleotide length as the PR sequences were selected. The sequences included in the analysis started at position 10096 and ended at position 10792, relative to the CHIKV isolate MY/06/37348 (Malaysia, 2006) reference sequence (accession number: FN295483) (31, 32). This fragment extends from position 142 to position 837 of the E1 glycoprotein gene sequence from the MY/06/37348 reference sequence. Positions with less than 95% site coverage were eliminated from the analysis. Nucleotide sequences were input in FASTA, which format was also used for

the output data. Sequence edition and sorting were performed using BioEdit (v.7.2.5), a sequence alignment editor (33). The multiple sequence alignment software MAFFT (v. 7.304b) was used for similarity-based methods, using the default penalties in which the software automatically selected the appropriate strategy from L-INS-i, FFT-NS-i, or FFT-NS-2, according to data size (30). Phylogenetic relationships were analyzed with MEGA software (v.6), using the maximum-likelihood method (34). The Tamura–Nei model was used to perform the analysis (35). To construct the initial tree for the heuristic search, we applied the neighbor-joining method to a pairwise distance matrix that had been computed using the maximum composite likelihood approach (34). Bootstrap analysis was performed on 50 replicates, and those with bootstrap values greater than 70 were indicated at the nodes. Nodes where no value was reported were not supported at this level. Evolutionary rate differences among sites were obtained using a discrete gamma distribution (5 categories [+G, parameter = 0.4311]). The CHIKV genotyping details were obtained using the Dengue, Zika & Chikungunya Viruses Typing Tool (v. 1.0, 2015), a tool designed to identify viral genotypes using BLAST and phylogenetic methods (36). The software tool Highlighter was used to highlight mismatches, matches, transition and transversion mutations, and silent and non-silent mutations in the nucleotide sequence alignments (37).

Statistical analysis

The chi-square test of independence was used for comparative statistical analyses of nucleotide and amino acid sequence data to identify correlations between sequence variations and distribution. A p-value of less than or equal to 0.05 was considered statistically significant.

Availability of data

The sequences detailed in this report are available from GenBank. The information regarding the accession numbers will be provided upon request.

