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Development of a quantitative one-step multiplex RT-qPCR assay for the detection of SARS-CoV-2 in a biological matrix

Jackson Alves da Silva Queiroz[a,b,1], Rita de Cássia Pontello Rampazzo[c,1], Edivá Basílio da Silva Filho[a,b], Gabriella Sgorlon Oliveira[a], Suyane da Costa Oliveira[a], Luan Felipe Botelho Souza[d], Soraya dos Santos Pereira[a,b], Moreno Magalhães de Souza Rodrigues[a], Adriana Cristina Salvador Maia[d], Cicileia Correia da Silva[d], Aline Linhares Ferreira de Melo Mendonça[d], Celina Aparecida Bertoni Lugtenburg[d], Francisco de Assis Araújo Aguiar[d], Rosiane de Souza Soares Rodrigues[d], Caio Henrique Nemeth Santos[e], Alice Paula Di Sabatino Guimarães[a], Fernando Rodrigues Máximo[f], Alcione de Oliveira dos Santos[a], Marco Aurélio Krieger[g], Juan Miguel Villalobos Salcedo[a,**], Deusilene Souza Vieira Dall'Acqua[a,b,*]

[a] *Fundação Oswaldo Cruz, FIOCRUZ, Unidade Rondônia, Porto Velho, Rondônia, Brazil*
[b] *Programa de Pós Graduação em Biologia Experimental, Fundação Universidade Federal de Rondônia– PGBIOEXP/UNIR, Porto Velho, Rondônia, Brazil*
[c] *Instituto de Biologia Molecular do Paraná – IBMP, Curitiba, Paraná, Brazil*
[d] *Laboratório Central de Saúde Pública do Estado de Rondônia – LACEN/RO, Porto Velho, Rondônia, Brazil*
[e] *Secretaria de Estado de Finanças de Rondônia – SEFIN RO, Porto Velho, Rondônia, Brazil*
[f] *Secretaria de Estado da Saúde de Rondônia – SESAU, Porto Velho, Rondônia, Brasil*
[g] *Instituto Carlos Chagas Fundação Oswaldo Cruz, FIOCRUZ-PR, Curitiba, Paraná, Brazil*

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A B S T R A C T

Introduction: Coronavirus disease-2019 (COVID-19) is a disease caused by Severe Acute Respiratory Syndrome Virus 2 (SARS-CoV-2) that 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 an analysis of 244 patients with known results selected by cross-section that 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 a proper patient management, because 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; an accurate diagnosis is critical information, whereas the current COVID-19 pandemic represents the biggest current global health problem.
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* Corresponding author at: Fiocruz Rondônia, Rua da Beira, 7176, Bairro Lagoa, 76812-245 Porto Velho, Rondônia, Brazil.
** Corresponding author.
E-mail address: deusilene.vieira@fiocruz.br (D.S.V. Dall'Acqua).
[1] The authors contributed equally to the study.

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Introduction

The *Coronavirus* genus comprises a variety of viral species that infect vertebrates, including human beings (Gorbalenya et al., 2020). 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) (Lim et al., 2016; Yuen et al., 2020) and three have been related to outbreaks and epidemics (SARS-CoV, MERS-CoV, and SARS-CoV-2) (Chafekar and Fielding, 2018; Fehr and Perlman, 2015; Guo et al., 2020).

Coronavirus disease-2019 (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 because of 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 (Guo et al., 2020).

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 failure, heart damage, liver dysfunction, and secondary infections are reported in about 20% of cases; 14% being considered serious and 5% considered critical (Guo et al., 2020; Cascella et al., 2020).

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 preexisting comorbidities and advanced age (Zheng et al., 2020). 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 (Yang et al., 2020), or even smoking, malignancies, and chronic kidney disease (Emami et al., 2020).

