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Highlights

- SARS-CoV-2 Quantification.
- Multiplex one-step reaction.
- High sensitivity and specificity assay.

ABSTRACT

Introduction: COVID-19 is a disease caused by Severe Acute Respiratory Syndrome Virus 2 (SARS-CoV-2) which emerged in China in late 2019. The rapid viral spread has made the disease a public health emergency of worldwide concern. The gold standard for diagnosing SARS-CoV-2 is reverse transcription followed by qualitative real-time polymerase chain reaction (RT-qPCR); however, the role of viral load quantification has not been thoroughly investigated yet.

Objective: The aim of this study was to develop a high-precision quantitative one-step RT-qPCR reaction using the association of the viral target and the human target in the same reaction.

Methods: The assay standardization involved the absolute quantification method with serial dilutions of a plasmid with the N gene in a biological matrix to build a standard curve.

Results and Discussion: The results demonstrated the possibility of quantifying as few as 2.5 copies/reaction and analysis of 244 patients with known results selected by cross-section revealed 100% agreement with a qualitative RT-qPCR assay registered by Anvisa. In this population, it was possible to quantify patients with between 2.59 and 3.5×10^7 copies per reaction and negative patients continued to indicate the same result.

Conclusion: This assay can be a useful tool for the proper patients management, since the level and duration of viral replication are important factors to assess the risk of transmission and to guide decisions regarding the isolation and release of patients also, an accurate diagnosis is critical information whereas the current COVID-19 pandemic represents the biggest current global health problem.

Keywords: SARS-CoV-2; Quantitative multiplex one-step RT -qPCR; COVID-19.

INTRODUCTION

The Coronavirus genus comprises a variety of viral species that infect vertebrates, including human beings (1). As of May 2019, approximately 2,505 species belonging to this genus have been cataloged; however, only seven of these have been isolated in humans, all of which were linked to respiratory infections. Four of these species are considered common worldwide and trigger mild conditions (HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1) (2,3) and three have been related to outbreaks and epidemics (SARS-CoV, MERS-CoV and SARS-CoV-2) (4–6).

COVID-19 is a disease caused by Severe Acute Respiratory Syndrome virus 2 (SARS-CoV-2) belonging to the *Coronaviridae* family. The virus was identified as the causative agent of a severe pneumonia outbreak in mid-December 2019 in Hubei province, China, and due to the impact of the disease on a global level, the World Health Organization (WHO) raised the state of COVID-19 to a pandemic in March 2020 based on its high rate of transmission and rapid global spread (6).

This disease causes mild respiratory symptoms in about 80% of those infected, where the basic recommendation is to stay home and observe social isolation. Severe cases with complications characterized by acute respiratory distress syndrome, arrhythmia, septic shock, acute kidney, heart damage, liver dysfunction, and secondary infections are reported in about 20% of cases; 14% being considered serious and 5% considered critical (6,7).

The viral dynamic of the new coronavirus is not fully understood; however, the severity of the disease may be related to numerous factors such as pre-existing comorbidities and advanced age (8). The vast majority of patients who develop severe conditions and require hospitalization have an association of at least one comorbidity, of which the main ones are hypertension, diabetes, cardiovascular diseases, and respiratory system diseases (9), or even smoking, malignancies and chronic kidney disease (10).

In the absence of vaccines and pharmaceutical products proven to be effective, the implementation of control measures is an alternative to battle SARS-CoV-2 infection (11). For this purpose, in view of the pandemic scenario, a sensitive and specific diagnosis performed in the right period to avoid false negative results, prove to be efficient in coping with COVID-19. The gold standard for the diagnosis of COVID-19 is reverse transcription followed by qualitative real-time polymerase chain reaction (RT-qPCR)

(12). This method is able to indicate whether or not the patient is infected; however, it is not able to measure viral load, whereas a quantitative RT-qPCR method is able to determine the viral particles number in an infected individual.

