

Circulation of Chikungunya virus East-Central-South Africa genotype during an outbreak in 2016-17 in Piauí State, Northeast Brazil

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ABSTRACT

Chikungunya virus (CHIKV) is an arbovirus that emerged in the Americas in 2013. Infection with CHIKV is symptomatic in most of the cases and patients can develop chronic arthralgia that lasts from months to years in over 40% of the cases. The East-Central-South Africa (ECSA) genotype was introduced in Brazil in 2014, in Bahia State. Here we report the circulation of the CHIKV ECSA genotype in Piauí State, Northeast Brazil, during the years 2016-2017. The phylogenetic analysis revealed a single introduction of this lineage probably in 2015 and its maintenance at least until 2017. This analysis has also demonstrated the proximity of this genotype with isolates from neighboring States, and its partial nucleotide sequence of the viral E1 gene revealed a synapomorphy synonyms. This finding highlights the spread of the ECSA genotype in Brazil and supports its circulation in the Brazilian Northeast.

KEYWORDS: Chikungunya virus. Brazil. ECSA genotype. Arbovirus. Togavirus. Alphavirus. Molecular surveillance. Epidemiology. E1 gene.

INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne, enveloped, positive single-stranded RNA virus of the family *Togaviridae*, genus *Alphavirus*. Its genome contains two open reading frames encoding four nonstructural (nsP1-4) and five structural proteins (C, E3, E2, 6K, E1)^{1,2}. Studies performed in Thailand, Malaysia, Mayotte and La Reunion islands have shown that 52 to 98% of the infected patients develop symptoms that range from fever, headache, myalgia, articular edema and rash to intense polyarthralgia and severe joint pain³⁻⁶. Furthermore, a variety of studies have reported a high prevalence of chronic arthralgia, which can last from months to years in approximately 40% of the infected people^{7,8}.

CHIKV was isolated for the first time in 1952-53 in Tanzania⁹. So far, four distinct lineages have been recognized: East-Central-South Africa (ECSA), Indian Ocean (IOL), Asian and West African lineages¹⁰. Until 2004, CHIKV had caused small and sporadic outbreaks at the African and Asian continents, however that year, the ECSA genotype caused massive outbreaks in the East Africa coast^{11,12}. This was followed by unprecedented epidemics in Indian Ocean islands and Asia as well as the first outbreak in a temperate region in Italy¹³. In 2013, the first cases of

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autochthonous transmission in the Americas were reported in the Caribbean island of Saint Martin, caused by the Asian genotype¹⁴. In late 2014, the Asian genotype was detected in the Amapa State, Northern Brazil¹⁵, whereas the ECSA genotype was later detected in Bahia State, Northeast Brazil¹⁶. According to the Brazilian Ministry of Health, between 2016 and 2018, 547,797 suggestive cases of CHIKV were reported in the country, with 361,640 laboratory confirmed cases and 387 CHIKV-associated deaths¹⁷⁻¹⁹. The Northeast region of the country accounted for 86.4% and 76,8% of the suggestive cases during 2016 and 2017, respectively^{17,19}.

Piauí State (PI) is located in the Northeast region of Brazil, and borders the States of Bahia, Ceara, Pernambuco and Maranhao and Tocantins (Figure 1). Until 2016, no cases of CHIKV (imported or autochthonous) were reported in Piauí State. However, in 2016, the incidence rate of suggestive CHIKV infections increased to 86.4 per 100.000 inhabitants, the lowest in the Northeast region¹⁹, and peaked in 2017 reaching 194.5 per 100.000 inhabitants¹⁷. In 2018, the incidence rate dropped to 17.4 per 100.000 inhabitants. In addition, deaths caused by CHIKV infection have been confirmed in Piauí State in 2016 and 2017^{17,18}. Considering that there has been an increase in the incidence rate of CHIKV in Piauí State, the aim of the present study was to investigate and to identify the circulating genotype in the State.