In the absence of vaccines and pharmaceutical products proven to be effective, the implementation of control measures is an alternative to combat SARS-CoV-2 infection (Ghaebi et al., 2020). 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) (Araujo-Filho J de et al., 2020). This method can indicate whether the patient is infected; however, it is not able to measure viral load, whereas a quantitative RT-qPCR method is able to determine the number of viral particles 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 the evaluation of cure rates after therapeutic intervention (Engstrom-Melnyk et al., 2015). 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 - 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 extracted 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 (Diseases and Control, 2020) was amplified and the PCR product was cloned using the pGEM®-T Easy Vector system (Promega®, Madison, Wisconsin, USA), then the plasmid was linearized by enzymatic restriction using the enzyme NcoI (New England Biolabs®, Massachusetts, USA)

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

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

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

according to the manufacturer's instructions. The linearized plasmid concentration was determined using UV spectrophotometry by NanoDrop® 2000 (ThermoFischer Scientific®, Massachusetts, USA) (Whelan et al., 2003). The plasmid was serially diluted in a biological RNA matrix obtained from SARS-CoV-2 negative human serum between 1×10^6 and 0.25 copies per microliter 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 process used for the reaction was as follows: 51 °C for 30 min for reverse transcription followed by PCR activation at 95 °C for 15 min and 45 subsequent cycles for 15 s at 95 °C and 1 min at 60 °C, the step where fluorescence was captured. The data were analyzed using a baseline of 3 - 15 for both targets (N1 and RNase P), threshold 0.2 for N1, and 0.1 for RNase P. 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.

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, and 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 on three singleplex reactions of one step real-time PCR (RT-qPCR), for the detection of two viral targets (N1 and N2 regions) 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 interquartile intervals 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 of 244 patients, presenting signs and symptoms as fever (67.6%), cough (62.3%), and sore throat (42.2%) at the time of nasal/oral swab collection. 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's 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), which presented 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, and 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 cycle thresholds (Cts) of 3 distinct curves that were the basis for determining the assay reproducibility and repeatability.

Reproducibility and Repeatability

The 3 assays were carried out with the standard dilutions evaluated individually and together, which presents 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.

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

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.

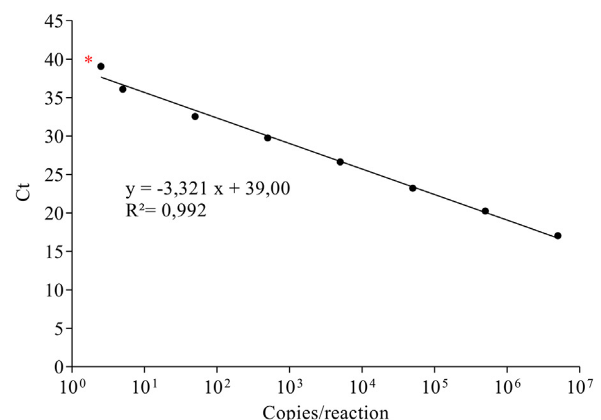

Figure 1. Quantification curve. Linear regression analysis of 9 serial dilutions using a recombinant plasmid with an N1 region diluted in RNA extracted from a human biological matrix, ranging from 1.25* to 5×10^6 copies per reaction, tested by RT-qPCR for SARS-CoV-2; Ct: cycle threshold.

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.

Standard (copies/reaction)	1 st run (Ct)	2nd run (Ct)	3rd run (Ct)	Mean	SD(±)	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
$5 \times 10^\circ$	36.11	36.17	36.30	36.19	0.10	0.27
$2.5 \times 10^\circ$	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**	-	-	-
R^2	**0.99**	**0.99**	**0.99**	-	-	-

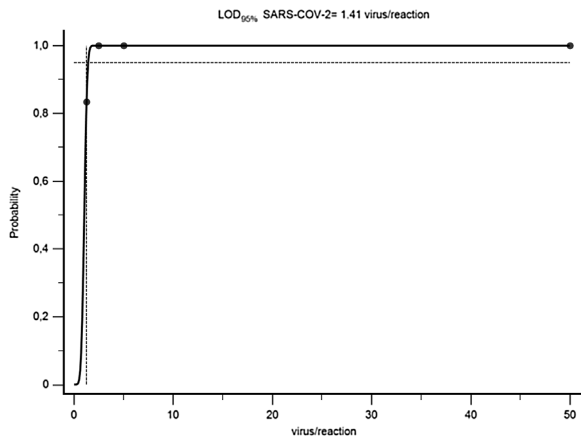

Figure 2. $LOD_{95\%}$ Determination. The analysis revealed a limit of detection at 1.41 copies/reaction.

