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Research paper

Two distinct lineages of chikungunya virus cocirculated in Aruba during the 2014–2015 epidemic

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ABSTRACT

Chikungunya virus (CHIKV), a positive-sense, single-stranded RNA virus in the family *Togaviridae,* is transmitted by *Aedes* mosquitoes. Of three known CHIKV genotypes, the Asian genotype was introduced into the Caribbean islands and rapidly spread throughout Central and South Americas. We previously found patients with symptoms compatible with chikungunya fever in 2014–2015 in Aruba, a Caribbean island of 180 km^2. We here describe the full genome sequences of eight CHIKV strains isolated from patient sera of the Aruban outbreak. Phylogenetic analysis revealed that two closely related but distinct lineages of Asian-genotype CHIKV circulated simultaneously during the epidemic in 2014–2015. These results suggested that CHIKV was introduced into Aruba more than once in a short period, reflecting the importance of Aruba as a travel hub within the region.

1. Introduction

Chikungunya virus (CHIKV) is transmitted by the bite from infected female *Aedes aegypti* mosquitoes and causes febrile illness with severe arthralgia in humans. The virus has been detected sporadically since the first report from Tanzania in 1952 (Lumsden, 1955). Although only one serotype of CHIKV exists, three genotypes have been reported based on and named after the geographical location where the respective genotypes were first recognized: East/Central/South/African (ECSA), West African (WA), and Asian. CHIKV belongs to the *Alphavirus* genus in the *Togaviridae* family and has a positive-sense, single-stranded RNA genome that is 11–12 kb in length with two open reading frames (ORFs) flanked by 5′ and 3′ untranslated regions (UTRs). CHIKV encodes for nonstructural and structural polyproteins that are separated by a subgenomic promoter (Chen et al., 2018). Since 2005, ECSA- and Asian-genotype CHIKVs have spread and caused large epidemics affecting the southwestern Indian Ocean region, India, and Southeast Asia (Weaver, 2014). Identified in 2005, the E1 glycoprotein A226V mutation of the ECSA genotype confers enhanced transmission by the *Aedes albopictus* mosquito and has been implicated in CHIKV's further spread in the last decade (Rianthavorn et al., 2010; Schuffenecker et al., 2006; Tsetsarkin et al., 2007). In late 2013, an Asian-genotype CHIKV was introduced into the Americas and rapidly spread through the Caribbean and South and Central Americas via *Aedes aegypti* mosquitoes (Chen et al., 2016; Leparc-Goffart et al., 2014; Munoz-Medina et al., 2018; Sahadeo et al., 2017; Stapleford et al., 2016; Tan et al., 2018).

The first transcontinental movement of Asian-genotype CHIKV was reported in October 2013, when CHIKV-positive sera were found in the Yap state of the Federated States of Micronesia (Lanciotti and Valadere, 2014). In the Americas, CHIKV was first observed in St. Martin on December 9, 2013, followed by the other Caribbean islands of Martinique (December 19, 2013) and Guadeloupe (December 28, 2013) (Cauchemez et al., 2014). In January 2014, sera from patients living in the British Virgin Islands and Dominica were also confirmed to be Asian-genotype CHIKV-positive (Leparc-Goffart et al., 2014). By June 2014, CHIKV cases were reported in 16 areas of the Caribbean

Abbreviations: CHIKV, Chikungunya virus; ECSA, Eastern/Central/Southern African; WA, West African; CO, Caribbean outbreak; ORF, open reading frame; UTR, untranslated region; MCC, maximum clade credibility; tMRCA, time of the most recent common ancestor; HPD, highest posterior density; PP, posterior probability; DR, direct repeat

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(Cauchemez et al., 2014). Finally, over millions of CHIKV-suspected cases were reported throughout the Americas from late 2014 to 2015 (PAHO, 2014b, 2015a, 2015c). Previous comprehensive phylogenetic analyses revealed that this Asian CHIKV reemergence in the Americas was characterized by an Asian Caribbean lineage (related to the recent South Pacific Asian CHIKVs), which consisted of two major and several minor clades, reflecting rapid and dynamic evolution of CHIKV (Chen et al., 2016; Sahadeo et al., 2017; Stapleford et al., 2016; Tan et al., 2018; Volk et al., 2010). In addition, an uncommon direct repeat insertion was found in 3′ UTR, which was specifically found in the Caribbean CHIKV lineage (Chen et al., 2016; Stapleford et al., 2016). However, whole-genome sequences of CHIKV from Aruba in the same period were not available.