In viral diagnostics, depending on the target or individual characteristic of each disease, quantitative RT-qPCR assay can be used in a number of ways, such as for screening, diagnosis or confirmatory testing, treatment decisions, monitoring or even evaluation of cure rates after therapeutic intervention (13). In this context, the objective of the study was to develop a quantitative one-step RT-qPCR reaction that is sensitive and specific to SARS-CoV-2.

MATERIALS AND METHODS

Ethical aspects

The study was carried out in accordance with the ethical principles stipulated by the 1975 World Medical Assembly and the Ministry of Health (Resolution 466). The project was evaluated and approved by the research ethics committee of the Center for Research in Tropical Medicine - CEPEM - Rondônia under protocol no. 4,000,086.

Study site and biological samples

The cross-sectional study was carried out at the Molecular Virology Laboratory of FIOCRUZ RO in collaboration with the Central Laboratory of Public Health of Rondônia - LACEN/RO in the period between March and June 2020, where 244 biological samples were selected. The samples were collected in basic health units and reference centers in different locations around the state using the combined swab method (nasal/oral) and the material was transferred to buffered saline (PBS pH 7.2), and transported at 4 °C to LACEN/RO, where qualitative molecular diagnosis was performed. The study included individuals who had compatible symptoms for COVID-19 within 3 to 5 days. Clinical and epidemiological data were collected from records contained in the Laboratory Environment Manager-GAL at LACEN/RO and E-SUS-NOTIFICA (Brazil, Ministry of Health).

Viral RNA extraction

Viral RNA was isolated from 200 μL of patient samples using the QIAamp Viral RNA Mini Kit (QIAGEN[®], Hilden, Germany) according to the manufacturer's instructions. RNA was and eluted in 60 μL .

Preparation of the Quantification Curve

Complementary DNA synthesis was performed using RNA extracted from a sample positive for SARS-CoV-2, associated with 0.5 μg of random primer using the SuperScript[™] III RT enzyme protocol (200 units/ μL) (Thermo Fisher Scientific[®], Massachusetts, USA).

The 943 bp coding sequence corresponding to the N1, N3 and N2 regions of the SARS-CoV-2 virus (14) was amplified and the PCR product was cloned using the pGEM[®]-T Easy Vector system (Promega[®], Madison, Wisconsin, EUA), then the plasmid was linearized by enzymatic restriction using the enzyme NcoI (New England Biolabs[®], Massachusetts, USA) according to the manufacturer's instructions. The linearized plasmid concentration was determined using UV spectrophotometry by NanoDrop[®] 2000 (ThermoFischer Scientific[®], Massachusetts, USA) (15). The plasmid was serially diluted in a biological RNA matrix get from SARS-CoV-2 negative human serum between 1×10^6 and 0.25 copies per microliter, in order to simultaneously amplify the viral target (N1) and the human endogenous control (RNase P).

Quantitative one-step Multiplex RT-qPCR Assay

The multiplex reaction was standardized using TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems[®], California, USA), 5 μL of each dilution from the curve, using 500 nM of each oligonucleotide and 250 nM for the probes (table 1). The cycling used for the reaction was: 51 °C for 30 minutes for reverse transcription followed by PCR activation at 95 °C for 15 minutes and 45 subsequent cycles for 15 seconds at 95 °C and 1 minute at 60 °C, the step where fluorescence was captured. The data were analyzed using a baseline of 3 to 15 for both targets (N1 and RNase P), threshold 0.2 for N1 and 0.1 for RNaseP. The tests were performed on a 7500 Real-Time PCR System (Applied Biosystems[®], California, USA) with results analyzed in the software 7500 System version 1.4.

Table 1. One-step Multiplex RT-qPCR primers and probes.

