EARLY ONLINE RELEASE

This article was posted on the Archives Web site as an Early Online Release. Note: Due to the extremely time sensitive nature of the content of this article, it has not been copyedited or formatted per journal style. Changes or corrections may be made to this article when it appears in a future print issue of the Archives. Early Online Release articles are citable by using the Digital Object Identifier (DOI), a unique number given to every article.

The DOI for this manuscript is doi: 10.5858/arpa.2021-0423-SA

The final published version of this manuscript will replace the Early Online Release version at the above DOI once it is available.

© 2021 College of American Pathologists
IDENTIFYING INCONCLUSIVE DATA IN THE SARS-CoV-2 MOLECULAR DIAGNOSTIC USING NUCLEOCAPSID PHOSPHOPROTEIN GENE AS TARGET

Raphael Contelli Klein, PhD; Mary Hellen Fabres Klein, PhD; Larissa Gomes Barbosa, BS; Lívia Vasconcelos Gonzaga Knnup, MD; Larissa Paola Rodrigues Venâncio, PhD; Jonilson Berlink Lima, PhD; Théo Araújo-Santos, PhD

Infectious Agents and Vectors Research Group at the Center for Biological and Health Sciences, Federal University of Western Bahia, Barreiras, Bahia, Brazil

Corresponding author:
Théo Araújo-Santos, PhD
Infectious Agents and Vectors Research Group
Center for Biological and Health Sciences
Federal University of Western Bahia
Rua da Prainha, 1326, Morada Nobre
Barreiras, Bahia, 47810047 Brazil
theo.santos@ufob.edu.br

The authors have no relevant financial interest in the products or companies described in this article.

Supplemental digital content can be found at the end of article.
**ABSTRACT**

**Context:** The gold standard test to identify the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in coronavirus disease 2019 (COVID-19) patients is the real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR), but the inconclusive data and presence of false positive diagnosis remain the major problem of this approach.

**Objective:** To compare the fitness of two primers sets to the SARS-CoV-2 nucleocapsid phosphoprotein (NP) gene in the molecular diagnosis of COVID-19, we verify the inconclusive data and confidence of high cycle threshold (Ct) values in the SARS-CoV-2 detection.

**Design:** The 970 patient samples were tested using United States Centers for Disease Control and Prevention protocol. We compared the fitness of two primers sets to two different regions of NP gene. In addition, we check the consistency of positive samples with high Ct values by retesting extracted SARS-CoV-2 RNA or by second testing of patients.

**Results:** The N1 and N2 displayed similar fitness during testing with no differences between Ct values. Then, we verified security range Cts related to positive diagnostic with Ct above 34 failing in 21/32 (65.6%) after retesting of samples. The samples patients with Ct above 34.89 that were doubly positive revealed a low sensitivity (52.4%) and specificity (63.6%) of the test in samples with Ct above 34.

**Conclusions:** It is secure to use one primer set to the NP gene to identify SARS-CoV-2 in samples. However, samples with high Ct values may be considered inconclusive and retested to avoid false positive diagnosis.
INTRODUCTION

Demands of molecular diagnosis to testing coronavirus disease 2019 (COVID-19) are fast growing around the world and costs and efficiency of the reverse transcription quantitative polymerase chain reaction (RT-qPCR) technique has been in check. In this context, different RT-qPCR kits are used to identify severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in patients’ samples are now available, using just one or multiple gene targets 1-3.

After six months in the COVID-19 pandemic, Brazil was between major countries affected by disease 4. Genome vigilance of the virus is now occurring and variants of the virus genome are disposable to genomic banks permitting check of the virus specificity of the disposable tests 5. Some studies are comparing sensitivity and specificity of different sets of probes/primers against different gene targets of the SARS-CoV-2. In this context, the conserved nucleocapsid phosphoprotein gene (NP) is the major target present in the disposable tests 1 and United States Centers for Disease Control and Prevention (US CDC) test includes two sets of primers with good sensitivity to virus detection 6. In addition, recent work demonstrates some mutation in the forward primer to NP gene target in Chinese Centers for Disease Control and Prevention test, suggesting US CDC protocol can be more appropriate to Brazilian testing 5.

