Detection of Chikungunya Virus in Saliva and Urine Samples of Patients from Rio de Janeiro, Brazil. A Minimally Invasive Tool for Surveillance

Tiago Souza Salles
Universidade Federal do Rio de Janeiro Instituto de Quimica
Thayane Encarnação Sá-Guimarães
Universidade Federal do Rio de Janeiro Instituto de Quimica
Victor Guimarães-Ribeiro
Universidade Federal do Rio de Janeiro Instituto de Quimica
Ana Claudia Amaral Melo
Universidade Federal do Rio de Janeiro
Davis Fernandes Ferreira
Universidade Federal do Rio de Janeiro
Monica Ferreira Moreira (monica@iq.ufrj.br)
Universidade Federal do Rio de Janeiro Instituto de Quimica https://orcid.org/0000-0002-3720-415X

Short report

Keywords: Chikungunya virus, saliva and urine samples, epidemiological surveillance of arbovirus, SYBR Green assay

Posted Date: February 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-178829/v1

Detection of Chikungunya virus in saliva and urine samples of patients from Rio de Janeiro, Brazil. A minimally invasive tool for surveillance

Tiago S. Salles[1,2], Thayane E. Sá-Guimarães[1], Victor Guimarães-Ribeiro[1], Ana Claudia A. Melo[1,2], Davis F. Ferreira[3,4] and *Monica F. Moreira[1,2]

1-Federal University of Rio de Janeiro, Laboratory of Biochemistry and Molecular Biology of Vectors, Institute of Chemistry, Rio de Janeiro, RJ, Brazil.
2-National Institute of Science and Technology in Molecular Entomology, Brazil.
3- Federal University of Rio de Janeiro, Institute of Microbiology, Rio de Janeiro, RJ, Brazil.
4- Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC, USA.

*Corresponding Author
Address: Laboratório de Bioquímica e Biologia Molecular de Vetores, Departamento de Bioquímica, Instituto de Química, Caixa Postal 68563, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, 21941-909, Brasil
Telephone Number: +55 21 3938 73 65
Fax Number: +55 21 3938 72 66
E-mail: monica@iq.ufrj.br

Abstract

In this study, we collected saliva and urine samples from individuals in the metropolitan region of Rio de Janeiro, Brazil, during the years of 2017 through 2019 and we were able to detect the presence of Chikungunya virus genome in these samples. Our findings reinforce the possibility to monitor Chikungunya virus circulation by analyzing saliva and urine from individuals during inter-epidemic periods.

Keywords: Chikungunya virus, saliva and urine samples, epidemiological surveillance of arbovirus, SYBR Green assay.

Chikungunya virus (CHIKV) is a mosquito-borne virus, transmitted in the urban area mainly by the *Aedes aegypti* and *Aedes albopictus* mosquitoes[1]. CHIKV belongs to family *Togaviridae* and genus *Alphavirus* and consists of a single-stranded RNA genome virus, about 70 nm diameter and a phospholipid envelope[2]. This virus was first described in Africa (Tanzania) in 1954 and later identified in Asia, and it was responsible for outbreaks in these two continents from the 1960s to the 1980s [3]. Currently, CHIKV is considered a real threat to countries localized in temperate and tropical zones that are infested by Aedes spp, such as Europe and the Americas [3,4].

Human CHIKV infection results in a spectrum of manifestations and it begins with a silent incubation period lasting 2–4 days on average (range 1–12 days) [5], which can evolve to either an asymptomatic and subclinical outcome or to clinical manifestations. Clinical onset is abrupt, with high fever, headache, back pain, myalgia, and arthralgia; the latter can be intense, affecting mainly the extremities (ankles, wrists, phalanges) but also large joints, referred to as the effect of the incapacitating arthralgia [6].

Our group collected saliva and urine from volunteers presenting symptoms compatible with the disease, except for one healthy individual (male) from which saliva was collected. The individuals that had saliva collected were one male and four females. The individuals that had urine collected were two males and four females. Three females donated both, saliva and urine. The samples were collected from individuals at the Federal University of Rio de Janeiro, using approved Protocol Ethics: 80709 HUCFF/FM/UFRJ from 2017 through 2019. Volunteers were students, student relatives and staff from the University Campus, and the ages were between 25 and 45 years old.

