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Short Communication

SARS-CoV-2 diagnostic diary: from rumors to the first case. Early reports of molecular tests from the military research and diagnostic institute of Rio de Janeiro

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ABSTRACT

According to the World Health Organization(WHO) viral diseases such as COVID-19 represent a serious threat to global health. Regarding this recent pandemic we present a historical timeline of last months in a diagnostic lab. Initially, an alert was issued to us on January 22 (D) about the COVID-19cases in Brazil. In line with international standards, 30 days after (D30), the primers and probes for quantitative Polimerase Chain Reactionrecommended by WHO were*in silico* tested and obtained by our institute. The first case of the new coronavirus in Brazil was confirmed in D36, on D44 the first case was confirmed in our state and on D56 our institute diagnosed the firstcase. Subsequently, on D66 the first two positive cases of members from our institute were reported and on D74 the first member of our molecular diagnosis team

(MDT) tested positive. Until D84 (~4%) of our institute members tested positive. In our conditions, after around 900 tests, we recommendthe combination ofN1/N2 primer sets (CDC protocol) for preliminary assays and mandatory confirmation for positive results using Charité protocol.Moreover, we recommend that every professional be tested before starting work, in addition to weekly tests for everyone involved preventing the reduction of ability to respond to the pandemic. For the future, the widespread utilizationof new sequencing technologiesmay be powerful tool in detecting the real pathogens in every sample, coinfections, multiple strains, phylogenomical, and phylogeographical interpretations and will contribute to faster and more accurate responses in epidemics.

key words: Coronavirus, SARS-CoV-2, qPCR, Global Pandemic, COVID-19

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INTRODUCTION

According to the World Health Organization(WHO), viral diseases continue to emerge and represent a serious threat toglobal health.[1-3]Viral epidemics such as Severe Acute Respiratory Syndrome(SARS), Middle East Respiratory Syndrome(MERS), and the recent coronavírus(COVID-19), all caused by coronavirus subtypes, clearly illustrate the grave danger posed by these pathogens.[4-6]

The tools used for the accurate diagnosis of these viral infections must have high sensitivity, specificity and preferably, affordability for the benefit of the entire population.[7]The diagnosis of pathologies related to respiratory viruses in Brazil is mostly clinical, with low requisition of confirmatory laboratory tests, which results in the underreporting of these infections.[8]Polymerase Chain Reaction(PCR)singleplex tests have emerged in recent times, but they have low resolvability due to the similarity

of clinical signs and symptoms shared by a large number of pathogens that cause respiratory viral infections.[9]Thus, these tests have gradually evolved into viral multiplex PCR panels with great prospects for diagnostic improvement. However, with the cost reachingup to $500 per patient, multiplex tests are expensive and inaccessibleto the majority population.[9]In addition to pathogens such as rhinovirus, influenza and H1N1, the limited panels currently available also screen for four subtypes of coronavirus already occurring in our environment: 229E, NL63, HKU1 e OC43.[10]

The Brazilian Armed Forces, represented by its health system and the Chemical, Biological, Radiological, and Nuclear defense system(CBRN),mobilize their resources to tackle relevant threats that reach national/international levels and require actions of biological defense. In our country, in accordance with the federal law,ensuring effective biosafety is one of the responsibilities of the Brazilian Army. The Army's role in this area makes it a key player in the public health services in peacetime and keeps it trained for providing medical aid in times of conflict. A part of this systemincludes theBrazilian Army Institute of Biology(IBEx), a clinical research institute closely associated withother Brazilian research institutions, such as Federal University of Rio de Janeiro(UFRJ) and Oswaldo Cruz Institute(IOC/Fiocruz). Our institute receives clinical samples of military personnel (active or retired) and their dependents from two most important Brazil cities (Rio de Janeiro and São Paulo). The objective of this Fast Track is to present a historical timeline of pandemic dynamics in a reference lab and contribute to diagnostic methods in face of the growing need for tests.