Aruba is an island of 180 km^2 in the Lesser Antilles, north of the South American country of Venezuela. Presence of *Aedes aegypti* in Aruba was confirmed in 1946 (Van Der Kuyp, 1948) and 2019 (personal communication with Luis Chong, Department of Public Health Aruba). There was no autochthonous CHIKV transmission until July 2014, when the first 3 cases were documented. Subsequently, autochthonous CHIKV transmission reports continued until November 2015 (PAHO, 2014a, 2015b). During October 2014–April 2015, Aruban patients suspected of having CHIKV infection were evaluated (Huits et al., 2018a). CHIKV-specific immunoglobulin M antibody testing and RT-PCR analysis identified 269 CHIKV patients (Huits et al., 2018b). From the 164 CHIKV RNA-positive sera, 12 were selected for virus isolation by inoculation into Vero cells, yielding 8 CHIKV isolates. In the present study, the full genomic sequences of these 8 isolates were determined. Phylogenetic analysis revealed that two closely related but distinct lineages of Asian-genotype CHIKVs were circulating simultaneously in Aruba during the epidemic in 2014–2015.

2. Materials and methods

2.1. Viruses

This study used eight CHIKV strains (ARUBA-15801056, -15801125, -15801136, -15801160, -15801358, -15801567, -15801654, and -15802650) that were isolated from the sera of patients in Aruba (Huits et al., 2018a; Huits et al., 2018b; Tuekprakhon et al., 2018a; Tuekprakhon et al., 2018b). For sequence determination, these viruses were propagated one additional time in Vero cells grown in Minimum Essential Medium (Gibco, USA) supplemented with 2% fetal bovine serum at 37 °C in a 5% CO_2 environment.

2.2. RNA extraction, reverse transcription, and amplification

Viral RNA was extracted from culture supernatants of Vero cells using QIAamp viral RNA mini kits (QIAGEN, Germany). Two overlapping cDNA fragments covering the full-length viral genome were synthesized using the SuperScript III first-strand synthesis system (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 4 μL of extracted viral RNA corresponding to approximately 10^5–10^7 copies were mixed with a primer designed in the present study (Table S1). After reverse transcription, two units of RNase H were added to each reaction. The resulting cDNA was amplified in a 50-μL PCR reaction containing 5 μL of reverse-transcribed template, 1.25 U of PrimeSTAR GXL DNA polymerase (Takara, Japan), dNTP mixture (200 μM each nucleotide), 0.2 μM each of forward and reverse primers (Table S1), buffer at a 1× final concentration, and nuclease-free water. PCR reactions were carried out in an ABI thermal cycler (Applied Bioscience, USA) under the following conditions: 94 °C for 1 min, followed by 35 cycles at 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 1 min/kb of expected amplification product. Resulting amplicons were purified with a NucleoSpin gel and PCR clean-up kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions, and the quality of the purified DNA product was assessed using a Nanodrop instrument (Thermo Fisher Scientific, USA).

2.3. Library construction and Illumina sequencing

Amplified double-stranded (ds) DNAs were quantified using a Qubit 2.0 fluorometer (Invitrogen, USA) and then normalized to 0.2 ng/μL. Both 3′- and 5′-amplicons of each viral isolate were pooled and processed for next-generation sequencing using an Illumina Nextera XT library preparation kit (Illumina, USA) to generate paired-end sequencing libraries. The normalized dsDNA amplicon underwent tagmentation using the transposome provided in the kit. Index 1 (i7), index 2 (i5), and adapters (P5 and P7) required for cluster formation were added to the tagmented DNA using a limited-cycle PCR. Sequencing-ready fragments were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics, USA) and the library concentration was quantified with a Qubit fluorometer. The library size was determined with a high-sensitivity DNA chip processed using a Bioanalyzer 2100 (Agilent Technologies, USA). The library was normalized to 4 nM and libraries with unique indices were mixed to generate pooled libraries. Pooled libraries were denatured with 0.2 N NaOH and then diluted with HT1 Hybridization buffer (Illumina, USA) to yield library concentrations of 8 pM. PhiX (Illumina, USA) was prepared in parallel and spiked into amplicon libraries at 5% for use as an internal control. The prepared sample was loaded onto a MiSeq v2 kit (500 cycles) reagent cartridge. Paired-end sequencing of 2 × 250 bp was processed on the Illumina MiSeq platform, and image analysis and base calling were generated as FASTQ files. During the run, quality was assessed using MiSeq control software. We used sequence data only when > 75% of the base calls had Q scores exceeding 30. To generate whole CHIKV genome assemblies, FASTQ files were imported into the CLC Genomics Workbench software version 9.5.3 (QIAGEN, 2016). Forward and reverse reads were aligned to LN898112-Martinique January 2014, a CHIKV that had emerged at the beginning of the Caribbean outbreak and which contains a novel genetic element in the 3′ UTR (Stapleford et al., 2016). The alignment was performed using map reads to the reference genome, and consensus sequences were extracted.