The saliva samples (n = 5) were obtained from five individuals with arbovirus-like symptoms and one asymptomatic individual. In the urine group (n = 6) all individuals were exhibiting arbovirus-like symptoms. The total number of samples, therefore, was eleven (n = 11) and the total number of individuals was eight (n=8). Individual samples were submitted to RNA extraction by the TRIzol

69 ™ method Reagent (Invitrogen, Carlsbad, California, USA), followed by a
70 Reverse Transcriptase (RT) assay by superscript IV (Invitrogen, Carlsbad,
71 California, USA), following the manufacturer recommendation. CHIKV RNA
72 detection was performed by using RT 2-step-qPCR and conventional PCR, using
73 the forward 5'-ctttggagccaacgctatcgctt-3' (SGCK-F) and reverse 5'-
74 tttgtccttgcactctgctgta-3' (SGCK-R) primers in the standardization SYBR Green
75 qPCR assays. For the conventional PCR, Nested methodology was used in the
76 first reaction with the forward 5'-taccgtataagactctagtc-3' (Nestalpha-F) and
77 reverse 5'-tgaatgtccccaaatcttccagg-3' (Nestalpha-R) primers, followed by the
78 second reaction with primers (SGCK-F/SGCK-R). The amplified viral material
79 was visualized on a 1.5% (v/v) agarose gel (Fig. 1D).
80
81 The standardization of the SYBR GREEN qPCR assay showed a efficiency of
82 93.1% and specific for the detection of CHIKV genetic material as shown in (Fig.
83 1A and 1B, Table 1A). The qPCR results showed that all urine samples were
84 positive for CHIKV RNA, including the sample from the asymptomatic individual.
85 Four saliva samples were positive, and one was negative. The negative saliva
86 sample was from the donor that had arbovirus-like symptoms but donated saliva
87 only. All two individuals that had symptoms and donated both saliva and urine
88 had both samples positive. Overall, we had ten positive samples for CHIKV and
89 one negative (Fig. 1E and Table 1B). The generic RT-PCR for alphaviruses
90 showed positive results, and direct sequencing of the viral amplicons showed a
91 CHIKV-specific sequence. The result of the Sanger sequencing was used as

query for BLAST searches of NCBI. In order to identify query sequences were used to search sequences on the CHIKV genome on the NCBI genome database, using BLASTn [7]. Sequences resulting from these searches, showing more than 90% identity were deemed as homologous. All the sequences from the homologous were then used as Datasets to the aligned using the MUSCLE software [8]and then pruned for removal of regions with a high frequency of indels using TrimAL using the "-gappyout" comman [9].

The maximum likelihood (ML) tree topology was inferred with the Iq-Tree 1.6 program [10]. Branch support was assessed by the ultrafast bootstrap implementation of IqTree using 1,000 replicates [11]. IqTree was executed via the command "iqtree -s infile -bb 1000". Because no outgroup was included in our analysis, rooting of the genome CHIKV genealogy was performed using the minimal ancestor deviation method of Tria at al. [12]. Fig 2 shows the phylogenetic tree indicating in red the RIO_2019_CHIKV genome sequenced in the work. Our results are similar to those of Musso *et. al.* [13] where they reported high number of CHIKV-positive samples during a period of high CHIKV prevalence, agreeing with the epidemiological data of the metropolitan region of Rio de Janeiro at the time of collection [4,14].

In the context of endemic co-circulation of other arbovirus diseases transmitted by *Ae. aegypti* such as dengue and zika fevers [14,15], a fast, reliable and noninvasive diagnostic method is important for surveillance and early detection of an increase in the number of infections. In this study, we show that it is possible

to detect CHIKV in saliva and urine samples of symptomatic and asymptomatic individuals. The time of sample collection in this study correlated to an outbreak in Rio de Janeiro city. Considering the endemicity of CHIKV in the state, our results strengthen the use of saliva (preferable) and urine as important tools for detecting asymptomatic individuals during inter-epidemic and pre-epidemic periods.