The major problem to break transmission is the early diagnosis of SARS-CoV-2 Infection. Recent data claim to the risk of false positive diagnosis to flexibilization of transmission cares 7-9. False positive RT-qPCR diagnosis is related to air droplets containing viral particles 10 and technical accuracy during sample processing 9.

In this article, we compared the fitness between both commercial NP gene targets present in the US CDC panel to SARS-CoV-2 and we identified equivalent
results using both targets. In addition, we verified that high cycle threshold (Ct) value in patient samples can show low confidence, suggesting that this Ct range can contribute to misinterpretation and false positive in the test results.

MATERIALS AND METHODS

Clinical Samples

Nasopharyngeal, nasal, and oropharyngeal swabs and sputum samples (n=970) collected during May and June of 2020 were obtained after SARS-CoV-2 detection in the Laboratory of Vectors and Infection Disease. Residual samples were de-identified samples and considered non-human subjects of the research. These samples were used to test the fitness profile of the US CDC 2019-nCoV_N1 and 2019-nCoV_N2 primer-probe sets as described in the following sections.

RNA Isolation

Sputum and Swabs obtained from patients reporting covid-19-like symptoms were processed to RT-qPCR SARS-CoV-2 detection. In brief, RNA isolation of samples was performed using commercial kits following supplier’s instructions, such as PureLink Viral RNA/DNA Mini Kit (ThermoScientific), Cellco (Cellco Biotec) and Biogene (Quibasa) and RNAs were resuspended in 60 µL of RNase-free water (GIBCO).

RT-qPCR

Reverse transcription quantitative PCR analysis was performed on QuantStudio 5 Real-Time PCR system (ThermoScientific, USA) using the primer set from 2019-nCOV RUO kit (IDT Coralville, IA). The PCR reaction mixture consisted of TaqMan
Fast Virus 1-Step Master Mix (ThermoScientific), 0.75 μL of primers and 2.5 μL of RNA in a final volume of 10 μL reaction. Cycling conditions were 50°C for 5 min and 95°C for 20 seconds, followed by 45 cycles at 95°C for 15 seconds and 58°C for 1 min. Alternatively, we used Kappa Probe Fast qPCR Master Mix (2X) Kit (Sigma-Aldrich), 0.75 μL of primers and 2.5 μL of RNA in a final volume of 10 μL reaction. Cycling conditions were 42°C for 5 min and 95°C for 3 minutes, followed by 45 cycles at 95°C for 5 seconds and 60°C for 1 minute. RNase P was used as a sample control. The primers and concentrations used in the experiment were as follows: 500 nM N1: Forward: 5′-GACCCCAAAATCAGCGAAAT-3′; 500 nM N1: Reverse: 5′-TCTGGTTACTGCCAGTTGAATCTG-3′; 125 nM N1-Probe FAM-ACCCCGCATACGTTTGGTGGACC-NFQ-MGB; 500 nM N2: Forward: 5′-TTACAAACATTGGCCGCAAA-3′; 500 nM N2: Reverse: 5′-GCGCGACATTCCCGAAGAA-3′ and 125 nM N2-Probe FAM-AACAATTGCCCCCAGCGCTTCAG-NFQ-MGB. Specificity of the PCR products of N1 and N2 amplification were confirmed by polyacrylamide gel electrophoresis with silver stain.

Single Nucleotide Polymorphisms in the NP gene

Single nucleotide polymorphisms (SNP) in the NP gene were identified using disposable data in the National Center for Biotechnology Information (NCBI) virus (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/scov2_snp, accessed 09/18/2021). Next, we measured the absolute and relative frequencies of the SNPs present in N1 and N2 regions or out of them in the NP gene of the reference genome of SARS-CoV-2 (NC_045512.2:28274-29533).
Statistical Analysis

Descriptive statistics were performed to determine the relative frequencies for categorical variables, as well as to obtain medians and their respective standard error values for continuous variables. Linear regression was built to compare N1 and N2 linearity profiles between both two targets to Ct value in all samples used in this study. A Receiver Operator Characteristics (ROC) curve analysis was performed to assess the sensibility and specificity of Ct value in a subset of samples. All data was analyzed using GraphPad Prism 5.0 (GraphPad Software Inc).