MATERIALS AND METHODS

Clinical samples and ethical statement

From April 2016 to November 2017, clinical samples were collected at the Urgency Medical Service Unit and State Hospital Dirceu Arcoverde in the city of Parnaíba, Piauí State, Brazil. Serum were obtained from whole blood samples collected from patients presenting two or more of the following symptoms: fever (>38.5 °C), headache, arthralgia, myalgia, rash and/or hemorrhagic manifestations. Informed consent was obtained from all the patients or their legal guardians, and a form with questions about age, days of symptoms, travel history and previous diagnostics was filled out. The collected samples were stored at 4 °C, transported to the research laboratory at the Federal University of Piauí within 5 h after collection and stored at -70 °C. When available, results of the serological tests for DENV and CHIKV performed by public health services were retrieved. The research was approved by the Human and Ethics Committee of the Federal University of Piauí, under the protocol N° CAAE 46111615.0.0000.5214.

Molecular investigation of Chikungunya infection

Viral RNA extractions were performed from serum of patients presenting three or more symptoms, less than

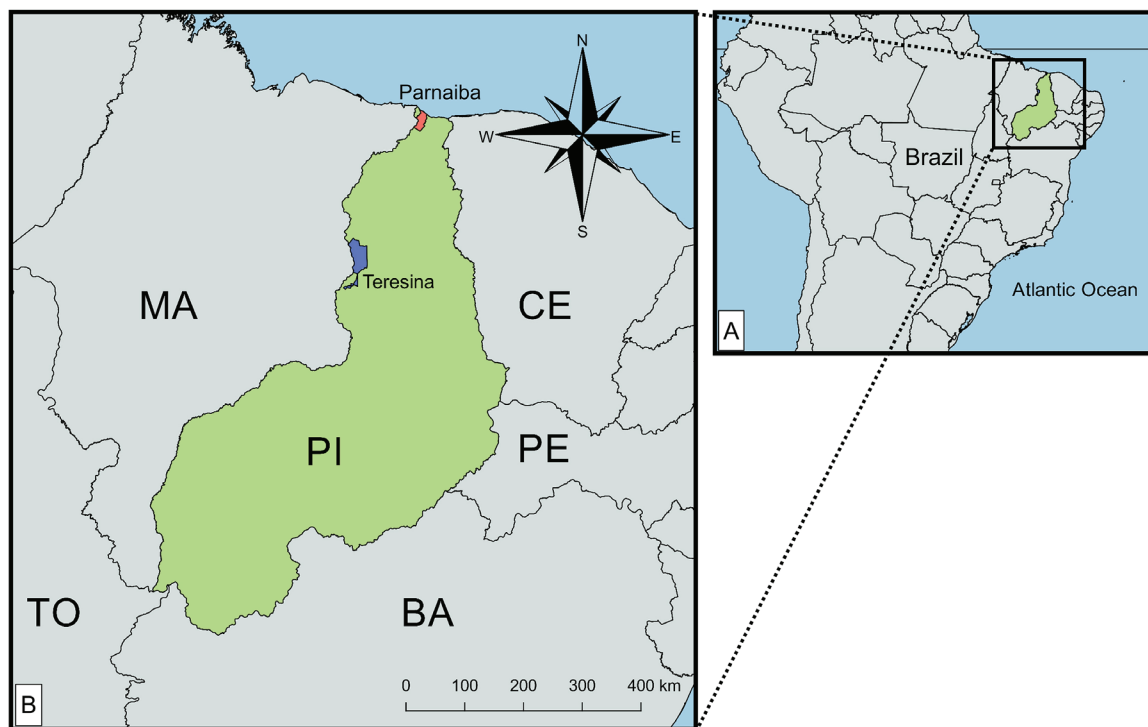


Figure 1 - The geographic location of Piauí State in Brazil. Teresina and Parnaíba municipalities are highlighted in blue and orange, respectively. BA: Bahia; CE: Ceara; MA: Maranhao; PE: Pernambuco; PI: Piauí TO: Tocantins.

5 days of the onset of symptoms and the presence of IgM antibodies against CHIKV, when available. The viral RNA extraction was performed using QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) or NucleoSpin® RNA Virus (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions.