TIMELINE OF METHODS DEVELOPMENT AND DIARY RESULTS

Initially, an alert was issued to us on January 22(D) about the imminent possibility of COVID-19 cases in Brazil. As a result,GenBank was searched for

sequences pertaining to the viral agent.Surprisingly, we found 5 complete genomes, some fragments, and a reference sequence for the new coronavirus(SARS-CoV-2) dated December 2019. The reference sequence was aligned (ClustalW) with the sequences of SARS, MERS, and four types of coronaviruses(229E, NL63, HKU1, and OC43), which were included in our multiplex PCR-based tests. From this, a phylogenetic tree(Figure 1) was built using the maximum likelihood method based on the Tamura-Nei model in MEGA software.[1]

The new viral sequences were very different from all others, however, they were found to be closer to SARS.[2]Moreover, based on the limited information available regarding the primers of commercial respiratory panels, we concluded that it would not be possible to detect the new SARS-CoV-2 using these panels.From January 29 (D8) onwards, we had access to the primer sequences released by Centers for Disease Control and Prevention(CDC)/WHO, intended to be used for the purpose of the detection of the virus(Table 1).

The preliminary similarity analysis using Primer-BLASTshowed that the disclosed primers were capable of detecting the SARS-CoV-2 genome, based on the sequences deposited in the NCBI. The specificity was predicted as ideal for the primer named N1. Thus, on February 20 (D30) to be in line with internationalstandards, the primers and probes recommended by the CDC/WHO protocol were procured (“N” Gene). B-actin gene served as an internal control to human DNA.Biological samples were obtained using three synthetic oropharyngeal swabs (nose – 2, throat – 1); and RNAs were extracted mainly using RNA Viral mini kit and QIAcube(QIAGEN, Hilden, Germany).

Two strategies were followed for the diagnosis of SARS-CoV-2 positive cases.In the first strategy, patients with similar signs and symptoms were tested for the presence of other viral agents using the 21 multiplex respiratory viruses panel by using real-time PCR(21VIR)(Mobius Life, Curitiba, PR)(Figure 2). If they were positive for any of the viruses in this assay, considering the hypothesis of co-detection, we assumed that there would be a decreased chance of infection by SARS-CoV-2. The second strategy included the active search for SARS-CoV-2 using the PCR direct detection test, in accordance with the CDC protocol.

The direct *in house* detection of SARS-CoV-2 followed the basic qPCR protocol of the commercial kit GoScript Probe 1-step qPCR Master Mix(Promega, Madison, WI) in StepOnePlus (Applied Biosystems, Foster City, CA) and CFX96 (Bio-Rad, Hercules, CA) thermocyclers. Anticipating a possible increase in demand for tests, we adapted the protocol to the “fast” format and decreased the processing time to forty minutes(Table 2).

On February 26(D36), the Brazilian Health Ministry confirmed the first case of the new coronavirus in Brazil. Upto that date, suspicious cases from military related personnelhad been referred to our institute for evaluation, all of which turned out to be negative.

On March 5(D44), the first case in our state(Rio de Janeiro) was confirmed by the Brazilian government.On March 12(D51), the first case of local transmission was confirmed in the city of Rio de Janeiro, with no history of travel to countries with community transmission.On March 12(D51), a presumptively positive virus sample was obtained from a Central State Laboratory(CSLab) in a suitable viral transport media. Surprisingly, the sample tested negative for 21VIR and our SARS-CoV-2test.On March

16(D55), an alternative diagnostic kit, manufactured in Brazil(Bio-Manguinhos/Fiocruz) and based on a second protocol from Charité, Berlim (E gene)was obtained together with new samples from the CSLab.From then on, our military institute began to provide diagnostic assistance forthe civilian samples fromCSLab. The virus sample obtained on March 12 was tested again and no virus was detected.

Our test for SARS-CoV-2 tested negative samples from civilian and military personnel until March 17(D56), when samples received from the CSLab were positive for the first time, as per the Charité protocol.[3]They were also confirmed as positive by our protocol. Additional tests and literature reports showed that N2 primers were not specific forSARS-CoV-2 virus.[4] Shortly afterwards, the CDC removed the sequences from its website and suggested commercial tests based on them. On thesame day, the first confirmed death by COVID-19 in Brazil was reported.

After March 18(D57), the diagnosis started to be based on the direct search for SARS-CoV-2, due to the low availability of 21VIR and the need to direct the lab workers to perform the COVID-19 tests. The 21VIR panels were used only for inpatients. Positive tests for COVID-19 were mandatorily confirmed by both protocols before being released.