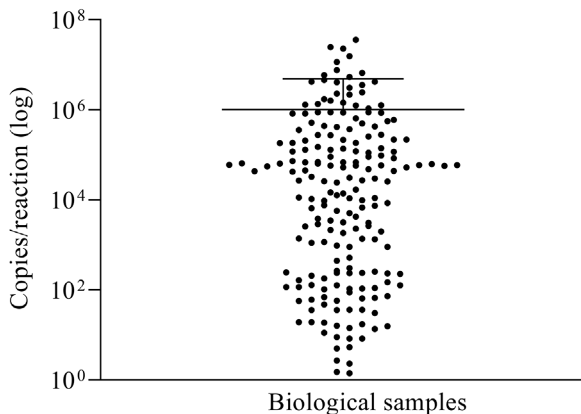

Figure 3. Viral load of positive samples tested with RT-qPCR for SARS-CoV-2. Schematic representation of the viral load detected in the positive samples by RT-qPCR for SARS-CoV-2. The minimum quantifiable viral load was 2.59 and the maximum was 3.5×10^7 copies per reaction in the tested population.

The comparison of 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%.

Discussion

Molecular tests are the gold standard for laboratory diagnosis of COVID-19 due to the short time gap for viral RNA detection (Cheng et al., 2020; Anon). 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 (Fomsgaard and Rosenstierne, 2020; Freire-Paspuel et al., 2020a,b; Wang et al., 2020; Ghaebi et al., 2020; van Kasteren et al., 2020; Gorbalenya et al., 2020; Guo et al., 2020; Han et al., 2020; Hu et al., 2020). The assays most commonly described in the literature involve the N, E, RdRP, and S genes for viral detection (Diseases and Control, 2020; Anon; Corman et al., 2020). The construction of this quantitative multiplex assay was based on the choice of the viral target N1 (Diseases and Control, 2020; Freire-Paspuel et al., 2020a), 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 to avoid false-negative results, thus preserving the reaction quality (Modarelli et al., 2018; Gonçalves-de-Albuquerque et al., 2014).

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, as the reactions can be quantified up to 2.5 copies/reaction and still have a $LOD_{95\%}$ of 1.41 copies per reaction (Lu et al., 2020; Corman et al., 2020; Li et al., 2020a; Pfefferle et al., 2020).

Regarding 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 (To et al., 2020; Yoon et al., 2020). The negative patients continued to demonstrate the same result, guaranteeing the test diagnostic accuracy, as 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, as this method permits the association of viral load and the development of obstructive pulmonary disease (Borg et al., 2003). 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 (Han et al. 2020; Zou et al., 2020; Chu et al., 2020; Chan et al., 2020).

The viral replication level and duration are important factors to assess the risk of transmission and to guide decisions regarding the isolation of patients (Zhou et al., 2020) 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 (Lou et al., 2020), with reports of viral transmission from post-isolation individuals to close contacts (Hu et al., 2020).

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 (Li et al., 2020b; Huang et al., 2020; Xie et al., 2020; Ai et al., 2020). Therefore, monitoring viral load through sensitive molecular tests is essential because 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 to understand 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 human health, due to 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., and M.A.K.; Molecular assays: J.A.S.Q; Data processing and analysis: R.C.P.R. and 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., and F.R.M.; Funding acquisition: D.S.V.D., F.R.M., M.A.K., and J.M.V.S.; Methodology: J.A.S.Q., R.C.P.R., S.S.P., and M.M.S.; Compiling patient profile data: G.S.O., S.C.O., and F.R.M..; Medical approach: F.R.M. and 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., and A.O.S.; Writing–review and editing: J.M.V.S., D.S.V.D.; S.S.P., and 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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Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

Ai T, Yang Z, Hou H, Zhan C, Chen C, Lv W, et al. Correlation of Chest CT and RT-PCR Testing for Coronavirus Disease 2019 (COVID-19) in China: a Report of 1014 Cases. Radiology [Internet] 2020;296(2 August), doi:http://dx.doi.org/10.1148/radiol.2020200642 E32–40. Available from:.