Ethic Statement

The Research Ethics Committee of Universidade Federal do Oeste da Bahia (UFOB) approved this study in 2020 (license number: 30629520.6.0000.0008). All clinical investigations were conducted according to the Declaration of Helsinki.

RESULTS

No Differences between N1 and N2 Primers Set Fitness in the SARS-CoV-2 Detection

Several tests use just one set of primers to detect SARS-CoV-2\(^2\). The efficiency or increment of different targets in the detection of SARS-CoV-2 was poorly addressed. Herein, we evaluate fitness of NP gene targets present in US CDC test diagnosis in 1029 population naso and oropharyngeal swabs or sputum samples. We verified N1 and N2 primers sets displayed similar Ct values for each sample (Figure 1). To verify the fitness in each Ct value range, we quantify the frequencies of results between N1 and N2 primers sets. We verify no differences of Ct value failure to detect viral RNA between both N1 and N2 primers sets (Table 1). In addition, we checked the relationship between patient data such as demographic and days of symptoms with Ct
values (Table 2). We observe no differences in Ct values between N1 and N2 by sex, age, or day of symptoms (Table 2). This data suggests just one primer set could be used to test patients with SARS-CoV-2 infection potentially reducing costs during molecular testing without diminish efficiency in the diagnosis accuracy.

**Accuracy of NP Gene Target in SARS-CoV-2 Detection**

The major challenge during presence/absence testing in molecular diagnosis is determinate the cut offs of testing. In this work, we verify using commercial templates of NP gene the limit of detection (LoD) and cut offs of each N1 and N2 primers sets (supplemental figure 1, [Supplemental Digital Content can be found at the end of article]). Both NP gene targets were able to detect 5 genome copies (GC) per microliter of reaction with Ct value with N1 (34.28±0.6841) and N2 (34.18±0.5382). In recent work, mock group usage shows high Ct values during US CDC N molecular test, highlighting the importance of establishing different cut offs that are proposed by standard protocols that recommend 40 Ct value 12,13. In this work, we verified a low reproducibility of high Ct values in patient samples using N1 and N2 US CDC primer sets (figure 2A). Also, we identified a usual amplicon size profile in the gel using both primers set in those samples (figure 2B-D). Our data suggest that traditional PCR method and acrylamide gels can alternatively be used in remote locals with poor access to molecular tools. SARS-CoV-2 antigen test are now disposable and they have lower cost than molecular methods and could be used to diagnostic COVID-19, but they present a less sensible and specific profile compared RT-qPCR14.

**High Ct Value Has Low Predictive Value to Diagnosis**

To verify the specificity of the US CDC test, we evaluated samples from the
same patient that were collected between 2 and 6 days after the first exam and we found that only 11/32 (34.4%) of the samples were negative, 10/32 (31.2%) maintained the Ct value and another 11/32 (34.4%) reduced the Ct value (figure 3A). The ROC curve analysis for patients with Ct above 34.9 that were doubly positive revealed a low sensitivity (52.4%) and specificity (63.6%) of the test in samples with Ct above 33 (figure 3B). Thus, our data suggest that samples tested with Ct close to the detection limit have a low predictive value and should not be considered for diagnosis before collecting a new sample and performing a second confirmatory test.