In the figure 3, the results of viruses detected from the 21VIR panel are summarized. Of the first 175samples analyzed (D30-D91), 118/2 were negative/inconclusive, and 55 were positive for 21VIR viruses. Among the viruses detected, a predominance of positive cases of Rhinovirus was found (~29%). But the great diversity of viruses circulating in our country showed that singleplextraditional tests would not be effective for diagnosis.

On March 18 (D57), the first military related patient with COVID-19 were diagnosed. The results and timeline are summarized in Figure 4. On March 27 (D66) the first positive case of a military officer from our institute was reported. Subsequently, on the same day (D66) a military officer assigned to our diagnostic team tested positive. On March 30 (D69), the first rapid immunological kits arrive in Brazil.

Until March 31 (D70), all results were similar between the two PCR-based diagnostic protocols. However, between April 1-14 (D71-D84), 28 tests were inconclusive to CDC protocol. So, the Charité protocol was very important to confirm the results. On March 31 (D70), upon receiving a request from the CSLab, we validated the new commercial 2019-nCoV Real-Time Reverse Transcriptase (RT)-PCR Diagnostic Panel suggested by the CDC in our laboratory [(5)], using 14 samples previously positive for our "*in-house*" CDC protocol and confirmed by the Charité protocol. All the results were consistent.

In our conditions, we suggest for now the use of a combination of N1/N3 or preferably N1/N2 primer sets (CDC protocol) for preliminary tests and mandatory confirmation of positive results using E gene from Charité protocol. Preferably N1/N2 because in March 15 (D54) CDC removed N3 primer set from its recommendation. Using this configuration, from around 1331 tests, we found 348 (26,5%) positives, 934 (71,1%) and only 31 (2,4%) remain inconclusive. Negative results may be obtained from CDC N1/N2 primers sets corroborating recent findings about higher sensitivity of N1 primer set. [(3,4)]

On April 04 (D74), the first member of our MDT tested positive for COVID-19. Until April 14 (D84), nine members of our institute (~4%) tested positive (two of our team). The first two positives from D66, retested one negative and the other remain

positive without symptoms, eighteen days after diagnostic. Finally, on April 21 (D91) the last day of our report, 27 Institute members (~10%) tested positive and were away from work.

RELEVANT DISCUSSION

An important aspect of COVID-19 is the fact that symptoms appear only a few days after close contact with an infected person or contaminated surfaces. Hence, the daily statistics of new cases represent people who may have had contact a few days before a positive diagnosis. Presymptomatic transmission in such cases is a possibility, and all of this should be taken into account while making epidemiological decisions.

Curiously, including 21VIR we found around 20% of codetection including rhinovirus (RV), around 15% of co-detection of RV/SARS-CoV-2 and a higher SARS-CoV-2 positivity in negative patients for 21VIR. Therefore the positive cases for 21VIR do not exclude the possibility of co-detection or co-infection by SARS-CoV-2.[5] This panels are a promising tool for understanding the cycles, seasonality and multitude of these viral respiratory pathologies, which are a major cause of clinical visits worldwide. [6]

The discrepancies observed between the two SARS-CoV-2 PCR protocols as well as the negative result of the first sample obtained from the CSLab may be due to some degree of degradation of the RNAs received, sensitivity levels discrepancies or inespecific amplifications recently described. But it is possible that multiple strains are circulating.[7]Subsequent sequencing data analysis willuncoverthis possibility. So, the molecular biology plasticity strategies related to genomes underline the importance of

involving professionals with therelevant expertise in providing technical, scientific and diagnostic support to clinical analysis.

The data referring to confirmed cases have a bias with regard to the time required for adaptation, planning, logistics, and definition of diagnostic protocols in national laboratories. Moreover, the objective of this quick report is to verify the molecular techniques and can change considerably in view of the distribution of other rapid diagnostic kits.[8]The first case in our institute was on D66. Our MDT technicians are experienced in the use of personal protective equipment(PPE) and in biological defense protocols. The temporal dynamics may be different in diagnostic laboratories with other characteristics.