Adapted from: Centers for Disease Control and Prevention. Accessed on: July 9th, 2020. (14)

Reproducibility and repeatability

Reproducibility and repeatability were measured through 3 assays with 5×10^6 ; 5×10^5 ; 5×10^4 ; 5×10^3 ; 5×10^2 ; 50; 5; 2.5; 1.25 copies/reaction executed on consecutive days, where each point was performed in technical octuplicates. This was the basis for determining analytical sensitivity.

Diagnostic sensitivity and specificity

Diagnostic sensitivity and specificity were assessed by testing 244 samples, 192 from patients known to be positive and 52 from patients known to be negative for SARS-CoV-2, with a result previously confirmed by LACEN/RO, using the SARS-CoV-2 Molecular Kit (BioManguinhos[®], Brazil). This qualitative kit is based in three singleplex reactions of one step real-time PCR (RT-qPCR), for detection of two viral targets (N1

| Name | Target Gene | Description | Sequence 5'-3' | Amplicon (pb) |
|----------------|-------------|-------------|---------------------------------------------------------------------|---------------|
| 2019-nCoV N1-F | | Sense | GACCCCAAATCAGCGAAAT | |
| 2019-nCoV N1-R | N | Antisense | TCTGGTTACTGCCAGTTGAATCTG | 73 |
| 2019-nCoV N1-P | | Probe | FAM - ACCCCGCATTACGTTTGGTGGACC - QSY (Applied Biosystems) | |
| RP-F | | Sense | AGATTTGGACCTGCGAGCG | |
| RP-R | RP | Antisense | GAGCGGCTGTCTCCACAAGT | 65 |
| RP-P | | Probe | HEX - TTCTGACCTGAAGGCTCTGCGCG - BHQ (LGC Biosearch Technologies) | |

and N2 region) and an internal human endogenous control for a specific gene RNase P.

Statistical Analysis

To summarize the information about the samples obtained, we used descriptive statistical techniques; in this case, we used proportions followed by their respective standard deviation for categorical variables and median with inter-quartile intervals (IQR) for numerical variables. To calculate the analytical sensitivity or Limit of Optical Detection ($LOD_{95\%}$), the repeatability data were exported to the software MedCalc[®]

version 19.4 and subjected to binomial regression analysis using the Probit statistical model. GraphPad Prism version 8.4.0[®] software was used to analyze diagnostic specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV).

RESULTS

Characterization of the samples obtained

The populational cohort was composed by 244 patients, presenting signs and symptoms as fever (67.6%), cough (62.3%), and sore throat (42.2%) at the moment of nasal/oral swab collect. The individuals were in an age range between 1 month and 90 years (39.1 years average, SD 14.4), with 129 male individuals (52.9%) and 115 female individuals (47.1%).

The cohort biological samples, collected between the third and the fifth day after the onset of symptoms, were characterized by LACEN using the Kit Molecular SARS-CoV-2 (BioManguinhos[®], Brazil), presenting 192 positive patients (78.7%) and 52 negative patients (21.3%). The developed assay confirmed the results, allowing the viral load quantification for the positive samples and validating the negative samples without amplification for viral target. The PPV and NPV were kept at 1.

Efficiency of the One-Step Quantitative RT-qPCR Assay

Quantification curve

The plasmid was diluted in intervals from 5×10^6 copies to 1.25 copies per reaction (5×10^6 ; 5×10^5 ; 5×10^4 ; 5×10^3 ; 5×10^2 ; 50; 5; 2.5; 1.25), to determine amplification efficiency and linearity (figure 1). All points on the quantification curve up to 2.5 copies per reaction had 100% amplification. The slope of the initial quantitative assay was -3.32 with an efficiency of 99.9%, a correlation coefficient of the line (R^2) equal to 0.99, and an amplification factor equal to 2. Table 2 shows the Cts of 3 distinct curves that were the basis for determining the assay reproducibility and repeatability.