2.4. Sequence determination of the 3′ UTR region by sanger sequencing method

Asian CHIKV has a complicated 3′ UTR region consisting of several duplications, which affected correct mapping of the Illumina FASTQ files to the reference. We therefore determined the 3′ UTR regions by Sanger sequencing. Briefly, 5 μL of 3′ cDNA used in Illumina sequencing was subjected to further PCR amplification of the 1.1-kb fragment of 3′ UTR by using ExTaq polymerase with corresponding primer pairs (Table S1). Amplified products were cloned into the pCR 2.1 vector (Invitrogen), and 4 clones of each product were employed for cycle sequencing with BigDye Terminator v3.1 (Applied Biosystems, USA) and analyzed by an ABI 3130XL sequence analyzer by using T7 and M13 reverse primers within the cloning vector to determine the sequence at both ends of amplified products. The consensus sequence among each quartet of clones was extracted using Genetyx-Mac ver.19. Resultant sequences were aligned using multiple alignment with LN989111.1 and KR046233.1, which were employed as reference sequences with or without duplication, respectively.

2.5. Sequence analysis

Full-length CHIKV sequences were assembled from nearly whole sequences obtained by Illumina sequencing and the 3′ UTR from Sanger sequencing; resulting sequences were deposited into the GenBank database as accession nos. LC500215–LC500222. These newly obtained full-length CHIKV sequences were combined with 231 reference sequences of Asian CHIKV, corresponding to epidemic Asian CHIKV sequences obtained from the outbreak in the Caribbean and the Americas

from late 2013 (Chen et al., 2016; Sahadeo et al., 2017; Stapleford et al., 2016; Tan et al., 2018; Volk et al., 2010) including the sequences that were recovered with high scores by BLAST searches with ARUBA-15801056 (KY680397-Dominican Republic-2014, KY435462-Jamaica-2014, LN898112-Martinique-2014, LN898110-Guadeloupe-2014, LN898093-Martinique-2013, KT327165-Mexico-2014) and ARUBA-15801358 (KY703969-Dominican Republic-2014, KY703963-Nicaragua-2015, KJ451624-British Virgin Islands-2014, KX702401-Haiti-2014). These reference sequences were downloaded from the NIAID Virus Pathogen Database and Analysis Resource (ViPR; http://www.viprbrc.org/) (Pickett et al., 2012) and GenBank (https://www.ncbi.nlm.nih.gov/genbank/). These reference sequences were selected from all geographical areas and CHIKV outbreaks. Duplicated sequences were removed. Among Asian CHIKVs from the Caribbean and Americas, only sequences with known collection year and month were included. The dataset was then tested with root-to-tip analysis and outliers were removed. All CHIKV sequence names are named in the format of country of isolation, accession number, and collection date (yyyy_MM) (Table S2). The Asian CHIKV dataset was aligned using MUSCLE (Edgar, 2004) in AliView v1.23 (Larsson, 2014) and ambiguous regions in UTRs were removed. Finally, 11,237 bp of the ORF alignment were obtained. Nucleotide sequences, along with corresponding predicted amino acid sequences, in Aruba CHIKV subclades were compared and annotated by comparison to LN898112-Martinique January 2014 conducted in AliView v1.23.