Real-time PCR-based SARS-CoV-2 detection.:https://preprints.scielo.org/index.php/scielo/prep.

Araujo-Filho J de AB, Sawamura MVY, Costa AN, Cerri GG, Nomura CH. Pneumonia por COVID-19: qual o papel da imagem no diagnóstico?. J Bras Pneumol 2020;46:1–2.

Borg I, Rohde G, Löseke S, Bittscheidt J, Schultze-Werninghaus G, Stephan V, et al. Evaluation of a quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases. Eur Respir J [Internet] 2003;21(6 June) 944–51, doi:http://dx.doi.org/10.1183/09031936.03.00088102 Available from:.

Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Napoli R Di. Features, Evaluation and Treatment Coronavirus (COVID-19) [Internet]. Features, Evaluation and Treatment Coronavirus (COVID-19). [cited 2020 Jul 17]. Available from:. 2020. https://www.ncbi.nlm.nih.gov/books/NBK554776/.

Chafekar A, Fielding BC. MERS-CoV: Understanding the Latest Human Coronavirus Threat. Viruses [Internet] 2018;10(2 February 24)93. . Available from: http://www.mdpi.com/1999-4915/10/2/93.

Chan JF-W, Yip CC-Y, To KK-W, Tang TH-C, Wong SC-Y, Leung K-H, et al. Improved Molecular Diagnosis of COVID-19 by the Novel, Highly Sensitive and Specific COVID-19-RdRp/Hel Real-Time Reverse Transcription-PCR Assay Validated In Vitro and with Clinical Specimens. McAdam AJ, editor. J Clin Microbiol [Internet] 2020;58(5 March 4). . Available from: https://jcm.asm.org/content/58/5/e00310-20.

Cheng MP, Papenburg J, Desjardins M, Kanjilal S, Quach C, Libman M, et al. Diagnostic Testing for Severe Acute Respiratory Syndrome-Related Coronavirus 2: A Narrative Review. Ann Intern Med. 2020;172(11):726–34.

Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, et al. Molecular Diagnosis of a Novel Coronavirus (2019-nCoV) Causing an Outbreak of Pneumonia. Clin Chem [Internet] 2020;66(4 April 1)549–55. . Available from: https://academic.oup.com/clinchem/article/66/4/549/5719336.

Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DKW, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Eurosurveillance. 2020;25(3):1–8.

Diseases R, Control D. 2019-Novel Coronavirus (2019-nCoV) Real-time rRT-PCR Panel Primers and Probes 2019-Novel Coronavirus (2019-nCoV) Real-Time rRT-PCR Panel Primer and Probes Note: Oligonucleotide sequences are subject to future changes as the 2019-Novel Coronavirus. 2020.

Emami A, Javanmardi F, Pirbonyeh N, Akbari A. Prevalence of Underlying Diseases in Hospitalized Patients with COVID-19: a Systematic Review and Meta-Analysis. Arch Acad Emerg Med [Internet] 2020;8(1)e35. . Available from: http://www.ncbi.nlm.nih.gov/pubmed/32232218.

Engstrom-Melnyk J, Rodriguez PL, Peraud O, Hein RC. Clinical Applications of Quantitative Real-Time PCR in Virology. p. 161–97. Available from:. 2015. https://linkinghub.elsevier.com/retrieve/pii/S0580951715000069.

Fehr AR, Perlman S. Coronaviruses: An Overview of Their Replication and Pathogenesis. Available from:. . p. 1–23. http://link.springer.com/10.1007/978-1-4939-2438-7_1.

Fomsgaard AS, Rosenstierne MW. An alternative workflow for molecular detection of SARS-CoV-2 – escape from the NA extraction kit-shortage, Copenhagen, Denmark, March 2020. Eurosurveillance [Internet] 2020;25(14 April 9). .

Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2020.25.14.2000398.

Freire-Paspuel B, Vega-Mariño P, Velez A, Castillo P, Gomez-Santos EE, Cruz M, et al. Cotton-Tipped Plastic Swabs for SARS-CoV-2 RT-qPCR Diagnosis to Prevent Supply Shortages. Front Cell Infect Microbiol [Internet] 2020a;10:. . Jun 23Available from: https://www.frontiersin.org/article/10.3389/fcimb.2020.00356/full.