Results

The CHIKV envelope sequence analyzed included 696 nucleotides of the total 1317 nucleotides from the complete E1 gene (Figure 1, panel A). The deduced amino acid sequence of the E1 glycoprotein region under study comprised domains I and II (Figure 1, panel B). The sequence analysis of the E1 gene using the Dengue, Zika & Chikungunya Virus Typing Tool revealed that the sequences from Puerto Rico belong to the Asian genotype, Caribbean clade (data not shown). PR sequences (bootstrap value = 99%) are similar to Asian/Pacific (Thailand, Malaysia, Singapore, Indonesia, and Philippines, among others), Caribbean (the Dominican Republic, Haiti, the British Virgin Islands, Martinique, and Saint Martin), Central American (Mexico, Nicaragua, and Panama), and South American (Colombia, Brazil, and French Guiana) sequences,
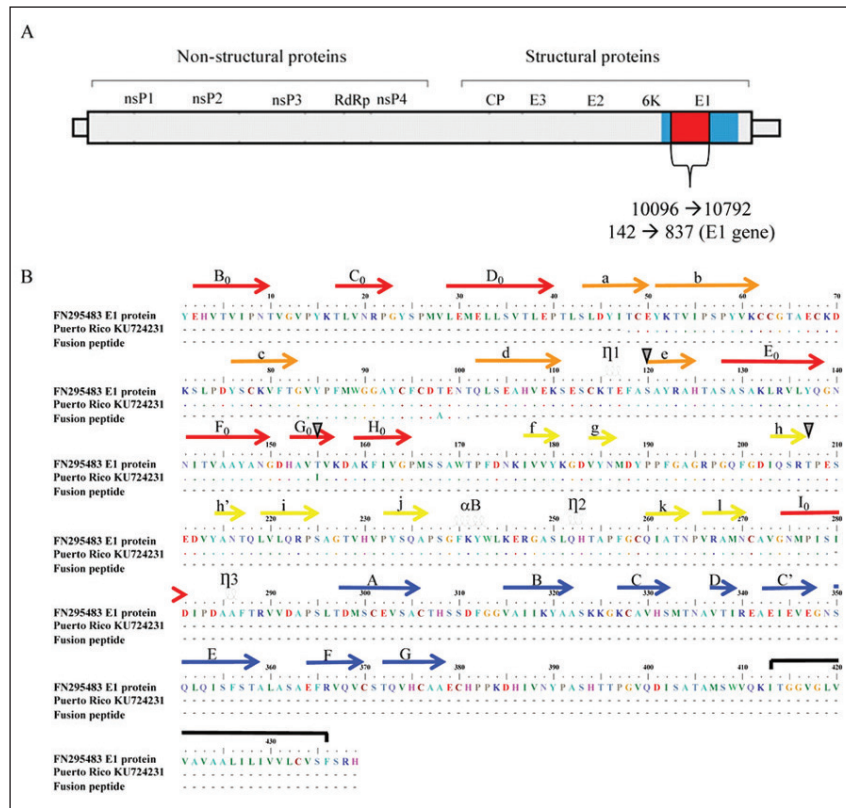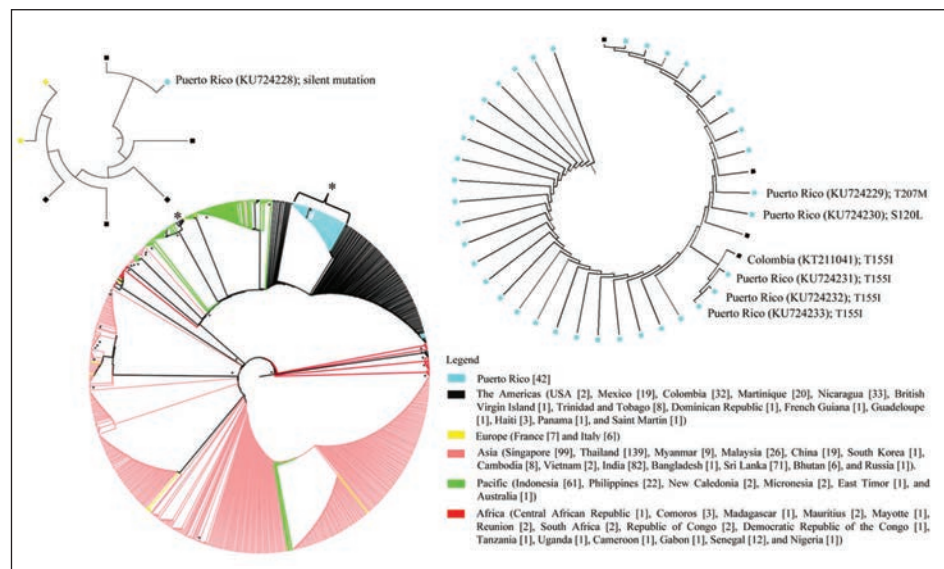
Figure 1. A schematic representation of the CHIKV genome and the E1 glycoprotein. Panel A: The position of the CHIKV full-length E1 gene and the 696 nucleotides analyzed in this study are denoted in reference to the complete genome. Panel B: The top line represents the CHIKV isolate MY/06/37348 (Malaysia, 2006) reference sequence FN295483. The second line is the PR sequence KU724231, which includes the mutation T155I as an example. The third line represents the E1 fusion peptide. The domains are identified according to Roussel et al. (52). The arrows represent the β sheets within each domain. Red arrows indicate E1 domain I; yellow arrows indicate domain II; and blue arrows indicate domain III. Orange arrows indicate domain II regions that are located within domain I. The loops indicate the α helices. The black line shows the transmembrane region. Sites of unique mutations detected in the sequences from PR are denoted with a grey triangle.

Figure 2. The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model. The bootstrap consensus tree was inferred from 50 replicates. The study involved 772 nucleotide sequences. Evolutionary analyses were conducted in MEGA v.6. The colors indicate world region and the bullets (•) indicate the branch with a bootstrap value ≥ 0.70. The number of sequences per country is shown in brackets. The asterisks (*) denote the region in the phylogenetic tree that contains the PR sequences with the mutations. These regions are provided as inserts for better visualization of the relationship of PR sequences to nearby sequences.
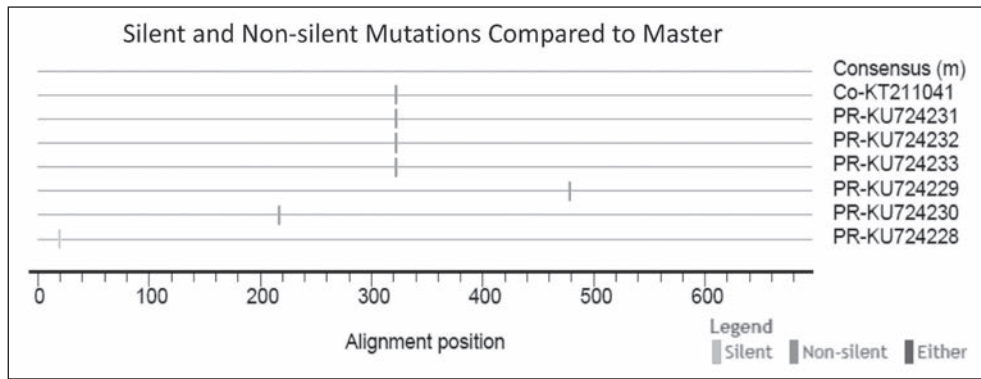

