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Letter to the Editor

Viral metagenomics in nasopharyngeal swabs of Brazilian patients negative for SARS-CoV-2 unveils the presence of Chikungunya virus infection

Dear editor,

We read with great interest the manuscript published by Le et al. reporting the detection of Rhinovirus and SARS-CoV-2 co-infection by viral metagenomics [1]. The application of metagenomics is useful for the management of COVID-19 patients and gives essential information for the presence of co-infections which additionally can worsen the clinical prognosis. The authors of the above-cited study tested exclusively patients with a SARS-CoV-2 confirmed infection. A syndromic surveillance data obtained in Brasilia, Brazil showed that only 33% of patients were SARS-CoV-2 positive. We therefore applied viral metagenomics on SARS-CoV-2 negative samples in order to characterize the co-circulating respiratory viruses in the Federal District of Brazil. Currently, pan-pathogen assays based on viral metagenomics uncover a variety of fungus, bacterial and viral co-infections in COVID-19 patients [2,3] which justifies the application of metagenomics in order to detect other circulating respiratory viruses. Moreover, there is no information on which respiratory viruses most commonly co-circulate in the examined Brazilian region.

In this study, samples were obtained from residents of the Cidade Estrutural, Federal District of Brazil, who sought primary healthcare for suspicion of COVID-19. The ecological and demographic characteristics of the examined region (Fig. 1A) favor the emergence and re-emergence of viral infections including respiratory, food- and arthropod-borne. Patients with COVID-19-like symptoms for 3 to 10 days who sought medical assistance at a primary health center at the Cidade Estrutural, between March and May 2020 were included. All of them signed a written informed consent approved by the Ethics Commission of the Faculty of Medicine, University of Brasília (CEP-FM/UnB, CAAE 39,892,420.7.1001.5558; CAAE 40,557,020.6.3001.5553) and Fundação de Ensino e Pesquisa em Ciências da Saúde (FEPECS/SES/DF, CAAE 40,557,020.6.3001.5553). Nasopharyngeal swabs (NS) were routinely collected for SARS-CoV-2 molecular diagnosis. The viral metagenomics was performed on 160 patients (58 males and 102 females; average age of 33±12.34 years of age) who presented negative SARS-CoV-2 RT-PCR results. The NS were initially centrifuged at low speed for cell depletion and pretreated with 20 U DNAse per sample (Ambion). Eight samples were then pooled and subjected to RNA extraction, reverse transcription and amplification as previously described [4]. Genomic libraries were prepared using Illumina DNA prep kit (Illumina) with the IDT for Illumina DNA/RNA UD indexes following the manufacturer's instructions. The sequencing was performed in Illumina NovaSeq 6000 platform using the NovaSeq 6000 S1 Reagent Kit (300 cycles) (Illumina). The virome abundance was accessed using a previously established bioinformatic pipeline [4]. In brief, the pipeline was composed of FastQC v.0.11.08, Trimmomatic v.0.3.9, Cutadapt v. 2.4, AfterQC v. 0.9.7, Kraken2 v.2.0.8, Spades 3.13.0, and Diamond 0.9.29 software. The sequence alignment and editing of the important contigs was performed using MAFFT v7.453 and Aliview programs. The maximum likelihood tree was reconstructed using IQ-TREE v1.6.12 with a statistical support of 1000 bootstrap replicates.

The metagenomic analysis of 160 NS samples assembled into 20 pools revealed Chikungunya virus (CHIKV) genomic reads into two pools (namely, 11 and 18). These two pools generated a total abundance of 10,654,714 reads from which just 3357 were classified as viral (0.03%), which was normal as viruses did not make part of the normal microbial composition of the nasopharynx. We classified as CHIKV 46 reads in pool 11 and 92 reads in pool 18. The identification of CHIKV reads in NS puzzled us and therefore we proceeded to test samples from the CHIKV-positive pools using Reverse Transcription PCR (RT-PCR). RT-PCR assays were carried out with two previously described sets of primers detecting both circulating in Brazil CHIKV genotypes [5] in Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System. Two samples of the NS showed RT-PCR positive results for CHIKV, one sample from each CHIKV-positive pool. The amplification threshold (Ct) of both samples demonstrated well-presented viral load (Cts were 28 and 26 respectively) (Fig. 1B). The positive samples belonged to patients with suspicion of SARS-CoV-2 infection. One of them was a female, 33 years old patient who reported fever, headache, jaundice, myalgia and earache. The other patient was male individual with 41 years of age, who reported fever, myalgia, retro-orbital pain, cough, headache, algesia, jaundice, anosmia, hyporexia and consciousness changes.