The cDNA synthesis was carried with random hexamers (20 µM, Qiagen, Hilden, Germany) and Moloney Murine Leukemia virus reverse transcriptase (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions. Using specific primers previously described for CHIKV *E1* (DVRChk-F 5' ACCGGCGTCTACCCATT CATGT 3'; DVRChk-R 5' GGGCGGGTAGTCCATGTTGTAGA 3')²⁰ and CHIKV *E2* (JM1 5' GCAGACGCAGAGAGGGCCAG 3'; JM2 5' CGTGCTGCAAGGTAGTTCTC 3'; JM3 5' GCTATTTGTAAGAACGTCAG 3'; JM4 5' TACCGTGCTGCGGTCGGGA A 3')²¹ genes, partial genome sequences were amplified. The expected products of 330 bp (*E1*) and 240 bp (*E2*) were separated by electrophoresis in 1.5% agarose gels stained with the intercalant agent GelRed™ (Biotium Inc., Fremont, California, USA) and visualized under U.V. lights. The expected bands were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) for nucleotide sequencing.

Nucleotide sequencing and phylogenetic analysis

PCR amplicons of the *E1* and *E2* genes were sequenced using 0.5 µM of each specific primer (forward and reverse) and approximately 10 ng of each DNA sample. The sequencing was performed in a ABI 3130 DNA Analyzer (Applied Biosystems) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), using the Sanger sequencing platform of Rene Rachou Institute (Fiocruz, Minas Gerais, Brazil). Raw data were analyzed and final contigs were assembled using the Geneious R9 version 9.1.8²². The partial sequences of *E2* and *E1* genes were concatenated in frame and aligned to 74 concatenated sequences of CHIKV genotypes retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>) using CLUSTALW, implemented on MEGA6²³. The nucleotide substitution model of Kimura 2-parameters with Gamma distribution (4 categories) (K2+G) was selected using jModelTest v 2.1.4²⁴. The maximum likelihood tree was reconstructed using MEGA 6²³ with a total of 88 sequences, the nucleotide substitution model K2+G with 1000 bootstrap replicates.

Bayesian inferences were performed using BEAST package 1.8.4²⁵ with Markov Chain Monte Carlo algorithms (MCMC). Input files for BEAST v.1.8.4 were created with BEAUTi v.1.8.4²⁶, using 88 concatenated *E2E1* sequences.

The best model was selected comparing the marginal likelihood estimations (MLE)²⁷. The estimates were performed using the nucleotide substitution model GTR, with gamma distribution (four categories), under the relaxed molecular clock and the Bayesian skyline demographic Model. Three hundred million chains were run, the first 30 million steps were discarded, and convergence of parameters was verified with Tracer v.1.5.0²⁸. The trees were sampled at every 10,000 steps and then summarized in a maximum clade credibility tree using TreeAnnotator v.1.8.2²⁹. The final tree was visualized in FigTree v.1.4.3³⁰.