suggesting a single introduction. Phylogenetic analysis revealed that the PR sequences clustered with countries in the Americas (Figure 2). The analysis of the E1 envelope glycoprotein gene identified variations at 4 nucleotides positions, compared to the to the consensus sequence from the Americas (Figure 3). Six PR sequences were different from the consensus sequence. Changes included both synonymous (n = 1) and nonsynonymous mutations (n = 5) (Figure 3). The nonsynonymous mutations were located within domains I and II of the E1 glycoprotein (Figure 1, panel B). Two nonsynonymous mutations, S120L and T207M, were unique to PR sequences. One nonsynonymous mutation, T155I, was found to be unique to Puerto Rico (n = 3) and Colombia (CO; n = 1). The PR sequences with the mutations clustered together with sequences from the Americas and the PR sequences that shared the mutation with CO were in the same node (Figure 2). We ran a chi-square analysis on the distribution of the changes at S120L, T155I, and T207M and compared PR sequences (group 1, n = 42) to sequences from around the world (group 2, n = 730). The analysis revealed that the changes at these positions had a statistically significant non-random distribution between the specified groups (Table 1).

Figure 3. Highlighter plot traces mismatched mutations as compared to the Asian/American consensus sequence. Silent (n=1) and non-silent mutations (n=5) are in 4 positions of the gene. One of the nonsynonymous mutations was found to be unique to the PR (n = 3) and CO (n = 1) strains.

Table 1. Positions within the multiple sequence alignment that significantly differ from the expected (random) distribution

Position*	Chi-square†	*p*-value	Degrees of freedom	Residue diversity
10313 (S120L)	17.416	5.80E-04	3	Group 1 (41 C, **1 T**) Group 2 (730 C)
10418 (T155I)	37.835	3.06E-08	3	Group 1 (39 C, **3 T**) Group 2 (729 C, **1 T**)
10574 (T207M)	17.416	5.80E-04	3	Group 1 (41 C, **1 T**) Group 2 (730 C)

*The positions are in relation to the CHIKV isolate MY/06/37348 (Malaysia, 2006) reference sequence (accession number: FN295483), which start at position 10096 and ends at position 10792. †The chi-square test of independence was performed with the Metadata-driven Comparative Analysis Tool for Sequences (meta-CATS) (51). Changes from the consensus sequences are in bold.

Discussion

The E1 glycoprotein is a fusion peptide and is one of the significant determinants of the evolutionary adaptation and dissemination of CHIKV (22, 38, 39). Recent studies have shown a possible relationship between nonsynonymous mutations and worldwide virus adaptation and spread. In the 2005 Indian Ocean outbreak (Reunion Island), *Aedes albopictus* was the primary vector responsible for the transmission of CHIKV (40–42). Molecular analysis identified an alanine to valine mutation at position 226 in the E1 glycoprotein (A226V). This E1-A226V mutation influenced the fitness of the virus in Aedes albopictus and affected the vector specificity and the duration of the epidemic (23, 28, 31). This mutation was related to the modulation of cholesterol in the cellular membrane. In the Semliki Forest virus (SFV), the fusion and exit of the virus is cholesterol-dependent (23, 43).

The mutation E1-A226V, which contributes to increased virus transmissibility by *Aedes albopictus*, was not detected in the E1 gene of the PR sequences included in this study. Its absence may in part be due to the CHIKV vector in Puerto Rico, *Aedes aegypti*. While the E1-A226V mutation has not been shown to be associated with *Aedes aegypti*, a single mutation can modify viral infectivity for a specific vector and expand the epidemic in a region that lacks the typical vector (23, 44). CHIKV sequence analysis in India showed 2 single nucleotide mutations that increased the virus's capacity to be transmitted by the *Aedes aegypti* mosquito (45). Even though the *Aedes aegypti* is the principal vector on the island, *Aedes albopictus* mosquitoes can also be found, albeit with less frequency (46). Thus, constant monitoring of the vector and virus is essential to assess the presence of mutations that may affect CHIKV transmissibility and pathogenesis.