2.6. Phylogenetic tree inferences and viral evolution rate analyses

Prior to investigation of evolutionary rate, the CHIKV sequence dataset was tested for the presence of recombination breakpoints using GARD implemented in the Datamonkey web server (https://www.datamonkey.org/) (Kosakovsky Pond et al., 2006). No evidenced of recombination was found. Then, initial maximum-likelihood (ML) trees of Asian CHIKV (Fig. S1) were generated in W-IQ-TREE (Trifinopoulos et al., 2016) obtained at http://iqtree.cibiv.univie.ac.at/. Specifically, we implemented GTR + F + G4 as the best-fit model selected with ModelFinder (Kalyaanamoorthy et al., 2017) using an ultrafast bootstrap (Hoang et al., 2018) of 1000 replicates, to determine the evolutionary temporal signal in the Asian CHIKV dataset using Tempest v1.5.3 (Rambaut et al., 2016). To reconstruct time-scaled phylogeny, Bayesian Markov Chain Monte Carlo (MCMC) sampling was analyzed in the BEAST v1.10.4 package (Suchard et al., 2018). For phylogeography, each sequence was tagged to a geographic region (Asia/Asia Pacific, Caribbean, North America, Central America, and South America) according to the collection country (Table S2). Our initial attempt using Bayesian Skyline failed because it showed poor convergence in the trace of parameters and effective sample size (ESS) of < 200, even after MCMC chains were run 150 million times. Therefore, 8 model combinations of SRD06 nucleotide substitution model (Shapiro et al., 2006), clock models of strict and relaxed uncorrelated log normal clock (UCLN) models, the Bayesian coalescent tree prior of Constant size, Exponential growth, Gaussian Markov random field (GMRF) Bayesian Skyride, and Bayesian Skygrid with 60 grid points (Gill et al., 2013) were compared by log marginal likelihood estimation (MLE) using path sampling (PS) and stepping-stone sampling (SS) methods with 50 path steps of 1 million iterations and log every 1000 (Baele et al., 2016). The result showed UCLN and Bayesian Skygrid coalescent tree prior was the best-fit model (Table S3). MCMC chains were run for 150 million generations with sampling every 15,000 generations to obtain ESSs over 200. Four independent runs were employed and combined in LogCombiner v1.10.4. The running speed was enhanced using BEAGLE (Suchard and Rambaut, 2009). The trace was accessed in Tracer v1.7.1. and then a 10% burn-in was removed to achieve good mixing of convergence with ESSs over 200. The maximum clade credibility (MCC) tree was generated using TreeAnnotator v1.10.4; 4000 trees were discarded (10% burn-in) and visualized in FigTree v1.4.3.

3. Results

3.1. Epidemiological history of Asian-genotype CHIKV in Aruba

To gain an understanding of evolutionary relationships of CHIKV Aruba strains used in this study and Asian CHIKV Caribbean outbreak strains, we conducted a Bayesian MCMC phylogenetic analysis. Our Asian CHIKV ORF dataset (n = 239), which was based on previous CHIKV epidemiological studies (Chen et al., 2016; Tan et al., 2018; Volk et al., 2010), exhibited a strongly positive temporal signal between divergence and time with $R^2 = 0.99$ (Fig. 1A). The time-calibrated phylogenetic tree (Fig. 1B) then was constructed using a UCLN and Bayesian Skygrid coalescent tree prior as the best-fit model (Table S3, Fig. S2). The estimated evolutionary rate of all viruses of the Asian CHIKV genotype was 4.66×10^{-4} substitutions per site per year (s/s/y) [95% highest posterior density (HPD): 4.19×10^{-4}–5.17×10^{-4} s/s/y]. The time of the most recent common ancestor (tMRCA) at the root was 1957.1 (95%HPD: 1955.8–1958.3). The Asian CHIKV MCC phylogeny revealed that the Caribbean outbreak (CO) lineage clade separated from KJ451622-Micronesia in October 2013 and from other viruses in the Asian genotype around 2011.7 (95%HPD: 2010.9–2012.5) with a posterior probability (PP) of 0.90. The monophyletic CO lineage clade tMRCA was 2013.2 (95%HPD: 2012.9–2013.5) with a PP of 1. The mean evolution rate among this lineage was 4.60×10^{-4} (95%HPD: 3.44×10^{-4}–6.05×10^{-4}). Three CO subclades were observed. Clade CO1 (PP = 1) contained viruses from the Caribbean (Guadeloupe, Martinique, Haiti, Trinidad and Tobago, and Jamaica) and Central America (Nicaragua) (Fig. 1C); clade CO2 (PP = 1) contained viruses from the Caribbean (Puerto Rico and Dominican Republic) and South America (Colombia) (Fig. 1D); and clade CO3 (PP = 1) contained Colombia and Nicaragua viruses (Fig. 1E). tMRCAs of CO1, CO2, and CO3 were 2013.5 (95%HPD: 2013.3–2013.7), 2013.7 (95%HPD: 2013.0–2013.9), and 2013.8 (95% HPD: 2013.6–2014.0), respectively.