Ethical approval

The Ethical Committee of RJ/BR Rio de Janeiro (80709 HUCFF/FM/UFRJ) has approved this study.

Declaration of interest

Declarations of interest: none

Author contributions

All authors collaborated in the study design; TSS, TESG, VGR, participated in diagnosing and managing these eight patients; TSS, ACAM, and MFM participated in epidemiological investigation; TSS, DFF, and MFM extracted and analyzed the clinical data; TSS and VGR prepared the first manuscript draft; DFF, ACAM and MFM modified the manuscript subsequently; all authors have reviewed and approved the final manuscript.

Acknowledgments

The authors are grateful to the Program for Technological Development in Tools for Health-RPT-FIOCRUZ for use of its facilities.

Formatting of funding sources

This work was supported by the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ-E-26/201.331/2016-REDE ZIKA), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT-EM/CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Reference

1. Sergon K, Njuguna C, Kalani R, Ofula V, Onyango C, Konongoi LS, et al. Seroprevalence of Chikungunya virus (CHIKV) infection on Lamu Island, Kenya, October 2004. American Journal of Tropical Medicine and Hygiene. 2008;78:333–7.
2. Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. Microbiological Reviews [Internet]. 1994;58:491 LP – 562. Available from: http://mmbr.asm.org/content/58/3/491.abstract
3. Simon F, Savini H, Parola P. Chikungunya: A Paradigm of Emergence and Globalization of Vector-Borne Diseases. Medical Clinics of North America. 2008. p. 1323–43.
4. Maljkovic Berry I, Rutvisuttinunt W, Sippy R, Beltran-Ayala E, Figueroa K, Ryan S, et al. The origins of dengue and chikungunya viruses in Ecuador following increased migration from Venezuela and Colombia. BMC Evolutionary Biology [Internet]. 2020;20:31. Available from: https://doi.org/10.1186/s12862-020-1596-8
5. Pialoux G, Gaüzère BA, Jauréguiberry S, Strobel M. Chikungunya, an epidemic arbovirosis. Lancet Infectious Diseases. 2007;7:319–27.
6. Enserink M. Massive Outbreak Draws Fresh Attention to Little-Known Virus. Science [Internet]. 2006;311:1085 LP – 1085. Available from: http://science.sciencemag.org/content/311/5764/1085.1.abstract
7. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. Database indexing for production MegaBLAST searches. Bioinformatics (Oxford, England). 2008;24:1757–64.

8. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research. 2004;32:1792–7.
9. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics (Oxford, England). 2009;25:1972–3.
10. Trifinopoulos J, Nguyen L-T, von Haeseler A, Minh BQ. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Research [Internet]. 2016;44:W232–5. Available from: https://doi.org/10.1093/nar/gkw256
11. Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. UFBoot2: Improving the Ultrafast Bootstrap Approximation. Molecular biology and evolution. 2018;35:518–22.
12. Tria FDK, Landan G, Dagan T. Phylogenetic rooting using minimal ancestor deviation. Nature ecology & evolution. England; 2017;1:193.
13. Musso D, Teissier A, Rouault E, Teururai S, de Pina JJ, Nhan TX. Detection of chikungunya virus in saliva and urine. Virology Journal [Internet]. Virology Journal; 2016;13:1–4. Available from: http://dx.doi.org/10.1186/s12985-016-0556-9
14. Ministério da Saúde. Monitoramento dos casos de Arboviroses urbanas transmitidas pelo Aedes (dengue, chikungunya e Zika). Boletim Epidemiológico Arboviroses [Internet]. 2019;51:1–13. Available from: https://portalarquivos2.saude.gov.br/images/pdf/2020/janeiro/20/Boletim-epidemiologico-SVS-02-1-.pdf
15. Salles TS, da Encarnação Sá-Guimarães T, de Alvarenga ESL, Guimarães-Ribeiro V, de Meneses MDF, de Castro-Salles PF, et al. History, epidemiology and diagnostics of dengue in the American and Brazilian contexts: a review. Parasites & Vectors [Internet]. 2018;11:264. Available from: https://doi.org/10.1186/s13071-018-2830-8

