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Short Communication

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[1,2], Thayane Encarnação Sá-Guimarães[1], Victor Guimarães-Ribeiro[1], Ana Claudia Amaral Melo[1,2], Davis Fernandes Ferreira[3,4] and Monica Ferreira Moreira[1,2]*

[1]*Federal University of Rio de Janeiro, Laboratory of Biochemistry and Molecular Biology of Vectors, Institute of Chemistry, Brazil*

[2]*National Institute of Science and Technology in Molecular Entomology, Brazil*

[3]*Federal University of Rio de Janeiro, Institute of Microbiology, Brazil*

[4]*Department of Molecular and Structural Biochemistry, North Carolina State University, USA*

***Corresponding author:** Monica Ferreira Moreira, 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

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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.

Abbreviations: CHIKV: Chikungunya Virus; qPCR: Quantitative Polymerase Chain Reaction; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; ML: Maximum Likelihood

Introduction

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™ method Reagent (Invitrogen, Carlsbad, California, USA), followed by a Reverse Transcriptase (RT) assay by superscript IV (Invitrogen, Carlsbad, California, USA), following the manufacturer recommendation. CHIKV RNA detection was performed by using RT 2-step-qPCR and conventional PCR, using the forward 5'-ctttggagccaacgctatcgctt-3' (SGCK-F) and reverse 5'-tttgtccttgcactctgctgta-3' (SGCK-R) primers in the standardization SYBR Green qPCR assays. For the conventional PCR, Nested methodology was used in the first reaction with the forward 5'-taccgtataagactctagtc-3' (Nestalpha-F) and reverse 5'-tgaatgtccccaaatcttccagg-3' (Nestalpha-R) primers, followed by the second reaction with primers (SGCK-F/SGCK-R). The amplified viral material was visualized on a 1.5% (v/v) agarose gel (Figure 1D).

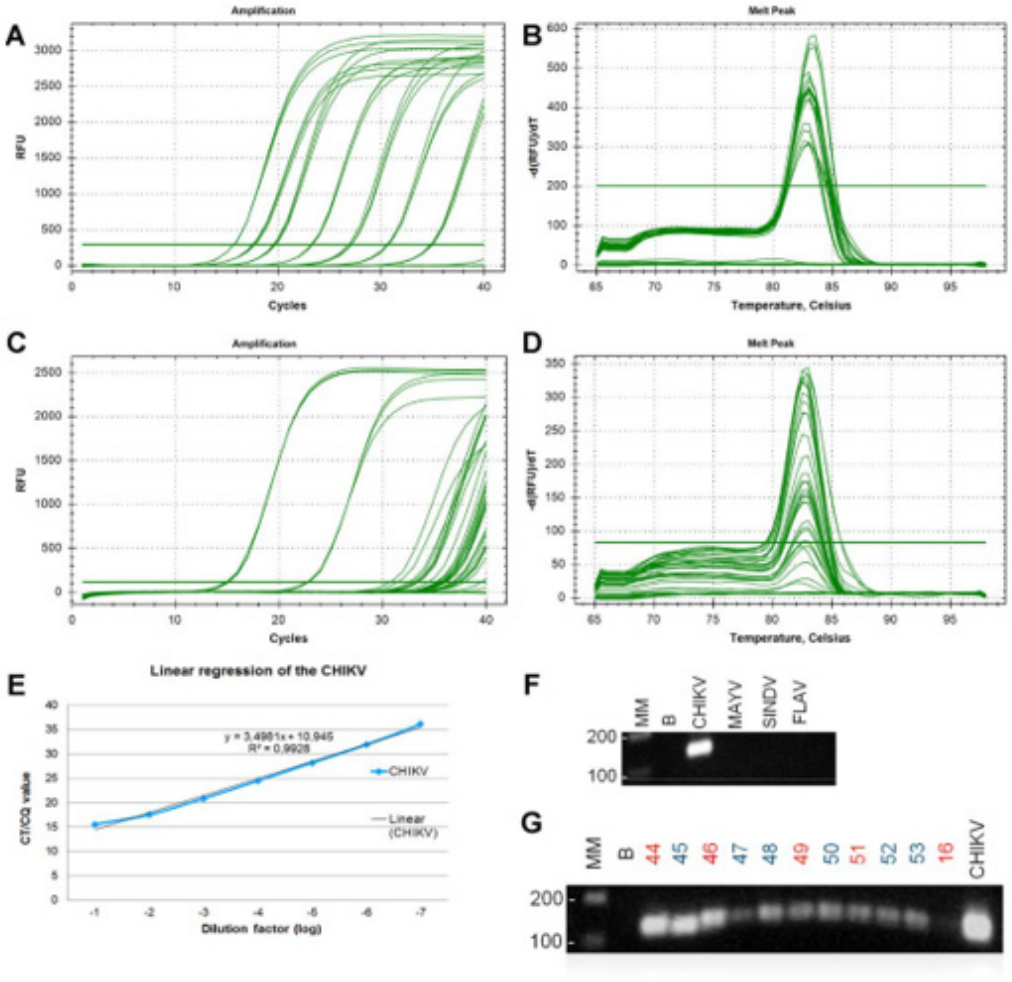

Figure 1: (A) Amplification curve for the qPCR standardization and sensitivity determination and (B) Melting pattern of the serial dilution curve for the CHIKV cDNA for the standardization with the SGCK-F / SGCK-R primers for qPCR with SYBR Green. (C) Amplification curve for the urine and saliva samples 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