Aruba CHIKV strains in early 2015 belonged to clades CO2 (ARUBA-15801056, -15801136, and -15801567) and CO3 (ARUBA-15801125, -15801160, -15801358, -15801654, and -15802650) with tMRCAs of 2014.6 (95%HPD: 2014.4–2014.9) and 2014.2 (95%HPD: 2013.9–2014.5), respectively. Aruba CO2 viruses clustered with viruses obtained in 2014 from elsewhere in the Caribbean (i.e., Dominican Republic and Puerto Rico), South America (i.e., Colombia, Brazil, and Venezuela), and USA (PP = 1) (Fig. 1D), while Aruba CO3 viruses were grouped as a small subclade with minor strains in Nicaragua and Colombia (PP = 0.98) (Fig. 1E). These results suggested that the introduction of CHIKV to Aruba occurred via at least two separate events at very close time points, subsequently spreading rapidly on the island.

3.2. Amino acid diversity of Aruba CHIKVs

All Aruba viruses analyzed in the present study retained the opal stop codon between nsP3 and nsP4. When we compared Aruba virus sequences with the CHIKV Caribbean strain (LN898112), eight non-synonymous substitutions were observed in the predicted protein sequences. Five of these substitutions occurred within a non-structural polyprotein (total length 2470 amino acids) and three were located within a structural polyprotein (total length 1248 amino acids) (Table 1). P131S in nsP1 was shared among CO2 ARUBA viruses, while Y543H and G720A in nsP2, along with L520P in nsP3, were shared among CO3 ARUBA viruses. By contrast, P369S in nsP3, K209R in C, and M299V and S408L in E1 were found only in ARUBA-15801654, -15802650, -15801160, and -15801136, respectively.

3.3. 3′ UTR duplication in Asian CHIKV Caribbean lineage

The CHIKV 3′ UTR showed differing numbers of nucleotides among various lineages (Filomatori et al., 2019; Hyde et al., 2015). 3′ UTRs of