With the increase in cases [9], new professionals will need to be recruited to the collection, diagnostic and medical care teams. However, it is suggested that every professional be tested before starting work, in addition to weekly tests for everyone involved. It should be noted that about 20% of responding health-care workers were infected in Italy. [10]In addition to individual risk, the withdrawal of professionals greatly reduces the ability to respond to the pandemic. New strategies for redeployment of personnel who have recovered from COVID-19 may be planned. Instead of the possibility of reinfection stays unclear, preliminary some reports suggest no recurrence after re-exposure of COVID-19 in non-human primate models.[11]

FUTURE PERSPECTIVE FOR TARGETED THERAPY

For the future, the widespread utilizationof new sequencing technologies, user-friendly bioinformatics tools and the appropriate use of sequencing tools in clinical diagnosis will aid in the development of targeted therapy. Sequencing approaches based

on targeted amplicons or metagenomics may be powerful toolindetecting the real pathogens in every sample, coinfections, multiple strains, phylogenomical, and phylogeographical interpretations and will contribute to faster and more accurate responses in epidemics.

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AUTHORS' CONTRIBUTION

Conceptualization - MD-R and CGMS; Formal analysis - MCC , NVGC, TLSN, EV EA and CGMS; Funding acquisition - MD-R and CGMS; Investigation – RS, EA, EV and CGMS; Methodology – CGMS; Project administration - MCC , NVGC, TLSN, EV, MD-R; Resources - EA and MD-R; Software – RS and CGMS; Supervision - NVGC, TLSN, MD-R; Writing-original draft -MCC and CGMS; Writing-review & editing - MCC and CGMS.

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Table 1 – Primers suggested by CDC previously.

NAME	SEQUENCE	CONC
2019-nCoV_N1Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'	20 μM
2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	20 μM
2019-nCoV_N1Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'	5μM
2019-nCoV_N2Forward Primer	5'-TTA CAA ACA TTG GCC GCA AA-3'	20 μM
2019-nCoV_N2 Reverse Primer	5'-GCG CGA CAT TCC GAA GAA-3'	20 μM
2019-nCoV_N2Probe	5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'	5μM
2019-nCoV_N3Forward Primer	5'-GGG AGC CTT GAA TAC ACC AAA A-3'	20 μM
2019-nCoV_N3 Reverse Primer	5'-TGT AGC ACG ATT GCA GCA TTG-3'	20 μM
2019-nCoV_N3Probe	5'-FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1-3'	5μM

Table 2 – Cycle conditions for SARS-Cov-2 PCR detection based in CDC primers

Steps	Cycles	Temperature	Time
Reverse transcription	1	45 °C	5 min
Reverse transcriptase inactivation and DNA Polymerase activation	1	95 °C	2 min
Denaturation	40	95 °C	3 sec
Annealing and extension		60°C	30 sec

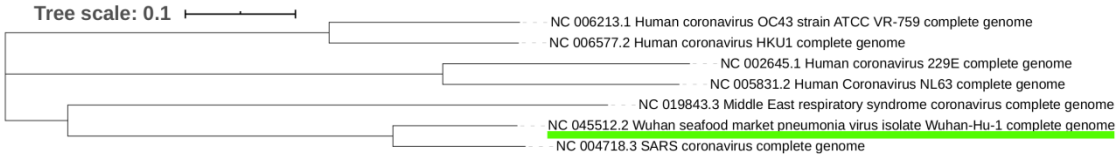

Figure 1 – Preliminary phylogenetic tree of simple reference genomes from important coronavirus subtypes. Highlighted, the sequence name referring to SARS-Cov-2 in January 22.

Human Adenovirus (HAdV) Human Bocavirus (HBoV) Human Coronavirus 229E (Cor229) Human Coronavirus HKU1 (HKU) Human Coronavirus NL63 (Cor63) Human Coronavirus OC43 (Cor43) Enterovirus (EV) Human Metapneumovirus A (HMPVA) Human Metapneumovirus B (HMPVB) Mycoplasma pneumoniae (Mpneu) Human Parechovirus (HPeV)	Human Rhinovirus (VR) Influenza A (FLUA) Influenza B (FLU B) Influenza A H1N1 (H1N1) Human Parainfluenza 1 (HPIV1) Human Parainfluenza 2 (HPIV2) Human Parainfluenza 3 (HPIV3) Human Parainfluenza 4 (HPIV4) Respiratory Syncytial Virus A (HRSVA) Respiratory Syncytial Virus B (HRSVB)

Figure 2 – 21VIR panel pathogens detectable in our Laboratory.