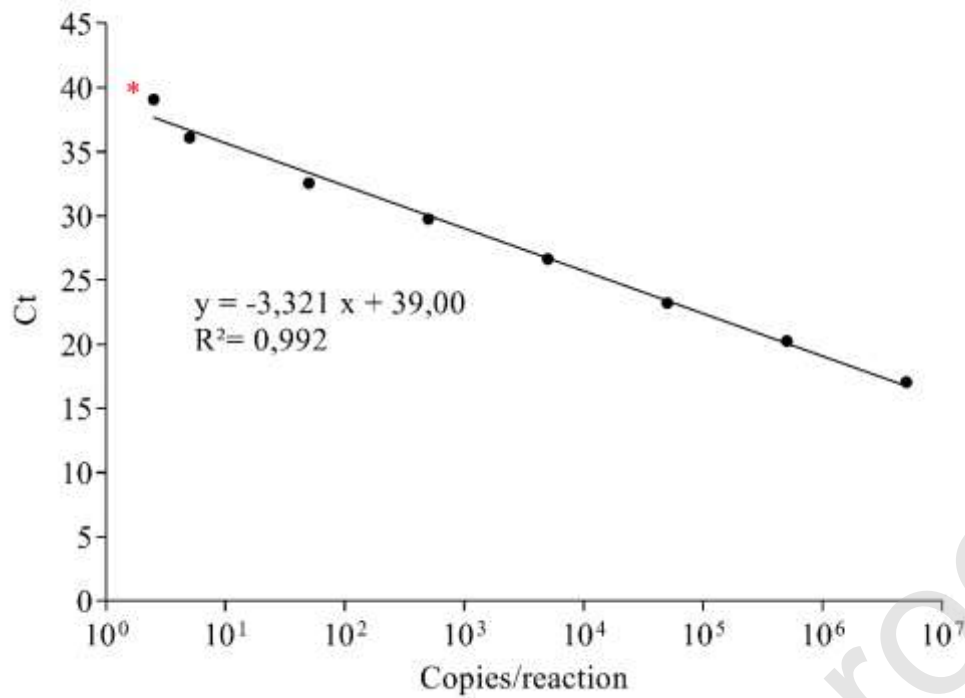


Fig 1. Quantification curve. Linear regression analysis of 9 serial dilutions using a recombinant plasmid with a N1 region diluted in RNA extracted from a human biological matrix, ranging from 1.25^* to 5×10^6 copies per reaction, tested by RT-qPCR for SARS-CoV-2; Ct: cycle threshold.

Reproducibility and Repeatability

The 3 assays were carried out with the standard dilutions evaluated individually and together presenting a Coefficient of Variation (CV%) less than 10%. The data for slope, intersection of the line and correlation between points are described in table 2.

| Standard (copies/reaction) | 1st run (Ct) | 2nd run (Ct) | 3rd run (Ct) | Mean | SD(\pm) | CV |
|-------------------------------|-----------------|-----------------|-----------------|-------|-------------|------|
| 5×10^6 | 17.05 | 17.24 | 17.34 | 17.21 | 0.15 | 0.86 |
| 5×10^5 | 20.24 | 20.24 | 20.17 | 20.22 | 0.04 | 0.20 |
| 5×10^4 | 23.22 | 23.25 | 23.26 | 23.24 | 0.02 | 0.09 |
| 5×10^3 | 26.65 | 26.60 | 26.55 | 26.60 | 0.05 | 0.19 |
| 5×10^2 | 29.75 | 29.82 | 29.91 | 29.83 | 0.08 | 0.27 |
| 5×10^1 | 32.56 | 32.71 | 32.64 | 32.64 | 0.08 | 0.23 |
| 5×10^0 | 36.11 | 36.17 | 36.30 | 36.19 | 0.10 | 0.27 |
| 2.5×10^0 | 39.08 | 39.54 | 38.87 | 39.16 | 0.34 | 0.88 |

| | | | | | | |
|----------------------|--------------|--------------|--------------|---|---|---|
| Slope | -3.32 | -3.35 | -3.30 | - | - | - |
| Intercept | 39.00 | 39.21 | 38.98 | - | - | - |
| R² | 0.99 | 0.99 | 0.99 | - | - | - |

Table 2. Repeatability and Reproducibility Assays. The reproducibility and repeatability assays were performed in three runs on alternate days with dilutions in technical octuplicates, with Coefficient of Variation (CV%) less than 10% between runs.