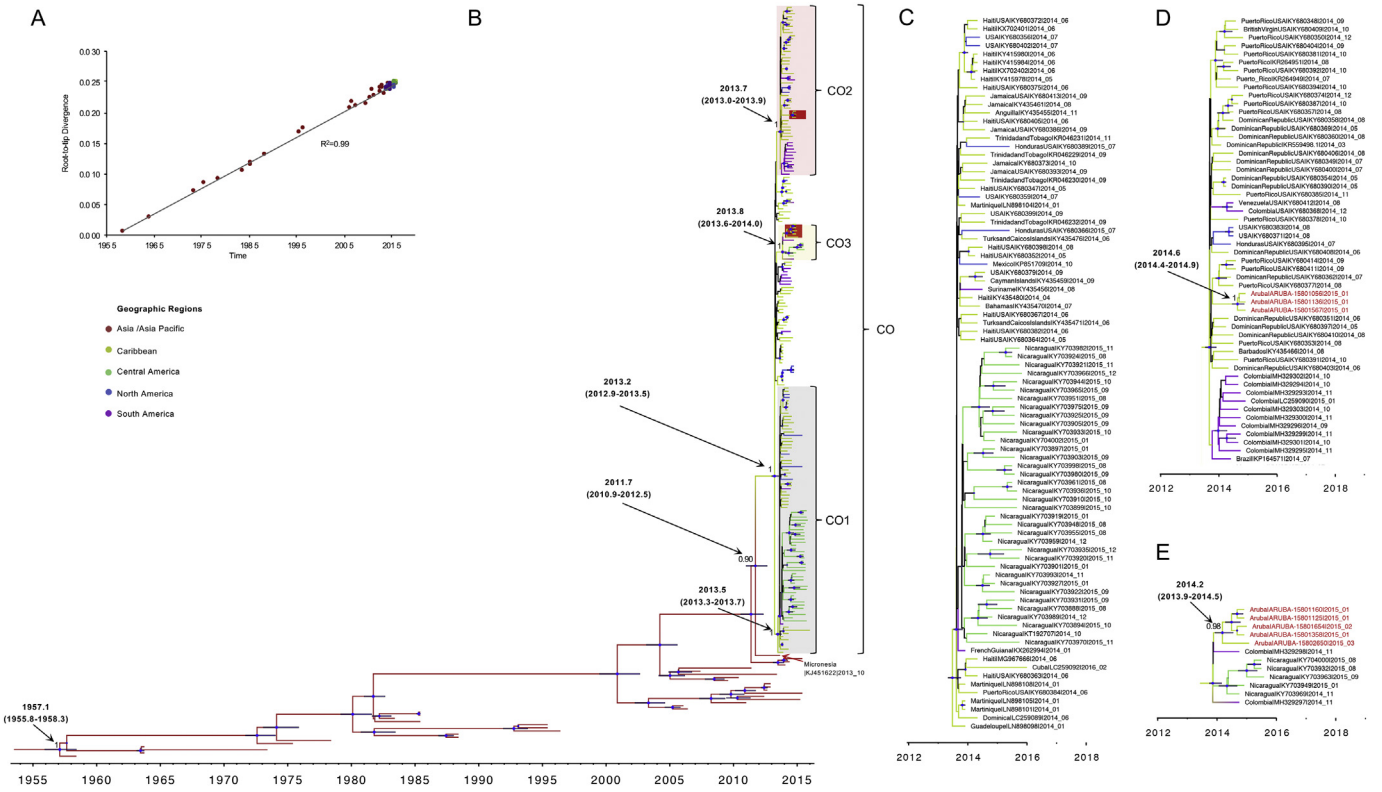

Fig. 1. Molecular clock analysis of Asian-genotype chikungunya virus. A) Temporal signal analysis of regression of root-to-tip divergence against date ($R^2 = 0.99$). B) Maximum clade credibility (MCC) tree of Asian chikungunya virus (CHIKV) open reading frames. Caribbean Outbreak (CO) lineages, clades CO1, CO2, and CO3, are indicated by vertical lines. CO1, CO2, and CO3 are highlighted in grey, pink, and yellow, respectively. Obtained Aruba sequences are highlighted in red. The time-scale in years is shown in the x-axis at the bottom. C) Magnification of clade CO1. D) Magnification of clade CO2. E) Magnification of clade CO3. Obtained Aruba sequences are labeled in red. The branch color corresponds to the geographic region indicated on the middle left. Time of the most recent common ancestor (tMRCA) values [95% highest posterior density (HPD) range] are indicated by black arrows and posterior probability (PP) support is shown adjacent to the key node. Blue horizontal node bars representing 95%HPD values of the node height and the blue node are shown only for those with PP > 0.9. The red arrow indicates KJ451622-Micronesia-October 2013. Sequences are designated in the format of country|Accession number|date of collection (year_month). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CHIKV genotypes ECSA and WA contain two types of direct repeats (DRs), consisting of one in which element 1 (DR1) appears twice and a second in which element 2 (DR2) appears three times. Asian-genotype CHIKVs including Caribbean-lineage viruses contain one more DR (DR3) consisting of the third (the most 3′ proximal) DR2 and the rest of the 3′ proximal region (Fig. 2). The re-emerging Asian CHIKV Caribbean lineage harbors a longer 3′ UTR than other lineages of Asian viruses, reflecting the presence of another DR of 177 nucleotides, which starts from the middle of the first (the most 5′ proximal) DR2 and ends near the end of the second DR2, spanning a complete DR1 (Fig. 2) (Chen et al., 2016; Filomatori et al., 2019; Hyde et al., 2015; Stapleford et al., 2016). This Caribbean-lineage-specific duplication was found in clinical samples from St. Martin, Guadeloupe, French Guiana, Mexico, Nicaragua, Dominican Republic, and Trinidad and Tobago, suggesting that this DR element was present in Asian CHIKV when the virus emerged in the Caribbean islands and the Americas. Because we experienced difficulties in mapping the Illumina FASTQ data even when we used a sequence (LN898112) that carried this duplication, we determined the sequences of the 3′ UTR region by the Sanger method. As shown in Fig. 2, we confirmed the presence of the 177-nucleotide duplication in the 3′ UTR of our Aruba sequences. Furthermore, we identified three polymorphic positions at bases 49, 230, and 302 (numbering from the beginning of the 3′ UTR). C and T nucleotides at position 230 were seen in CO2 and CO3 Aruba viruses, respectively.

Table 1
Amino acid diversity of Aruban chikungunya viruses.

Polyproteins	Nucleotide change[a]	Protein	Amino acid change[b]	Strains carrying the change
Non-structural protein	C391T	nsP1	P131S	ARUBA-15801056, 15801136, and 15801567
	T3232C	nsP2	Y543H	ARUBA-15801125, 15801160, 15801358, 15801654, and 15802650
	G3764C	nsP2	G720A	ARUBA-15801125, 15801160, 15801358, 15801654, and 15802650
	C5104T	nsP3	P369S	ARUBA-15801654
	T5368C	nsP3	L520P	ARUBA-15801125, 15801160, 15801358, 15801654, and 15802650
Structural polyprotein	A626G	Capsid	K209R	ARUBA-15802650
	A3322G	E1	M299V	ARUBA-15801160
	C3650T	E1	S408 L	ARUBA-15801136

[a] Numbers indicate nucleotide numbering from the first nucleotide in each open reading frame.
[b] Numbers indicate amino acid numbering from the first amino acid in each protein.