Table 1 - standardization and amplification of samples by qPCR SYBR GREEN assay

A) performance and Standardization for the SYBR GREEN assay using Chikungunya virus with specific primers (SGCK-F / SGCK-R)	
Virus dilution #	***Average CT in triplicate***

1:10	15,54
1:100	17,48
1:1000	20,85
1:10000	24,47
1:100000	28,19
1:1000000	31,92
1:10000000	36,79
H_2O	**N/A**

B) amplification for the SYBR GREEN assay using Chikungunya virus, saliva and urine samples with the specific primers (SGCK-F / SGCK-R)

Sample code	***Sample types***	***Average CT in triplicate***
16	saliva	N/A
44	saliva	31,74
45	urine	33,41
46	saliva	33,11
47	urine	36,69
48	urine	35,88
49	saliva	36,02
50	urine	35,61
51	saliva	34,65
52	urine	35,84
53	urine	35,85
Positive control #	1:10	14,97
Positive control #	1:1000	22,71

Negative control #	-	**N/A**
H_2O	-	**N/A**

* cDNA from the Vero cell supernatant

* cDNA from the CHIKV suspension

Efficiency of the qPCR assay equals to 93.1% ($E=10^{(-1/\alpha)-1}$)

199

200

201 **Legend**

202 **Fig.1**:(A) Amplification curve for the qPCR standardization and sensitivity
203 determination and (B) Melting pattern of the serial dilution curve for the CHIKV
204 cDNA for the standardization with the SGCK-F / SGCK-R primers for qPCR with
205 SYBR Green. (C) Amplification curve for the urine and saliva samples and (D)
206 Melting pattern of the serial dilution curve for the CHIKV cDNA (positive
207 control), saliva and urine cDNA samples for quantification with the SGCK-F /
208 SGCK-R primers for qPCR. (E) Linear regression for the serial dilution curve
209 with the CHIKV cDNA for standardization with the SGCK-F / SGCK-R primers in
210 qPCR with SYBR Green, which made it possible to determine the efficiency of
211 the reaction in the value of 93,1%. (F) qPCR gel for the cDNAs standard CHIKV
212 cDNA with the SGCK-F / SGCKR primers; MM: molecular mass; B: blank;
213 CHIKV, Chikungunya virus; MAYV, Mayaro virus; SINDV, Sindbis virus; FLAV,
214 pool of flavivirus (DENV1-4, YFV, ZIKV). (G) qPCR gel for the CHIKV cDNAs
215 (saliva and urine) and standard CHIKV cDNA with the SGCK-F / SGCKR
216 primers; MM: molecular mass; B: blank; red: saliva; blue: urine samples

217

Fig 2. Phylogenetic tree using the sequences searched in BLASTn as dataset to alignment in MUSCLE software and using IQTREE to the construction of the phylogenetic tree, highlight in red the RIO2019CHIKV genome sequenced this work.