The analytical sensitivity was determined using the limit of optical quantification (LOQ) at 2.5 copies per reaction and limit of optical detection (LOD_{95%}) at 1.41 copies per reaction; this is the minimum viral load value for qualitative detection (figure 2).

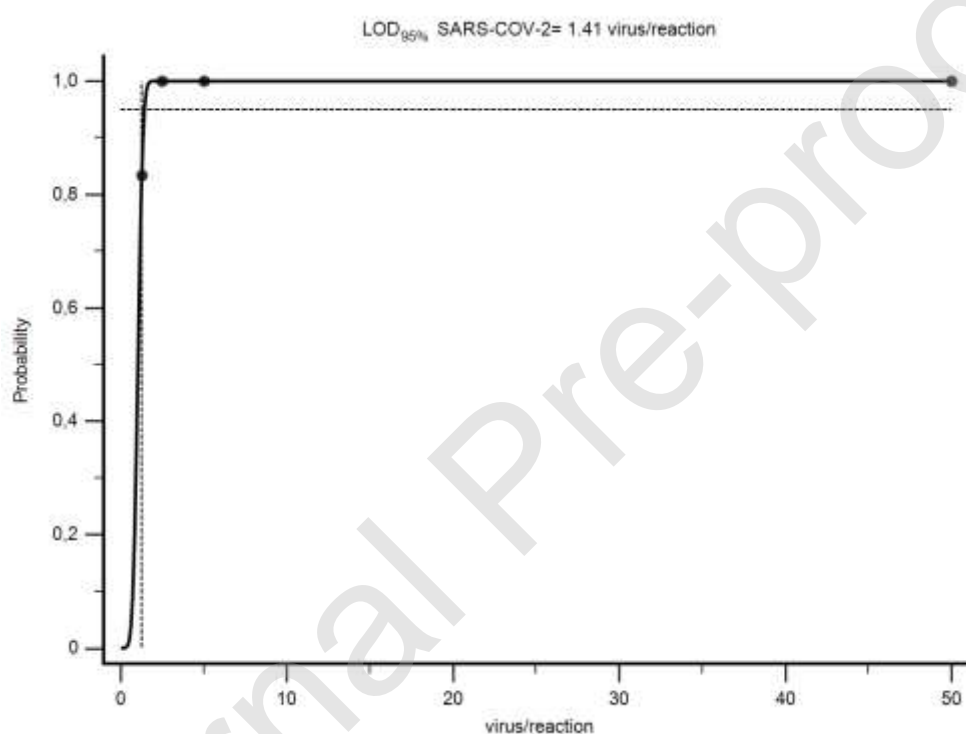


Fig 2. LOD_{95%} Determination. Analysis revealed a limit of detection at 1.41 copies/reaction.

Diagnostic sensitivity and specificity

Biological samples from the 244 selected patients indicated 192 positive results, of which 189 were quantifiable and 3 were between LOD (1.41 copies/reaction) and LOQ (2.5 copies/reaction) values; the viral load of patients ranged from 2.59 to 3.5×10^7 copies per reaction (Figure 3), while the 52 negative samples remained negative.

The results comparison results from the qualitative test, performed in the reference laboratory, with the assay developed in this study, the one-step RT-qPCR proposed for

SARS-CoV-2, presented sensitivity (95% CI: 0.98-1.00), specificity (95% CI: 0.93-1.00), PPV (95% CI: 0.98-1.00) and NPV (95% CI: 0.93-1.00) of 100%.