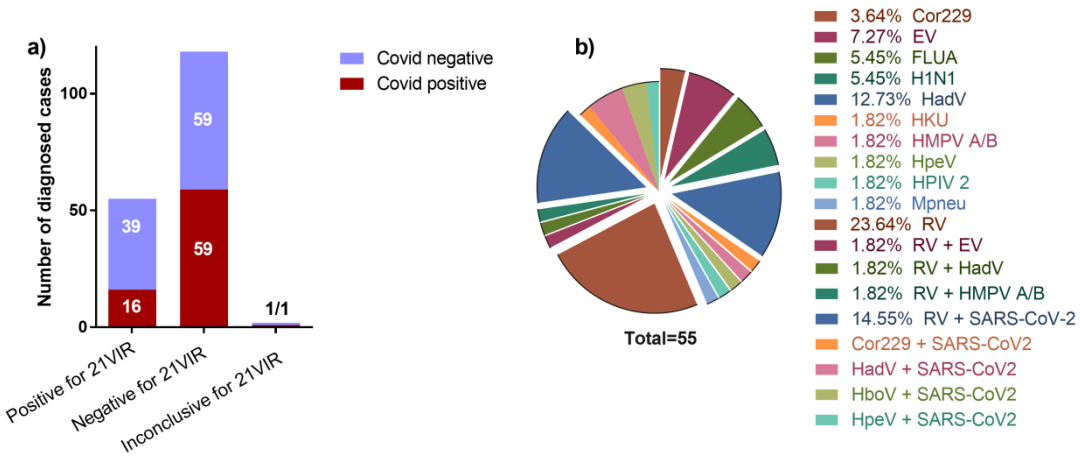

Figure 3 – A) Diagnosed cases of 129 tests of 21VIR related to SARS-CoV-2 results. **B)** Proportion of respiratory virus detected and codetected in our investigation. RV – rhinovirus, EV – enterovirus, FLUA – Influenza A, H1N1 – Influenza A(swine), HadV – adenovirus, Cor229 – coronavirus subtype 229, HKU - coronavirus subtype HKU1, HMPV A/B – metapneumovirus subtypes A or B, HPIV2 – parainfluenza 2, Mpneu – Micoplasma pneumoniae(bacteria)*, HpeV–parecovirus, SARS-CoV2 – Coronavirus COVID-19.

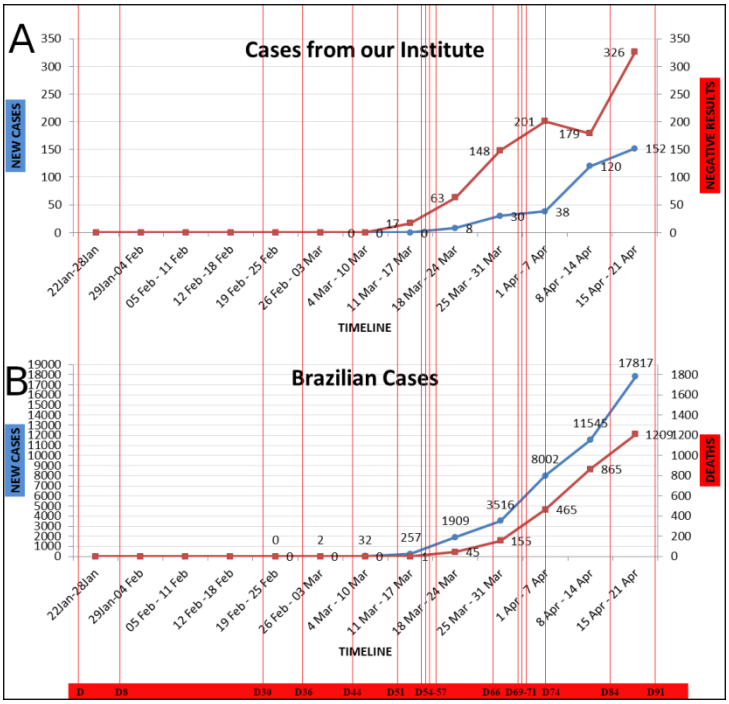

Figure 4 – A) Timeline of new cases diagnosed and negative results by our Institute. **B**) Timeline of new cases diagnosed and deaths obtained from Brazilian Health Ministery.