The finding that 2 amino acid substitutions were unique to PR CHIKV sequences and that 1 mutation was detected only in PR (n = 3) and CO sequences (n = 1) (compared to sequences from around the world) suggests that autochthonous cases are emerging in Puerto Rico. Previous studies have shown that single amino acid changes in the E1 glycoprotein can affect how the virus fuses with the target cells (47). While at this time the implication of these mutations remains unclear, the 3 mutations are changes from small, non-polar amino acids to hydrophobic amino acids, similar to mutation A226V. Whether these mutations have the potential to alter E1's biological activity, including fusogenicity, remains to be elucidated. Additional epidemiological information will help us to better understand the role of these amino acid substitutions in CHIKV pathogenesis. Since these mutations are unique to the PR CHIKV sequences, we suggest that these mutations may represent a signature from the island. However, additional research regarding CHIKV evolution in Puerto Rico is necessary to confirm this. In addition, the virus gene flow between Puerto Rico and CO should be monitored to understand the role of CHIKV mutations in disease severity and chronicity in the 2 countries.

Recent data suggest that around 25% of the blood donors in Puerto Rico acquired CHIKV during the 2014 epidemic, meaning that approximately 800,000 individuals in this population were infected (16). Nevertheless, the number of new cases appears to have dropped in the last few years, possibly as a result of the establishment of strategies for vector control (*Aedes aegypti*) and an increased awareness of mosquito-borne diseases. However, according to ArboNET, in 2016, 100% of the transmitted cases in the United States and its territories occurred in Puerto Rico (48). Puerto Rico is a United States Commonwealth, and because of the high level of travel between

Puerto Rico and the United States (49), how the virus evolves and adapts to new hosts remains a crucial issue under study. After the first case was identified, the expansion of CHIKV in Puerto Rico decreased dramatically (50), likely resulting from long-lasting immunity to the virus. However, the viral mutation may lead to the re-emergence of chikungunya in Puerto Rico, and thus it is essential to carefully monitor autochthonous transmission for the emergence of mutations that might alter CHIKV transmissibility and pathogenesis.

Resumen

Objetivo: El virus chikungunya (CHIKV) es un *Alphavirus* transmitido a los humanos principalmente a través de los mosquitos *Aedes*. En Puerto Rico, las primeras infecciones transmitidas localmente se notificaron en mayo de 2014. Aunque el virus en Puerto Rico está relacionado con el linaje asiático/americano, muchos casos autóctonos han surgido recientemente en la región del Caribe, lo que plantea la pregunta: ¿Cómo CHIKV evolucionará y se adaptará en Puerto Rico? Dado el papel de la glicoproteína de la envoltura (E1) en la evolución y transmisión del virus, analizamos la diversidad genética y las relaciones filogenéticas entre las secuencias del gen E1 en Puerto Rico y otras partes del mundo. Materiales y métodos: Para analizar la variación genética, se obtuvieron 772 secuencias de nucleótidos del gen E1 de la base de datos "Virus Pathogen Resource (ViPR)". Se realizó un análisis de máxima verosimilitud para observar las relaciones filogenéticas entre las secuencias de Puerto Rico y las secuencias de cuarenta y ocho países alrededor del mundo. Resultados: Análisis del gen E1 identificó variantes en cuatro posiciones de nucleótidos, que incluyen mutaciones sinónimas y no sinónimas. Además, dos cambios de aminoácidos no sinónimos, T207M y S120L, son únicos en las secuencias de PR, y se encontró que T155I se compartió entre las cepas PR (n = 3) y Colombia (n = 1). Conclusión: Nuestro análisis del gen E1 reveló nuevas marcas moleculares en las secuencias de Puerto Rico, una de las cuales también se encontró en Colombia. Si bien los estudios han demostrado posibles relaciones entre T98A y A226V con la adaptación y propagación viral, ninguna secuencia de Puerto Rico contenía estas mutaciones adaptativas a los vectores. Por lo tanto, el monitoreo constante del virus sigue siendo un factor importante en el establecimiento de estrategias de control para monitorear la propagación viral.

Acknowledgments

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