RESULTS

Clinical samples and serological results

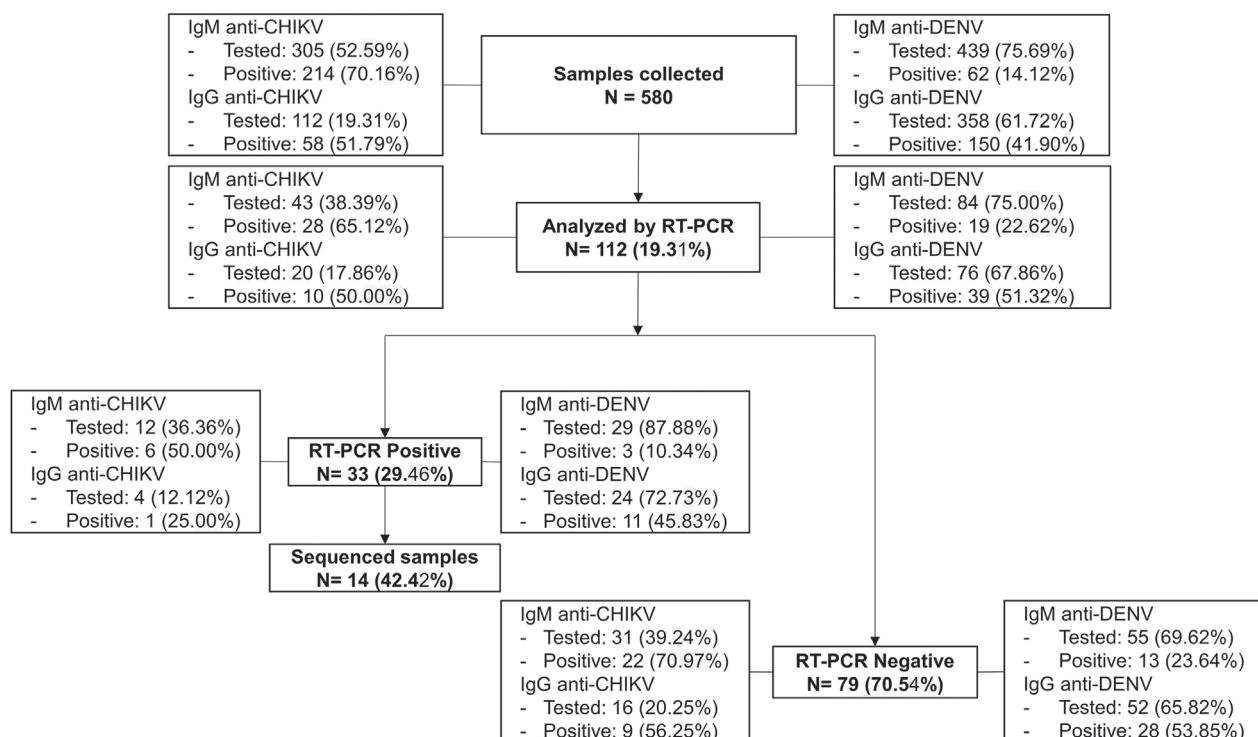
During April/2016 and November/2017, 580 patient samples were collected. 40.2% (233/580) of samples were collected during 2016, whereas 59.8% (347/580) were collected during 2017. The majority of the patients were woman (66.4%). The main clinical signs were fever (84.65%), arthralgia (82.06%), headache (77.75%) and myalgia (71.38%) (Table 1). However, 0.86% (5/580) of patients have also presented neurological complications, such as encephalitis. Of the 580 samples collected, serological tests for CHIKV IgM were performed on 52.59% (305/580) samples, CHIKV IgG on 19.31% (112/580), DENV IgM on 75.69% (439/580) and DENV IgG on 61.72% (358/580). Of these, 70.16% (214/305) were positive for CHIKV IgM, 14.12% (62/439) for DENV IgM and 36 (6.21%) were positive for both CHIKV and DENV IgM tests (Figure 2).

RT-PCR for CHIKV

From the total of collected samples, RNA extraction were performed on 19.31% (112/580) samples. Of these, 56.25% (63/112) were from 2016 and 43.75% (49/112) from 2017. Of the analyzed samples, 29.46% (33/112) were positive for CHIKV by PCR, with 57.58% (19/33) from 2016 and 42.42% (14/33) from 2017. Of the positive samples, CHIKV specific IgM tests were available for 36.36% (12/33) patients, of which 50% (6/12) were CHIKV IgM positive (Figure 2). 87.88% (29/33) of the PCR-positive CHIKV samples had DENV IgM tests performed, and 10.34% (3/29) of samples were positive. Only one of the CHIKV-positive samples with serological tests results presented both DENV and CHIKV IgM antibodies. Of the 33 CHIKV-positive samples, one (3.03%) patient of 11 years old presented clinical signs of encephalitis as well as fever, vomiting, headache, myalgia and eye

Table 1 - Demographic and clinical characteristics of suspected cases of CHIKV infection during April/2016 to November/2017 in Piauí, Brazil, according to CHIKV laboratory test results (RT-PCR).