**Variability of N1 and N2 Regions from NP Gene**

Since fails of RT-qPCR detection are related with mutations in the targets used in molecular diagnostic, we studied the single nucleotide polymorphism (SNP) identified in the NP gene (Figure 4A and B). We observed 96/1362 (7.05%) SNPs in N1 region compared to 40/1362 (2.94%) in N2. However, we identified a small number of mutation sites when compared to the 1226 SNPs, described outside the target region in the NP gene (Figure 4C). To verify the impact of the described SNPs in the molecular diagnostic of the COVID-19, we compared sequences N1 and N2 regions of NP gene from different variants of SARS-CoV-2, P.1, B.1.1.7, B.1.617.2, B.1.351 obtained from different world continents (supplemental figure 2A-C). Surprisingly, just one mutation was found in the N1 region of NP gene (supplemental figure 2B). The data suggest that NP gene continuous being a suitable target to diagnostic news variants of SARS-CoV-2. However, more studies will be needed to answer the impact of the mutations on the efficiency of the different targets in face of the news variants of the virus.
Performance analysis of SARS-CoV-2 virus detection tests was performed worldwide\textsuperscript{12,13}. The primers and probe set’ performance for virus detection was evaluated, but there are still few studies that assess the sensitivity and specificity in the critical range of detection of the RT-PCR technique\textsuperscript{2}. Among the most sensitive primer and probe sets are those for target N available by the US CDC\textsuperscript{6,13}. In this study, we evaluated the performance of the two sets of primers and probes used by the CDC and identified that both presented the same diagnostic performance, suggesting that only one of the targets could be used in the molecular diagnosis of COVID-19, as with other tests that use just one molecular target reducing costs\textsuperscript{1,2}. Liu et al. 2020 evaluated the performance of primer and probes sets from different RT-qPCR diagnostic kits for COVID-19, finding results similar to those of our study\textsuperscript{12}. Here, we assessed the performance of the US CDC primer set on sputum and swabs patient samples and noted a similar performance between the two N1 and N2 targets for virus detection. Some studies have compared the sensitivity between specimens for the detection of SARS-CoV-2 and identified that saliva samples may have a similar or superior sensitivity to swab samples\textsuperscript{13,15,16}.

The RT-qPCR is the gold standard technique for detecting the SARS-CoV-2 virus and it has been used to validate alternative diagnostic methods for COVID-19\textsuperscript{1,2,17}. However, data concerning about sensitivity and specificity of the technique for the higher Ct ranges are still scarce\textsuperscript{2}. In this sense, a study evaluated the Ct value of health workers who underwent two tests and found an increase in the Ct value in an interval of 21 days between exams\textsuperscript{18}. Most commercial diagnostic tests recommend that Ct values below 40 be considered as the cutoff point for a positive diagnosis for coronavirus\textsuperscript{12,13,19}. Nevertheless, studies that evaluated the detection limit, as well as, our study have shown a low predictive value of RT-qPCR in samples with Ct above 35 using the US
CDC protocol 12,13. We found that patients with Ct samples above 34, when retested in a brief period, may have the test result drastically altered, suggesting that high Ct values have a low positive predictive value. Due to the COVID-19 pandemic and the growing need for molecular testing, many laboratories have not been able to assess the efficiency of the tests made available for use and they are using Ct value below 40 as positive diagnoses for SARS-CoV-2. We identified, by repeating two or more times the sample extraction with high Ct, that these samples have low reproducibility, suggesting the need to perform a new US CDC test for greater diagnostic security. We identified that the low reproducibility of samples in the Ct range above 34 for US CDC primer sets could happen for 3 reasons: (i) cross contamination of the samples during processing; (ii) low viral load in the samples due to the final or initial stage of infection; (iii) presence of low viral load close to the detection limit of the technique20.

Few cases of reinfection have been reported in different countries around the world 21–24. The reinfection data report a case of mild infection with low viral load and high Ct value followed by a period without positive serology for SARS-CoV-2 infection and a second infection with high viral load and severe symptoms and followed by serology positive 21,22. We consider that the authors should exercise caution in stating cases of reinfection based on high Ct values in either of the two episodes reported in the same patient. In addition, a definitive study on humoral response demonstrated a robust long-term production of neutralizing antibodies against SARS-CoV-2 in patients infected only once 25. Evidence of reinfection should consider cases in which there was a clear viral load in both episodes and could rule out the presence of cross-contamination between samples during the analyzes, which could explain both the high Ct values between the samples and the genetic diversity observed. Then, any case report presented viral load below Ct value 30 in both episodes of infection should be
considered reinfection 21–24.