Figures

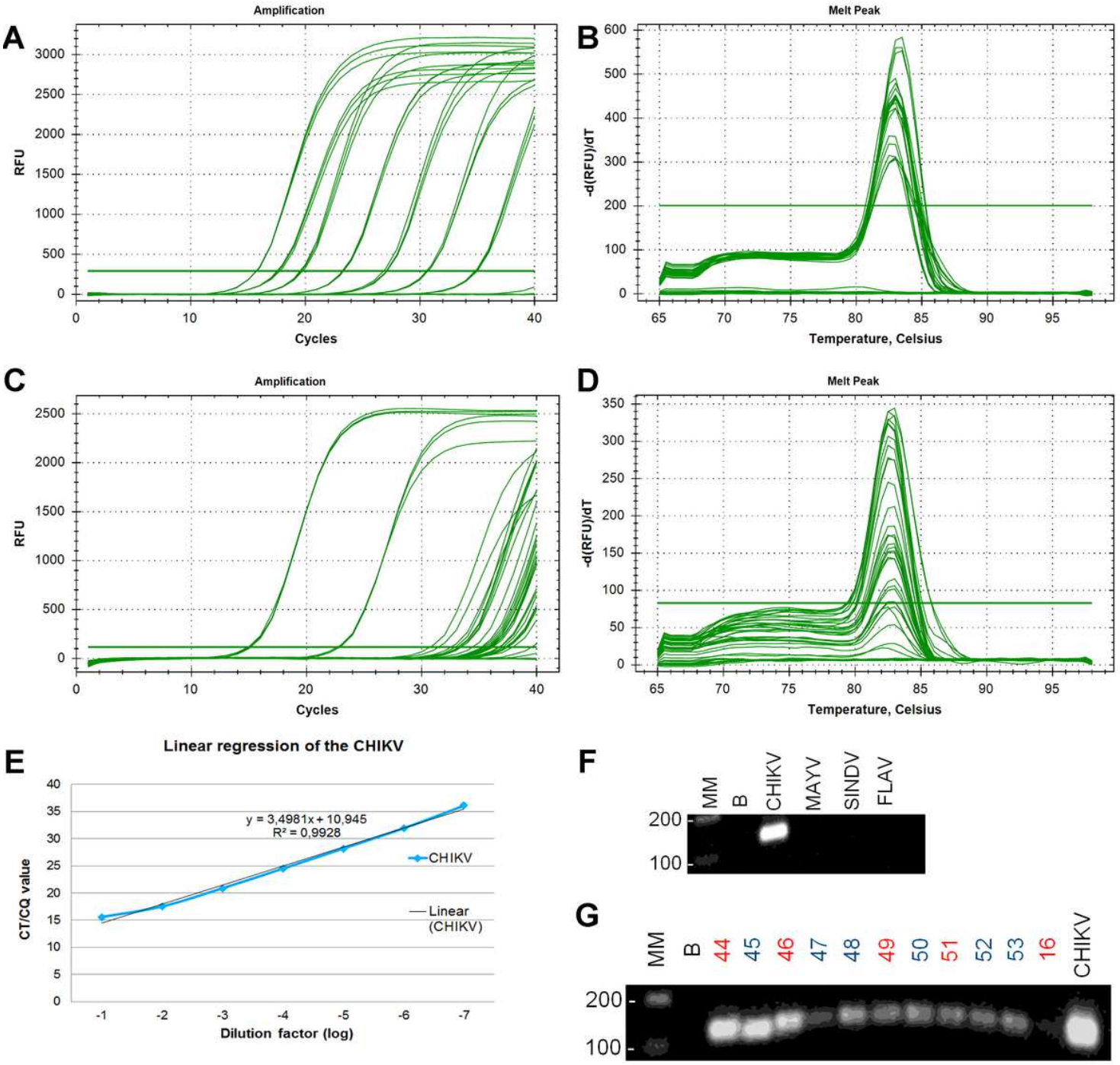

Figure 1

(A) Amplification curve for the qPCR standardization and sensitivity determination and (B) Melting pattern of the serial dilution curve for the CHIKV cDNA for the standardization with the SGCK-F / SGCK-R primers for qPCR with SYBR Green. (C) Amplification curve for the urine and saliva samples and (D) Melting pattern of the serial dilution curve for the CHIKV cDNA (positive control), saliva and urine cDNA samples for quantification with the SGCK-F / SGCK-R primers for qPCR. (E) Linear regression for the

serial dilution curve with the CHIKV cDNA for standardization with the SGCK-F / SGCK-R primers in qPCR with SYBR Green, which made it possible to determine the efficiency of the reaction in the value of 93,1%. (F) qPCR gel for the cDNAs standard CHIKV cDNA with the SGCK-F / SGCKR primers; MM: molecular mass; B: blank; CHIKV, Chikungunya virus; MAYV, Mayaro virus; SINDV, Sindbis virus; FLAV, pool of flavivirus (DENV1-4, YFV, ZIKV). (G) qPCR gel for the CHIKV cDNAs (saliva and urine) and standard CHIKV cDNA with the SGCK-F / SGCKR primers; MM: molecular mass; B: blank; red: saliva; blue: urine samples