Here, the potential of metagenomics to detect unsuspected viral agents in any type of clinical sample has been demonstrated, while it was also revealed that arthropod-borne viruses (arboviruses) were largely neglected during the SARS-CoV-2 pandemic. While the NS is a common procedure to diagnose influenza, SARS, MERS-CoV, COVID-19 and other respiratory infections, the sampling of blood is the procedure of choice for arboviral diagnosis [6]. Although saliva has also been a suitable clinical sample for CHIKV RNA detection during the first week after symptoms onset [7,8], to our knowledge this is the first report showing the metagenomic evaluation and confirmation of CHIKV RNA in NS samples. The detection of CHIKV RNA was only possible due to the application of metagenomics, since CHIKV infection was not suspected as a causative agent of the reported symptoms. The presence of multiple viruses co-circulating in a symptomatic population may hide the presence of less-expected viral agents, mainly during the COVID-19 pandemic, which was our case. Therefore, viral metagenomics is a powerful diagnostic tool not only for analysis of the viral diversity in clinical samples but also provides important information regarding epidemiological surveillance and circulating

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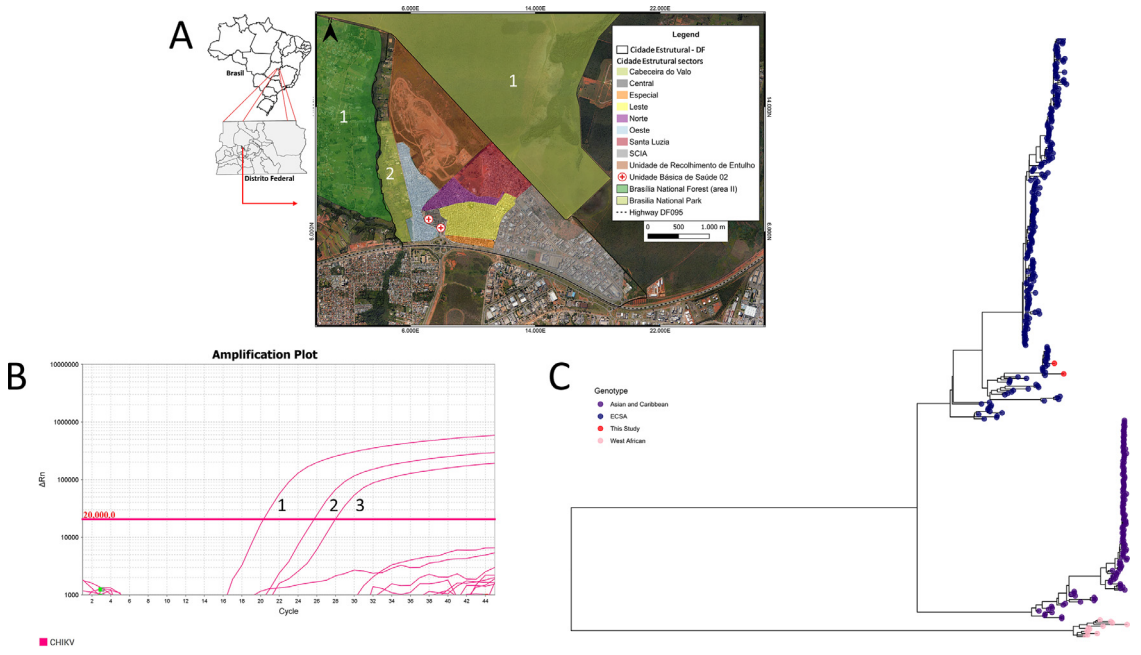

Fig. 1. Estrutural City location map, CHIKV confirmatory RT-qPCR assay and maximum likelihood tree. A) The Cidade Estrutural has 40,000 inhabitants of whom 4000 are waste pickers and has the lowest HDI of the Federal District of Brazil. The city borders the Brasilia National Forest, which is an environmental protection area of 423.6 km^2 characterized by the typical wooded savanna (Cerrado) (landmark 1), and Cabeceira do Valo, where residents often farm vegetables (landmark 2); B) Representative amplification plot of confirmatory RT-qPCR assay showing the CHIKV positive control (observe 1), positive sample from pool 18 (observe 2) and positive sample from pool 11 (3). C) Approximate maximum likelihood tree of the obtained Chikungunya virus (CHIKV) contigs during the metagenomic analysis of nasopharyngeal swabs (2 contigs of 655 and 718 bp belonging to the nonstructural polyprotein). In the phylogenetic reconstruction 397 complete CHIKV genomes obtained from the GenBank were used under the GTR+G4+*F* nucleotide substitution model with a statistical support of 1000 bootstrap replicates. The phylogenetic tree showed 3 major clades comprising the CHIKV genotypes. Our samples (red dots) were clustered along the CHIKV East-Central-South African genotype, which by far is the most common genotype in Brazil.

viruses [9]. In support of this, we performed phylogenetic analysis of the obtained viral contigs, which identified the circulation of ECSA CHIKV genotype, the most widely spread CHIKV genotype in Brazil (Fig. 1C).

In summary, our study demonstrates the use of viral metagenomics for identification of unsuspected viral agents in NS of patients showing respiratory symptoms, but negative for SARS-CoV-2 RNA. This investigation draws the attention to the circulation of viruses, which are clinically important but have been largely neglected/unsuspected during the SARS-CoV-2 pandemic but must be included in the differential diagnosis of the patients. Despite the significant advantages of the metagenomics for virus identification, some issues like cost-efficiency, need of high-cost equipment and laboratory expertise must be carefully analyzed in regards to metagenomic application for diagnostic purposes especially in resource-limited countries.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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