Nevertheless, this study has potential limitations. The short number of patients examined twice since it was difficult to get samples of the same in the moment of the pandemic in which testing was scaring to the population. In addition, we did not sequence the patient samples and we could not to affirm that those Ct failures are related to mutations in the N1/N2 targets, which must be very unlikely, considering that during the period of the pandemic in which the tests were conducted, no cases of variants have been identified in Brazil. Patients enrolled in this study was in distinct stages of the disease. However, we did not access to complete data of the patients to conclude whether Ct value used in diagnostic display a cut off enough to stablish a correlation between clinical forms of COVID-19 diagnosis and viral load. Moreover, we recommend more studies using others molecular targets to detect SARS-CoV-2 to verify if our data can be extrapolated to non-N1/N2 assays.

Together, our data show that both N1 and N2 sets of probes and primers can be used individually for the diagnosis of COVID-19. In addition, we found that the RT-qPCR technique involving US CDC primers should be used with caution in the diagnosis of patients whenever the Ct values are close to the detection limit established by each one laboratory services. We recommend that samples with Ct above the detection limit be re-extracted and reanalyzed. In cases of doubt, the patient sample may be tested again by an alternative qPCR protocol, or a new examination must be performed on the patient before the diagnosis can be released as positive. The data from this and study has an impact on the interpretation of future data about the COVID-19 pandemic and on the conduct of sample analysis using the NP gene as a target.
REFERENCES

1. Pizzol JLD, Hora VP da, Reis AJ, et al. Laboratory diagnosis for Covid-19: A mini-review. *Rev Soc Bras Med Trop.* 2020;53:e20200451. doi:10.1590/0037-8682-0451-2020

2. Mathuria JP, Yadav R, Rajkumar. Laboratory diagnosis of SARS-CoV-2 - A review of current methods. *J Infect Public Health.* 2020;13(7):901-905. doi:10.1016/j.jiph.2020.06.005

3. Yüce M, Filiztekin E, Özkaya KG. COVID-19 diagnosis —A review of current methods. *Biosens Bioelectron.* 2021;172:112752. doi:10.1016/j.bios.2020.112752

4. Hallal PC, Hartwig FP, Horta BL, et al. SARS-CoV-2 antibody prevalence in Brazil: results from two successive nationwide serological household surveys. *Lancet Glob Health.* 2020;8(11):e1390-e1398. doi:10.1016/S2214-109X(20)30387-9

5. Candido DS, Claro IM, de Jesus JG, et al. Evolution and epidemic spread of SARS-CoV-2 in Brazil. *Science.* 2020;369(6508):1255-1260. doi:10.1126/science.abd2161

6. Etievant S, Bal A, Escuret V, et al. Performance Assessment of SARS-CoV-2 PCR Assays Developed by WHO Referral Laboratories. *J Clin Med.* 2020;9(6):1871. doi:10.3390/jcm9061871

7. Roy S. COVID-19 Reinfection: Myth or Truth? *SN Compr Clin Med.* 2020;2(6):710-713. doi:10.1007/s42399-020-00335-8

8. Roy S. Physicians’ Dilemma of False-Positive RT-PCR for COVID-19: a Case Report. *SN Compr Clin Med.* 2021;3(1):255-258. doi:10.1007/s42399-020-00655-9
9. Lin L, Carlquist J, Sinclair W, Hall T, Lopansri BK, Bennett ST. Experience With False-Positive Test Results on the TaqPath Real-Time Reverse Transcription-Polymerase Chain Reaction Coronavirus Disease 2019 (COVID-19) Testing Platform. *Arch Pathol Lab Med.* 2020;145(3):259-261. doi:10.5858/arpa.2020-0612-LE

10. Scheier T, Shah C, Huber M, et al. Do we cause false positives? An experimental series on droplet or airborne SARS-CoV-2 contamination of sampling tubes during swab collection in a test center. *Antimicrob Resist Infect Control.* 2021;10(1):51. doi:10.1186/s13756-021-00920-z

11. Centers for Disease Control and Prevention. 2019-novel coronavirus (2019-nCoV) real-time RT-PCR primer and probe information. Published online 2020. https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html Accessed November 3, 2020.