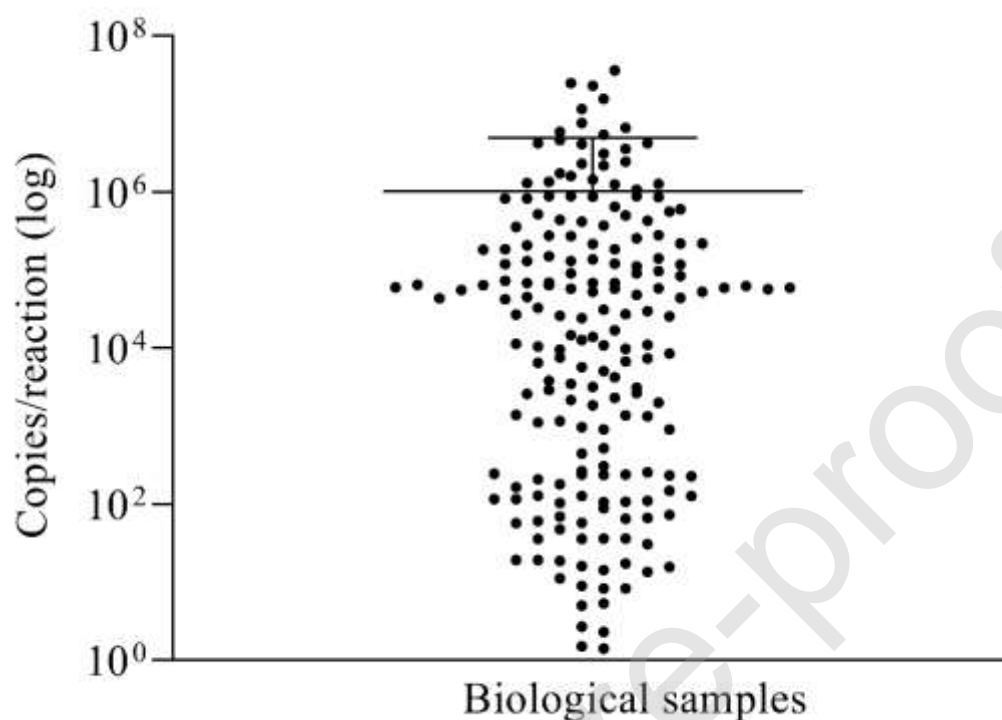


Fig 3. Viral load of positive samples tested with RT-qPCR for SARS-CoV-2. Schematic representation of the viral load detected in the positive samples by RT-qPCR for SARS-CoV-2. The minimum quantifiable viral load was 2.59 and the maximum was 3.5×10^7 copies per reaction in the tested population.

DISCUSSION

Molecular tests are the gold standard for laboratory diagnosis of COVID 19 due to the short time gap for viral RNA detection (16,17). This study developed a quantitative assay based on one-step RT-qPCR for the detection of SARS-CoV-2 in a biological matrix using 244 symptomatic patients with qualitative molecular diagnosis, selected by cross-section.

Most published RT-qPCR assays for the diagnosis of SARS-CoV-2 are qualitative (18–23). The assays most commonly described in the literature involve the N, E, RdRP, and S genes for viral detection (14,17,24). The construction of this quantitative multiplex assay was based on the choice of the viral target N1 (14,21), with the addition of an

endogenous human control, a single copy gene RNase P, as a reference gene to monitor the nucleic acids extraction and the RT-qPCR assay in order to avoid false-negative results, thus preserving the reaction quality (25,26).

Regarding the sensitivity of the developed method, the current published assays for SARS-CoV-2 detection by RT-qPCR report LOD_{95%} values between 2.9 and 275.72 copies per reaction, which confirms that the developed assay has high levels of analytical sensitivity, since the reactions can be quantified up to 2.5 copies/reaction and still have a LOD_{95%} of 1.41 copies per reaction (20,24,27,28).