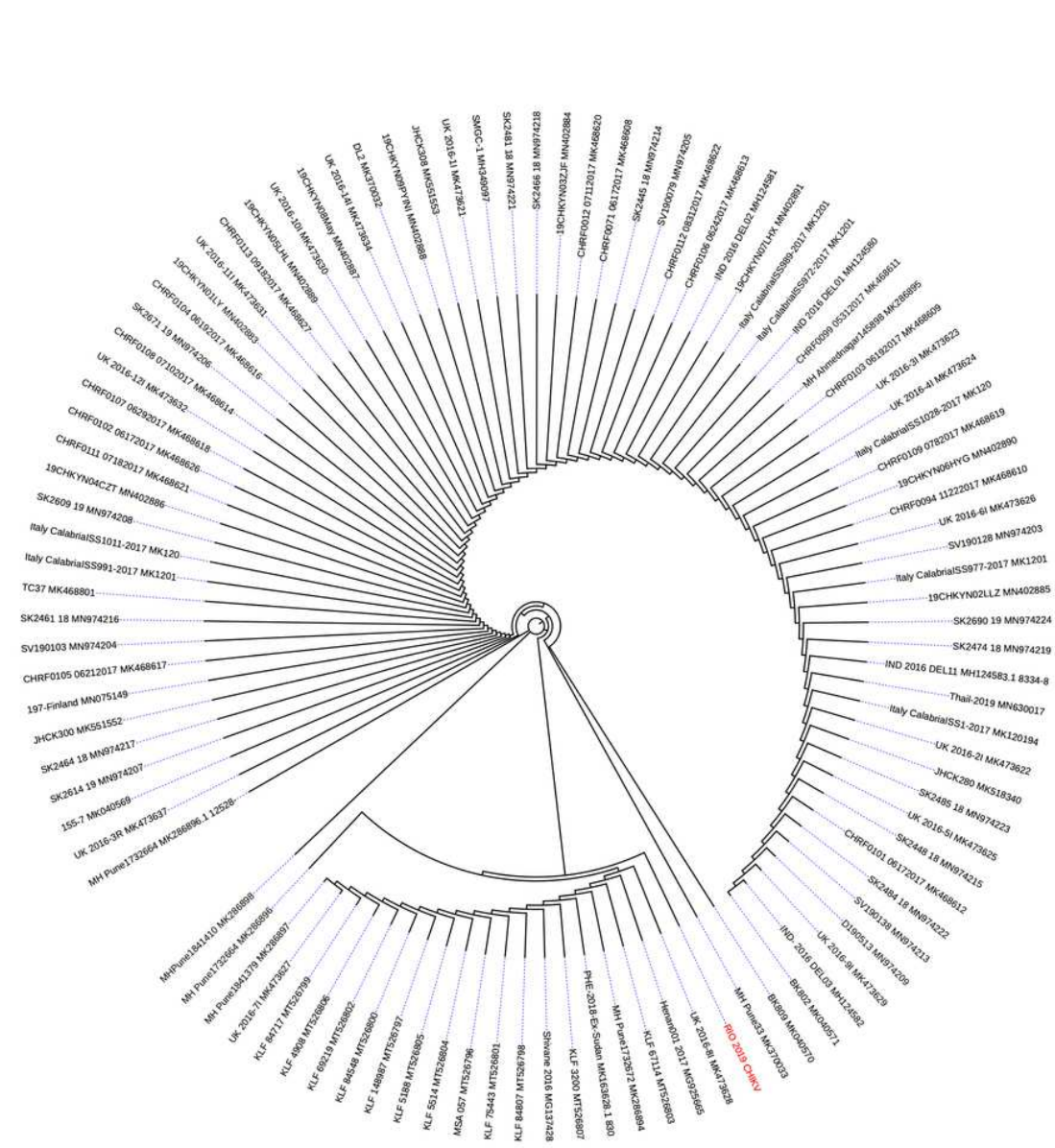

Figure 2

Phylogenetic tree using the sequences searched in BLASTn as dataset to alignment in MUSCLE software and using IQTREE to the construction of the phylogenetic tree, highlight in red the RIO2019CHIKV genome sequenced this work.