| | All Samples (n=580) | Suspected cases (n=112) | Positive (n=33) | Negative (n=79) | Sequenced (CHIKV ECSA) (n=14) |
|----------------------------|------------------------|----------------------------|--------------------|--------------------|-------------------------------------|
| Sex | | | | | |
| Female | 66.4% | 62.5% | 54.55% | 65.82% | 57.14% |
| Age (years) | | | | | |
| Median(range) | 33(1-89) | 29(3-85) | 35(6-85) | 27(3-76) | 37(7-73) |
| Clinical Symptoms | | | | | |
| Fever | 84.65% | 85.71% | 93.94% | 81.01% | 100% |
| Arthralgia | 82.06% | 82.14% | 90.91% | 78.48% | 92.86% |
| Headache | 77.75% | 74.11% | 81.82% | 69.62% | 92.86% |
| Myalgia | 71.38% | 66.96% | 75.76% | 63.29% | 100% |
| Exanthema | 63.79% | 66.07% | 57.57% | 69.62% | 71.43% |
| Prostration | 54.14% | 56.25% | 48.48% | 59.49% | 57.14% |
| Eye problems | 52.07% | 52.68% | 45.45% | 55.70% | 42.86% |
| Nauseas | 48.02% | 42.86% | 54.54% | 37.98% | 57.14% |
| Vomits | 25.52% | 26.78% | 21.21% | 27.89% | 28.57% |
| Articular Edema | 23.45% | 25.89% | 39.39% | 20.25% | 57.14% |
| Diarrhea | 18.79% | 16.94% | 12.12% | 18.99% | 14.29% |
| Neurological Complications | 0.86% | 4.46% | 3.03% | 5.06% | 7.14% |

**Figure 2** - Flowchart showing the total number of patient samples collected and laboratory tested (RT-PCR) to CHIKV during April/2016 to November/2017.

complications. The presence of CHIKV IgM antibodies was detected in this sample, but DENV IgM and IgG were not detected. Among the PCR negative samples (79/112),

CHIKV IgM tests were performed on 39.34% (31/79) of the patients. Of these, 70.97% (22/31) of the samples presented CHIKV IgM antibodies (Figure 2).

Nucleotide sequencing and phylogenetic analysis

The partial sequence of *E2* and *E1* genes of 14 samples from 2016 and 2017 were determined (GenBank accession N° MK510154-MK510181). The partial sequences of *E2* (208 nt) and *E1* (120 nt) genes were concatenated in frame (328 nt; corresponding to positions 8,804 to 8,923 and 10,253 to 10,460 compared to the nucleotide sequence of CHIKV strain BHI3745/H804709, isolated in Feira de Santana, Bahia (GenBank accession N° KP164570). The phylogenetic analysis based on the Maximum likelihood (Supplemental Figure 1) and Bayesian methods revealed that all sequenced isolates belonged to the ECSA genotype and clustered together, forming a monophyletic group, called CHIKV/PI/2016-2017 (supported by a posterior probability (PP) equal to 0.96). This group clustered with

other CHIKV isolates, circulating from 2014 to 2016, in Bahia¹⁶, Rio de Janeiro³¹, Sergipe³², Alagoas³³, Paraíba and Pernambuco (PP=1) (Figure 3).

The isolates from CHIKV/PI/2016-2017 had the most recent common ancestor (MRCA) estimated in 2015 (95% Bayesian credible interval = 2014 to 2016). The nucleotide sequences within CHIKV/PI/2016-2017 were identical to each other. When sequences within CHIKV/PI/2016-2017 were compared to sequences from other Brazilian isolates (collected during 2014-2016), a nucleotide similarity of 99.3% was observed. Analysis of nucleotide and predicted amino acid sequences of CHIKV/PI/2016-2017 compared to sequences belonging to others Brazilian CHIKV/ECSA genotypes, indicated a synonymous nt substitution characterized as synapomorphy (position 87 of the E1 gene of CHIKV/PI/2016-2017 sequences).