12. Liu X, Feng J, Zhang Q, et al. Analytical comparisons of SARS-COV-2 detection by qRT-PCR and ddPCR with multiple primer/probe sets. *Emerg Microbes Infect.* 2020;9(1):1175-1179. doi:10.1080/22221751.2020.1772679

13. Vogels CBF, Brito AF, Wyllie AL, et al. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT–qPCR primer–probe sets. *Nat Microbiol.* 2020;5(10):1299-1305. doi:10.1038/s41564-020-0761-6

14. Boum Y, Fai KN, Nikolay B, et al. Performance and operational feasibility of antigen and antibody rapid diagnostic tests for COVID-19 in symptomatic and asymptomatic patients in Cameroon: a clinical, prospective, diagnostic accuracy study. *Lancet Infect Dis.* 2021;21(8):1089-1096. doi:10.1016/S1473-3099(21)00132-8
15. Güçlü E, Koroglu M, Yürümez Y, et al. Comparison of saliva and oro-nasopharyngeal swab sample in the molecular diagnosis of COVID-19. Rev Assoc Médica Bras. 2020;66(8):1116-1121. doi:10.1590/1806-9282.66.8.1116

16. Wyllie AL, Fournier J, Casanovas-Massana A, et al. Saliva or Nasopharyngeal Swab Specimens for Detection of SARS-CoV-2. N Engl J Med. 2020;383(13):1283-1286. doi:10.1056/NEJMc2016359

17. Ai T, Yang Z, Hou H, et al. Correlation of Chest CT and RT-PCR Testing for Coronavirus Disease 2019 (COVID-19) in China: A Report of 1014 Cases. Radiology. 2020;296(2):E32-E40. doi:10.1148/radiol.2020200642

18. Cariani L, Orena BS, Ambrogi F, et al. Time Length of Negativization and Cycle Threshold Values in 182 Healthcare Workers with Covid-19 in Milan, Italy: An Observational Cohort Study. Int J Environ Res Public Health. 2020;17(15):5313. doi:10.3390/ijerph17155313

19. on behalf of the SARS-CoV-2 Foch Hospital study group, Farfour E, Lesprit P, et al. The Allplex 2019-nCoV (Seegene) assay: which performances are for SARS-CoV-2 infection diagnosis? Eur J Clin Microbiol Infect Dis. 2020;39(10):1997-2000. doi:10.1007/s10096-020-03930-8

20. Layfield LJ, Camp S, Bowers K, Miller DC. SARS-CoV-2 detection by reverse transcriptase polymerase chain reaction testing: Analysis of false positive results and recommendations for quality control measures. Pathol - Res Pract. 2021;225:153579. doi:10.1016/j.prp.2021.153579

21. Prado-Vivar B, Becerra-Wong M, Guadalupe JJ, et al. A case of SARS-CoV-2 reinfection in Ecuador. Lancet Infect Dis. 2021;21(6):e142. doi:10.1016/S1473-3099(20)30910-5
22. Tillett RL, Sevinsky JR, Hartley PD, et al. Genomic evidence for reinfection with SARS-CoV-2: a case study. *Lancet Infect Dis*. 2021;21(1):52-58. doi:10.1016/S1473-3099(20)30764-7

23. To KKW, Hung IFN, Ip JD, et al. Coronavirus Disease 2019 (COVID-19) Re-infection by a Phylogenetically Distinct Severe Acute Respiratory Syndrome Coronavirus 2 Strain Confirmed by Whole Genome Sequencing. *Clin Infect Dis*. 2021;73(9):e2946-e2951. doi:10.1093/cid/ciaa1275