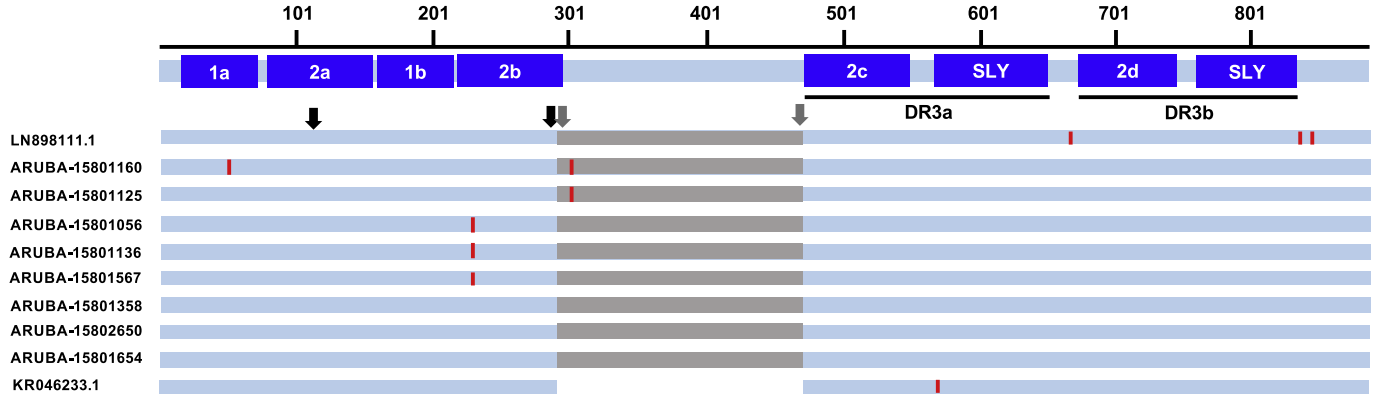

Fig. 2. Schematic illustration of the 3′ UTR of Aruban chikungunya strains. 3′ UTR sequence alignment of ARUBA-15801056, -15801125, -15801136, -15801160, -15801358, -15801654, and -15802650 (obtained in the present study) and reference strains of Caribbean LN898111.1 and non-Caribbean KR046233.1. The Caribbean-CHIKV-specific insertion of 177 nucleotides is shown as grey bars between two vertical grey arrows, which is formed by duplication of the preceding regions (between the two black arrows). Nucleotide polymorphisms are labeled in red. Direct repeats 1 (1a and 1b) and 2 (2a, 2b, 2c, and 2d) and a Y-shaped stem-loop structure (SLY) are shown in blue boxes. Direct repeats 3 (DR3a and DR3b) are indicated by black horizontal lines. Nucleotide numbers in the 3′ UTR are shown at the top. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The CHIKV epidemic in the Caribbean islands started in late 2013 and rapidly spread to the South American mainland in 2014. We sequenced 8 isolates obtained from patient sera collected in early 2015 in Aruba. Our estimation showed that Asian-Caribbean CHIKV was introduced to the Caribbean in mid-2013 and spread to the Caribbean and Americas with a substitution rate of 4.60×10^{-4} s/s/y. The overall Asian CHIKV substitution rate was 4.66×10^{-4} s/s/y, which was similar to the previously reported values of 4.71×10^{-4} s/s/y (95%HPD: 3.84×10^{-4}–5.65×10^{-4} s/s/y) (Nunes et al., 2015), 4.6×10^{-4} s/s/y (95%HPD: 4.1×10^{-4}–5.2×10^{-4} s/s/y) (Sahadeo et al., 2017), and 4.33×10^{-4} s/s/y (95%HPD: 3.54×10^{-4}–5.15×10^{-4} s/s/y) (Tan et al., 2018). The Asian-genotype CHIKV tended to show a slower evolution rate than the ECSA-genotype CHIKV, as the ECSA Brazilian lineage circulating during 2014–2017 showed a substitution rate of 7.15×10^{-4} s/s/y (95%HPD: 5.04×10^{-4}–9.55×10^{-4} s/s/y) and ECSA Indian Ocean lineage strains obtained during 2004–2008 outbreak showed a substitution rate of 8.46×10^{-4} s/s/y (95%HPD: 5.78×10^{-4}–10.09×10^{-4} s/s/y) (Naveca et al., 2019; Volk et al., 2010).