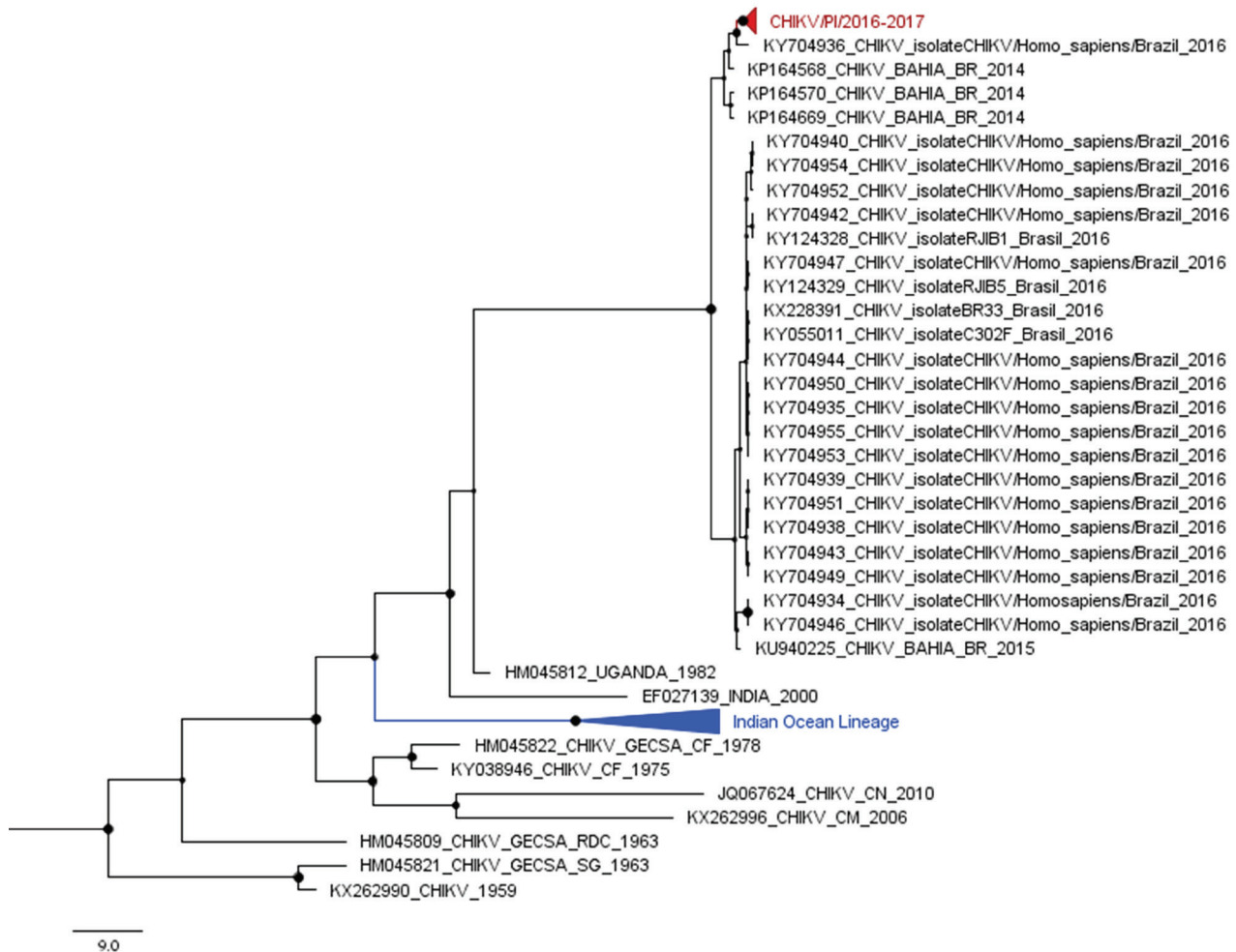


Figure 3 - Bayesian phylogenetic analysis of Chikungunya virus. A subtree of the ECSA genotype, from the maximum clade credibility tree inferred using 88 Chikungunya virus sequences (328 nt) is shown. The posterior probability values are represented by circles drawn in scale in the nodes. Clades containing strains from Piauí (2016/2017) and the Indian Ocean Lineage are shown in red and blue, respectively. Branch lengths are drawn to scale of years. The tree was reconstructed using the nucleotide substitution model GTR with gamma distribution (four categories), under the relaxed molecular clock and the Bayesian skyline demographic model. PI: Piauí. BR: Brazil. GECSA: Genotype East-Central South African. CHIKV: Chikungunya virus.