Freire-Paspuel B, Vega-Mariño P, Velez A, Castillo P, Cruz M, Garcia-Bereguiain MA. Evaluation of nCoV-QS (MiCo BioMed) for RT-qPCR detection of SARS-CoV-2 from nasopharyngeal samples using CDC FDA EUA qPCR kit as a gold standard: An example of the need of validation studies. J Clin Virol [Internet] 2020b;128:104454. . Jul Available from: https://linkinghub.elsevier.com/retrieve/pii/S1386653220301967.

Ghaebi M, Osali A, Valizadeh H, Roshangar L, Ahmadi M. Vaccine development and therapeutic design for 2019-nCoV/SARS-CoV-2: Challenges and chances. J Cell Physiol [Internet] 2020;. . Jun 18;jcp.29771. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jcp.29771.

Gonçalves-de-Albuquerque S da, Pessoa e Silva R, de Morais RC, Trajano-Silva LA, Régis-da-Silva C, Brandão-Filho S, et al. Tracking false-negative results in molecular diagnosis: proposal of a triplex-PCR based method for leishmaniasis diagnosis. J Venom Anim Toxins Incl Trop Dis [Internet] 2014;20(1)16. . Available from: http://jvat.biomedcentral.com/articles/10.1186/1678-9199-20-16.

Gorbalenya A, Baker S, Baric R. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol [Internet] 2020;5(4 April 2)536–44. . Available from: http://www.nature.com/articles/s41564-020-0695-z.

Guo Y-R, Cao Q-D, Hong Z-S, Tan Y-Y, Chen S-D, Jin H-J, et al. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak – an update on the status. Mil Med Res [Internet] 2020;7(1 December 13)11. . Available from: https://mmrjournal.biomedcentral.com/articles/10.1186/s40779-020-00240-0.

Han MS, Byun J-H, Cho Y, Rim JH. RT-PCR for SARS-CoV-2: quantitative versus qualitative. Lancet Infect Dis [Internet] 2020;. . May; Available from: https://linkinghub.elsevier.com/retrieve/pii/S1473309920304242.

Hu Z, Song C, Xu C, Jin G, Chen Y, Xu X, et al. Clinical characteristics of 24 asymptomatic infections with COVID-19 screened among close contacts in Nanjing, China. Sci China Life Sci [Internet] 2020;63(5 May 4)706–11. . Available from: http://link.springer.com/10.1007/s11427-020-1661-4.

Huang P, Liu T, Huang L, Liu H, Lei M, Xu W, et al. Use of Chest CT in Combination with Negative RT-PCR Assay for the 2019 Novel Coronavirus but High Clinical Suspicion. Radiology [Internet] 2020;295(1 April)22–3, doi:http://dx.doi.org/10.1148/radiol.2020200330 Available from:.

Li Y, Li J, Zhang Y, Dai L, Li L, Liu J, et al. Development of an automatic integrated gene detection system for novel severe acute respiratory syndrome-related coronavirus (SARS-CoV2). Emerg Microbes Infect [Internet] 2020a;9(1 January 1)1489–96, doi:http://dx.doi.org/10.1080/22221751.2020.1782774 Available from:.

Li Y, Yao L, Li J, Chen L, Song Y, Cai Z, et al. Stability issues of RT-PCR testing of SARS-CoV-2 for hospitalized patients clinically diagnosed with COVID-19. J Med Virol [Internet] 2020b;92(7 July 5)903–8, doi:http://dx.doi.org/10.1002/jmv.25786 Available from:.

Lim Y, Ng Y, Tam J, Liu D. Human Coronaviruses: A Review of Virus–Host Interactions. Diseases [Internet] 2016;4(4 July 25)26. . Available from: http://www.mdpi.com/2079-9721/4/3/26.

Lou B, Li T-D, Zheng S-F, Su Y-Y, Li Z-Y, Liu W, et al. Serology characteristics of SARS-CoV-2 infection since exposure and post symptom onset. Eur Respir J [Internet] 2020;, doi:http://dx.doi.org/10.1183/13993003.00763-2020 May 19;2000763. Available from:.