We estimated that Asian-Caribbean CHIKV was introduced into Aruba around mid-2014. More than half of clinically suspected cases (269 of 498 cases) were confirmed as having CHIKV infection during the outbreak (Huits et al., 2018a). Our phylogenetic analysis revealed that the eight Aruba viruses can be separated into two distinct subclades that encode proteins featuring unique amino acid substitutions. Our results suggest that CHIKV was introduced into Aruba via at least two independent routes.

Of the eight Aruban strains that we analyzed, five strains belonged to CO3. Aruban viruses in CO3 were either closely related to sequences from Central American viruses from Nicaragua or South American viruses from Colombia and encoded proteins sharing the unique amino acid substitutions of Y543H and G720A in nsP2, and L520P in nsP3. Nicaragua is situated on the western rim of the Caribbean Sea, and Colombia sequences were collected in the Andean and Pacific regions of Colombia (Villero-Wolf et al., 2019). It is thus difficult to reconstruct a detailed route for the introduction of CO3 CHIKV to Aruba. It is possible that the virus was carried to Aruba directly by travelers returning from Nicaragua or Columbia and that one of these viruses seeded autochthonous transmission in Aruba.

The remaining three Aruban strains clustered in CO2. The topology of the phylogenetic tree suggested that Aruban viruses in CO2 are closely related to KY680362, which was obtained from a USA traveler returning from the Dominican Republic in July 2014 (Tan et al., 2018). Considering that the phylogenic tree of the entire CO2 clade also was rooted among viruses obtained from USA travelers returning from the Dominican Republic, it is highly likely that the Aruban CO2 viruses also originated from the Dominican Republic.

One of the major limitations of the present study is the lack of travel history data of the patients from whom viral strains were isolated. We therefore cannot exclude the possibility that the three CO2 Aruba viruses derived from three imported cases and are not the result of autochthonous transmission in Aruba. However, we consider this hypothesis unlikely, given that the CO2 Aruban viruses formed a distinct cluster that encoded nsP1 displaying a unique P131S amino acid substitution. Another limitation is a lack of sufficient data from Venezuela, the country closest to the island of Aruba. It is apparent that data from Venezuela would be required to exclude possible routes of CHIKV invasion into Aruba from South America. It also would be interesting to see whether there were any differences in patients' clinical symptoms or disease severity between the two subclades of Aruban viruses.

Since the 2014–2015 outbreak, virtually no CHIKV case in Aruba has been reported thus far (CARPHA, 2015; PAHO, 2015c). However, ECSA-genotype CHIKV is still circulating in Brazil (Naveca et al., 2019; Nunes et al., 2015), and ECSA genotype CHIKV was detected in a female *Aedes albopictus* pool in Haiti in 2016 (White et al., 2018) It is thus important to continue surveillance of ECSA-genotype CHIKV in Aruba.

Our Aruba CHIKV sequences carried a typical 3′ UTR duplication characteristic of the CHIKV Asian Caribbean lineage (Chen et al., 2016; Stapleford et al., 2016), which exhibited an extended-length (177-bp) DR. Variations in the 3′ UTR length are commonly found in Alphaviruses. Precise roles of variable numbers of repeated sequence elements in the Alphavirus life cycle remain largely unclear, but it is essential for virus adaptive growth in mosquito (Hyde et al., 2015; Ou et al., 1982; Pfeffer et al., 1998; Rupp et al., 2015). The deletion of two copies of both DR1 and DR2 in ECSA-genotype CHIKV and the deletion of 177-bp duplication in Caribbean CHIKV impaired viral replication in a mosquito cell line but not in a mammalian cell line (Chen et al., 2013; Morley et al., 2018; Stapleford et al., 2016). Consistently, the numbers of DR copies in Caribbean CHIKV positively correlated with virus replication capability in mosquito rather than in mammalian cells (Filomatori et al., 2019). It has been suggested that RNA recombination in highly divergent CHIKV 3′ UTR facilitated adaptation of CHIKV in mosquitoes. In our sequences, we observed polymorphisms in the 3′ UTR that might be linked to or restricted within the subclades of Caribbean viruses.

In summary, we have shown that two distinct lineages of CHIKVs

were cocirculating during the Aruba epidemic in 2014–2015, suggesting at least two independent introductions. This work complements the publicly available full genome sequence of CHIKV from the Caribbean outbreaks in 2014, including 3′ UTR sequences that are not easily determined by Illumina sequencing.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.104129.

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