DISCUSSION

In this study, we performed a molecular investigation of CHIKV infection in symptomatic patients in Piauí State, Northeast Brazil, during 2016 and 2017. By using molecular tests, CHIKV infections were confirmed in 33 patients out of 112 tested samples. As the presence of acute symptoms such as fever, arthralgia, myalgia and headache lasting less than 5 days after the onset of the symptoms was a criterion of inclusion, our symptoms data should be carefully considered. However, it is noteworthy to add that one of the CHIKV-positive patients developed encephalitis, the most common neurological complication reported during outbreaks in La Reunion³⁴, Guadeloupe and Martinique³⁵ islands.

It is difficult to estimate the incidence of neurological symptoms among infected patients, however, encephalopathies are the most common neurological disorders associated with CHIKV infection³⁶. On the other hand, symptoms such as fever, arthralgia, headache and myalgia are better described in CHIKV infected patients. These symptoms were the most prevalent in our positive samples, and were also observed in patients from Alagoas³³ and Sergipe³⁷ States, Northeast Brazil.

After the partial nucleotide sequencing of *E1* and *E2* genes, phylogenetic analysis indicated that the Piauí CHIKV isolates presented in this study were clustered within the ECSA genotype and formed a monophyletic clade with other Brazilian samples from 2014 to 2016. Based on the evolutionary analysis, our extracted viral RNAs share the most common recent ancestor dating from 2015, suggesting the introduction of CHIKV ECSA genotype into Piauí probably in that year. Studies performed in Pernambuco³⁸ and Alagoas³³ States, also located in the Brazilian Northeast region, have suggested that CHIKV was also introduced in these States in 2015.

Moreover, the interval between the introduction of CHIKV in Piauí in 2015, and the report of its detection made only in 2016 by the surveillance system, demonstrated a silent circulation and maintenance of the virus in the region^{18,39}. These data corroborate the finding from Costa *et al.*³³, who estimated the introduction of CHIKV in Alagoas in mid-April 2015, but the virus was detected by the surveillance system a few months later³³. Furthermore, a recent study on isolates from Rio de Janeiro, Southeast Brazil, has shown that the introduction of CHIKV ECSA genotype in the State may have taken place almost a year after its detection by the surveillance system⁴⁰. Altogether, these data corroborate our finding that CHIKV may have been circulating in Piauí up to a year before its detection.

CONCLUSION

In conclusion, we provided the first genotype surveillance of CHIKV cases during 2016/2017 in Piauí State, Northeast Brazil. Our study corroborates the circulation of the ECSA genotype in other Brazilian Northeastern States during this period, such as Bahia⁴¹, Sergipe³² and Alagoas^{33,42}. Our data also indicate the introduction of CHIKV in Piauí in 2015, and its maintenance up to 2017. Since previous studies have shown a high incidence of chronic symptoms^{7,8}, which could have social and economic impacts, more research is needed to evaluate the prevalence and impact of acute and chronic CHIKV infections in the region.

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AUTHORS' CONTRIBUTIONS

FDC and GPF conceived and designed the study. IMR, LS, NIOS and BPD carried out experiments and performed phylogenetic analyses. PAA sequenced the PCR fragments. FDC, ELTB and TCCSG collected the patient samples and performed the experiments. JOS, ACTCP and EGK contributed with reagents and materials. FDC, IMR, BPD and GPF analyzed the data and wrote the paper.