As for the cross-section, it was found that all 244 patients evaluated presented 100% agreement with the qualitative results, with the lowest quantified viral number at 2.59 copies/reaction ($0.4 \log_{10}$ copies/mL), and the highest at 3.5×10^7 copies/reaction ($7.6 \log_{10}$ copies/mL). The high value of the detected viral load is similar to the viral levels reported in other studies using samples from the respiratory tract (29,30). The negative patients continued to demonstrate the same result, guaranteeing the test diagnostic accuracy, since these patients also had clinical symptoms for respiratory diseases.

The quantitative RT-qPCR technique has been applied to other respiratory viruses and has shown advantages for the management of patients with respiratory syncytial virus, since this method permits the association of viral load and the development of obstructive pulmonary disease (31). In some studies with COVID-19 the subject is still controversial and, in some cases, the infected patients monitoring uses qualitative assays mistakenly considering the quantitative methods for monitoring these patients, based only on the Ct values used as a viral load quantification unit, in addition to the absence of a standard curve as reference material and internal control (32–35).

The viral replication level and duration are important factors to assess the risk of transmission and to guide decisions regarding the isolation of patients (36) and, consequently, to establish measures to control the virus spread; thus, the quantitative assay could be used as a strategy for assessing the viral load of patients in this situation, considering that IgM and IgG antibodies are often not able to be detected after the 14-day period (37), with reports of viral transmission from post-isolation individuals to close contacts (38).

In some studies on the diagnosis of COVID-19, it was observed that the first qualitative RT-qPCR assays from patients showing signs and symptoms of the disease,

such as high fever and severe pulmonary alterations, showed negative results, and only became positive after subsequent tests during hospitalization (39–42). Therefore, monitoring viral load through sensitive molecular tests is essential since these patients could suffer from inadequate management and even compromise hospitals by favoring the virus transmission.

Among the limitations faced during the study development, the choice of an adequate human endogenous control stands out, to maintain the same sensitivity of the singleplex reaction after its addition. For this, adjustments were needed in the concentrations of primers, probes of both targets, and annealing temperature. In addition, the quantitative RT-qPCR method can be useful in understanding the real role of viral load in the severity of the disease and in the transmissibility period of SARS-CoV-2.

CONCLUSION

The ongoing pandemic of the new Coronavirus (SARS-CoV-2) represents the biggest current global health problem, and has seriously threatened humanity health, due to the high morbidity and mortality associated with severe infections. Despite the medical importance of COVID-19, no licensed vaccines or proven effective specific drugs are available against the virus. Thus, immediate isolation measures for positive cases, social distancing, aiming to reduce viral circulation, accompanied by specific and sensitive tests such as quantitative RT-qPCR represent a strategy for immediately coping with the disease.

Author contributions

Conceptualization: J.M.V.S, D.S.V.D., S.S.P., M.M.S., M.A.K.; Molecular assays: J.A.S.Q; Data processing and analysis: R.C.P.R. M.A.K.; Panel data sample selection: E.B.S.F., L.F.B.S., A.C.S.M., C.C.S., A.L.F.M.M., C.A.B.L., F.A.A.A., R.S.S.R., C.H.N.S, A.P.S.G., A.O.S., F.R.M.; Funding acquisition: D.S.V.D., F.R.M., M.A.K., J.M.V.S.; Methodology: J.A.S.Q., R.C.P.R., S.S.P., M.M.S.; Compiling patient profile data: G.S.O., S.C.O., F.R.M.; Medical approach: F.R.M., J.M.V.S.; Writing–original draft: J.A.S.Q., R.C.P.R., E.B.S.F., G.S.O., S.C.O., S.S.P., A.O.S.; Writing–review & editing: J.M.V.S., D.S.V.D.; S.S.P., M.M.S., Coordination: D.S.V.D.; All authors have read and agreed to the published version of the manuscript.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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