Lu R, Wu X, Wan Z, Li Y, Jin X, Zhang C. A Novel Reverse Transcription Loop-Mediated Isothermal Amplification Method for Rapid Detection of SARS-CoV-2. Int J Mol Sci [Internet] 2020;21(8 April 18)2826. . Available from: https://www.mdpi.com/1422-0067/21/8/2826.

Modarelli JJ, Ferro PJ, Esteve-Gasent MD. Development and application of a canine endogenous internal positive control for use in real-time PCR assays. J Vet Diagnostic Investig [Internet] 2018;30(5 September 22)789–92, doi:http://dx.doi.org/10.1177/1040638718795206 Available from:.

Pfefferle S, Reucher S, Nörz D, Lütgehetmann M. Evaluation of a quantitative RT-PCR assay for the detection of the emerging coronavirus SARS-CoV-2 using a high throughput system. Eurosurveillance [Internet] 2020;25(9 March 5). . Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2020.25.9.2000152.

To KK-W, Tsang OT-Y, Leung W-S, Tam AR, Wu T-C, Lung DC, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect Dis [Internet] 2020;20(5 May)565–74 Available from: https://linkinghub.elsevier.com/retrieve/pii/S1473309920301961.

van Kasteren PB, van der Veer B, van den Brink S, Wijsman L, de Jonge J, van den Brandt A, et al. Comparison of seven commercial RT-PCR diagnostic kits for COVID-19. J Clin Virol [Internet] 2020;128:104412. . Jul Available from: https://linkinghub.elsevier.com/retrieve/pii/S1386653220301542.

Wang Y, Kang H, Liu X, Tong Z. Combination of RT-qPCR testing and clinical features for diagnosis of COVID-19 facilitates management of SARS-CoV-2 outbreak. J Med Virol [Internet] 2020;92(6 June 11)538–9, doi:http://dx.doi.org/10.1002/jmv.25721 Available from:.

Whelan JA, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods [Internet] 2003;278(1–2 July) 261–9. . Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022175903002230.

Xie X, Zhong Z, Zhao W, Zheng C, Wang F, Liu J. Chest CT for Typical Coronavirus Disease 2019 (COVID-19) Pneumonia: Relationship to Negative RT-PCR Testing. Radiology [Internet] 2020;296(2 August), doi:http://dx.doi.org/10.1148/radiol.2020200343 E41–45. Available from:.

Yang J, Zheng Y, Gou X, Pu K, Chen Z, Guo Q, et al. Prevalence of comorbidities and its effects in patients infected with SARS-CoV-2: a systematic review and meta-analysis. Int J Infect Dis [Internet] 2020;94:91–5. . May Available from: https://linkinghub.elsevier.com/retrieve/pii/S1201971220301363.

Yoon JG, Yoon JG, Song JY, Yoon S-Y, Lim CS, Seong H, et al. Clinical Significance of a High SARS-CoV-2 Viral Load in the Saliva. J Korean Med Sci [Internet] 2020;35 (20). . Available from: https://jkms.org/DOIx.php?id=10.3346/jkms.2020.35.e195.

Yuen K-S, Ye Z-W, Fung S-Y, Chan C-P, Jin D-Y. SARS-CoV-2 and COVID-19: The most important research questions. Cell Biosci [Internet] 2020;10(1 December 16)40. . Available from: https://cellandbioscience.biomedcentral.com/articles/10.1186/s13578-020-00404-4.

Zheng Z, Peng F, Xu B, Zhao J, Liu H, Peng J, et al. Risk factors of critical & mortal COVID-19 cases: A systematic literature review and meta-analysis. J Infect [Internet] 2020;. . Apr; Available from: https://linkinghub.elsevier.com/retrieve/pii/S0163445320302346.

Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet [Internet] 2020;395(10229 March)1054–62. . Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673620305663.

Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. N Engl J Med [Internet] 2020;382(12 March 19)1177–9, doi:http://dx.doi.org/10.1056/NEJMc2001737 Available from:.