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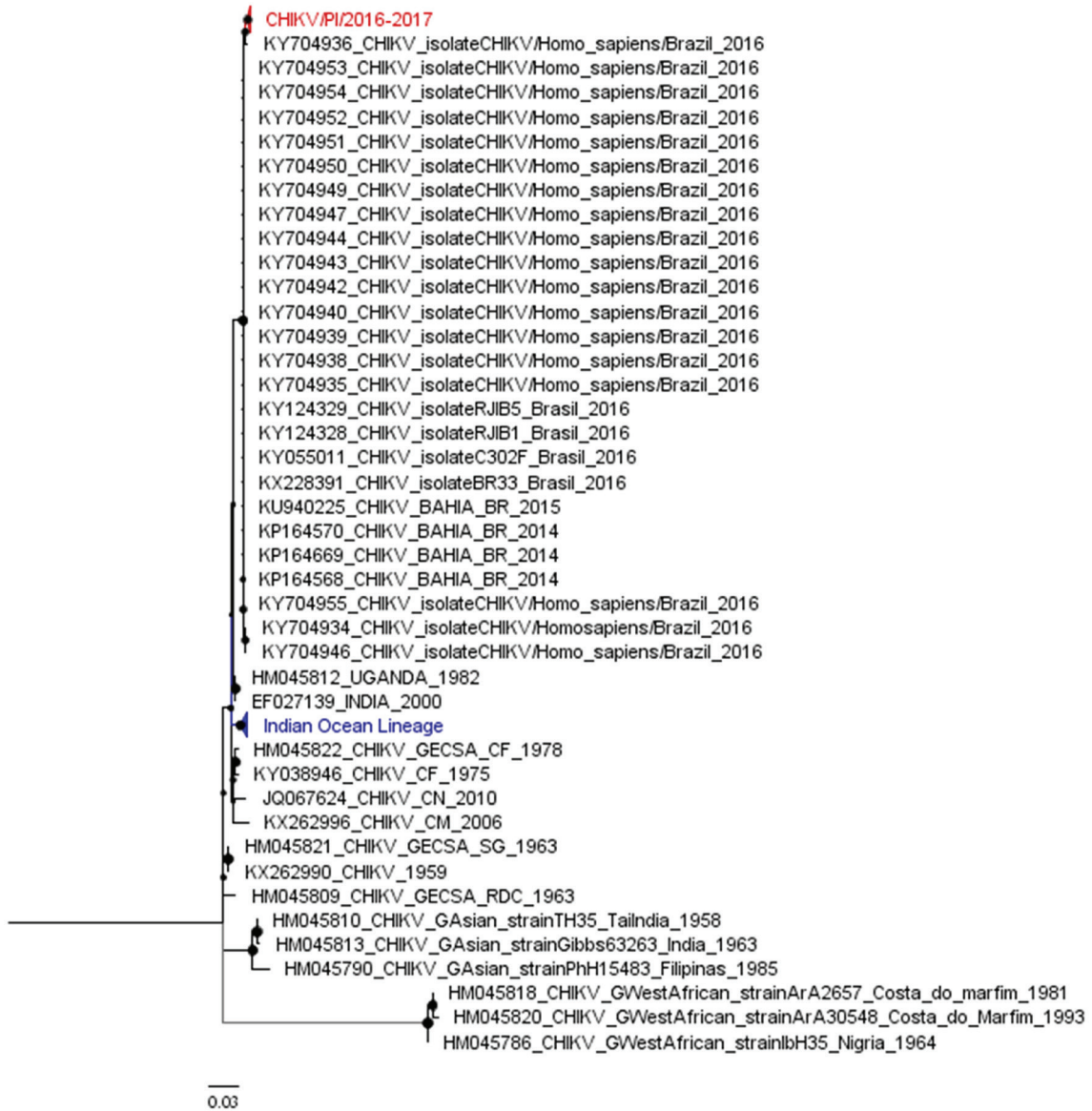
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Supplemental Figure 1 - Maximum likelihood analysis of Chikungunya virus. The maximum likelihood tree was inferred using 88 Chikungunya virus sequences (328 nt). The bootstrap values are represented by circles drawn in scale in the nodes. Clades containing strains from Piauí (2016/2017), and Indian Ocean Lineage are shown in red and blue, respectively. Branch lengths are drawn to scale or the number of substitutions per site. The tree was reconstructed using the nucleotide substitution model Kimura-2-parametters with gamma distribution (four categories) (K2+G). PI: Piauí State. GECSA: Genotype East-Central South African. Gwest African: Genotype West African. GAsian: Genotype Asian. CHIKV: Chikungunya virus.