The standardization of the SYBR GREEN qPCR assay showed a efficiency of 93.1% and specific for the detection of CHIKV genetic material as shown in (Figure 1A & 1B) (Table 1). The qPCR results showed that all urine samples were positive for CHIKV RNA, including the sample from the asymptomatic individual. Four saliva samples were positive, and one was negative. The negative saliva sample was from the donor that had arbovirus-like symptoms but donated saliva only. All two individuals that had symptoms and donated both saliva and urine had both samples positive. Overall, we had ten positive samples for CHIKV and one negative (Figure 1E) (Table 1). The generic RT-PCR for alphaviruses showed positive results, and direct sequencing of the viral amplicons showed a CHIKV-specific sequence. The result of the Sanger sequencing was used as query for BLAST searches of NCBI.

Table 1: Standardization and amplification of samples by qPCR SYBR REEN assay.

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

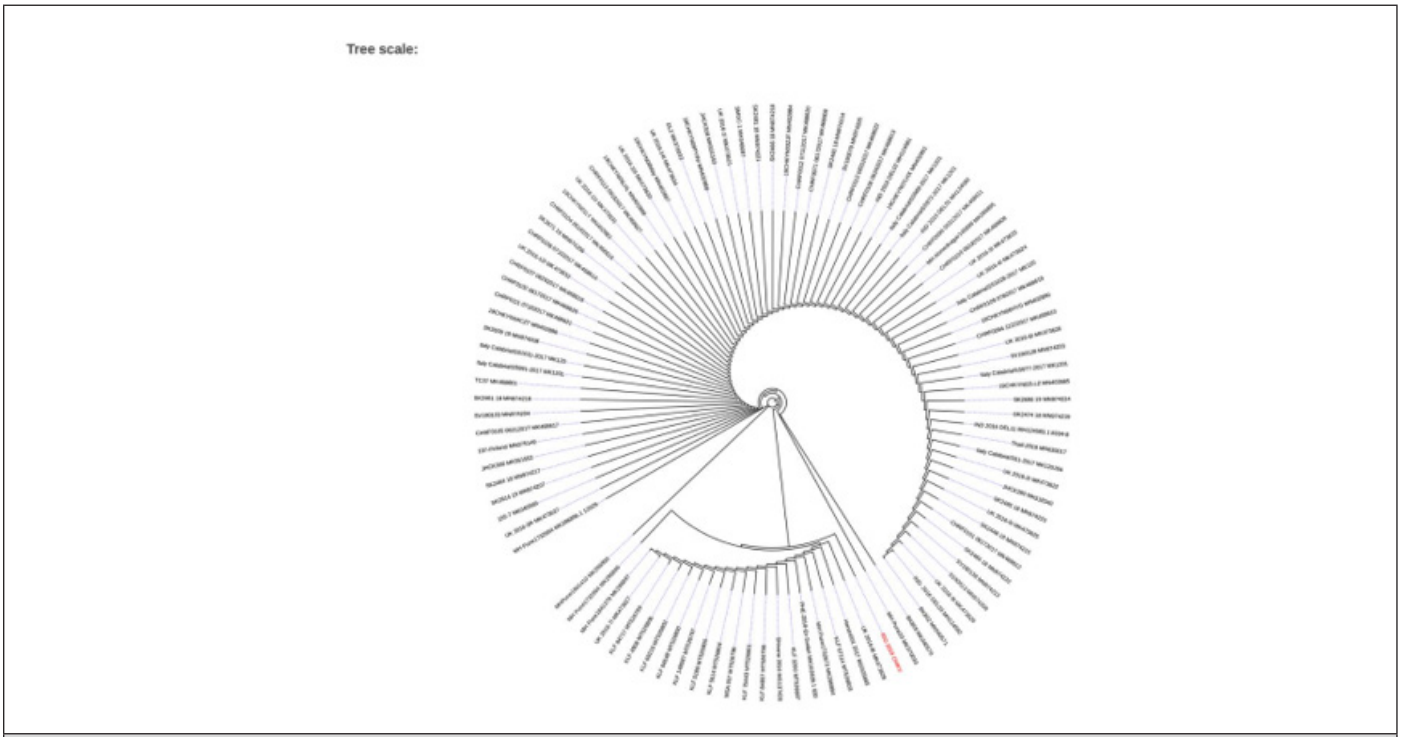

Figure 2: Phylogenetic tree using the sequences searched in BLASTn as dataset to alignment in MUSCLE software and using IQTREE to the construction of the phylogenetic tree, highlight in red the RIO2019CHIKV genome sequenced this work.

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 et al. [12]. Figure 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 with the new primers presented by the SYBR GREEN assay. 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 literature data when the use of saliva (preferable) and urine as important tools for detecting asymptomatic individuals during inter-epidemic and pre-epidemic periods.

Ethics Approval and Consent to Participate

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

Availability of data and material

All the supporting data generated for this manuscript and its conclusions is available in the text. No other supporting material is required.

Competing Interests

The authors declare that they have no competing interests.

Consent for publication

Written consent was obtained from the patients / family for publication.

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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.

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