24. Van Elslande J, Vermeersch P, Vandervoort K, et al. Symptomatic Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) reinfection by a phylogenetically distinct strain. *Clin Infect Dis*. 2021;73(2):354-356. doi:10.1093/cid/ciaa1330

25. Wajnberg A, Amanat F, Firpo A, et al. Robust neutralizing antibodies to SARS-CoV-2 infection persist for months. *Science*. 2020;370(6521):1227-1230. doi:10.1126/science.abd7728
Figure legends

**Figure 1. Comparison between nucleocapsid phosphoprotein (NP) gene targets N1 and N2 Ct values during severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection in patient samples.** Data on the graphs indicate N1 and N2 Ct values comparison for each patient samples of (A) swab and (B) sputum by linear regression (left panels) or paired analysis (right panels). $P < .001$ for linear regression and paired t test $P > .99$.

**Figure 2. Checking nucleocapsid phosphoprotein (NP) gene targets N1/N2 accuracy by cycle threshold (Ct) value using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and polyacrylamide gel.** Patient samples were completely reanalyzed to check the accuracy to N1 and N2 gene targets. (A) Contingency graphs display percentages of agreement between two testing in different cycle threshold (Ct) groups. Representative (B) polyacrylamide gel and amplification plots (C) in log and (D) linear representations. Arrowheads indicate nonspecific amplicons. Und. - undetermined Ct.

**Figure 3. Double testing analysis of patient samples with high cycle threshold (Ct) value.** (A) Patient samples were completely reanalyzed after new patient’s testing until six days after first test. Blue dots indicate reduction and red dots indicate an increase of Ct value after second test. Red dashed line indicates the US CDC cut off to positives samples. (B) A receiver operator characteristics (ROC) curve analysis was performed to assess the sensibility and specificity in the comparison between double positive samples and just first positive sample (presumptive negative). AUC: area under curve.
Figure 4. Current mutations in the N1 and N2 regions of nucleocapsid phosphoprotein (NP) gene. Single nucleotide polymorphisms (SNP) were identified in the (A) N1 and (B) N2 regions from NP gene. Data on the graph (C) represent relative frequencies of SNPs on N1 and N2 regions. Total of 1362 mutations were identified in hole NP gene until September 18th, 96/1362 (7.05%) in N1 and 40/1362 (2.94%) in N2 regions.
| Outcomes<sup>a</sup> | All samples (n=970) | Sputum (n=174) | Swabs (n=796) |
|----------------------|---------------------|----------------|---------------|
|                      | N1                  | N2             | N1            | N2            | N1            | N2            |
| Undetermined         | 466 (48.1%)         | 466 (48.0%)    | 83 (47.7%)    | 83 (47.7%)    | 383 (48.2%)   | 383 (48.1%)   |
| Ct < 30              | 250 (25.8%)         | 249 (25.7%)    | 55 (31.6%)    | 53 (30.5%)    | 195 (24.5%)   | 196 (24.6%)   |
| 30 ≤ Ct < 34         | 71 (7.3)            | 63 (6.5%)      | 11 (6.3%)     | 11 (6.3%)     | 60 (7.5%)     | 52 (6.5%)     |
| 34 ≤ Ct < 40         | 137 (14.2%)         | 142 (14.6%)    | 17 (9.7%)     | 23 (13.2%)    | 120 (15.1%)   | 119 (14.9%)   |
| 40 ≤ Ct failure      | 45 (4.6%)           | 50 (5.15%)     | 8 (4.6%)      | 4 (2.3%)      | 37 (4.7%)     | 46 (5.8%)     |

Ct indicates cycle threshold.

<sup>a</sup>Values on the table represent the absolute and relative frequencies.
| Variables                | Ct undetermined | Ct < 30 | 30 ≤ Ct < 34 | 34 ≤ Ct < 40 | 40 ≤ Ct (failure) |
|--------------------------|-----------------|---------|--------------|--------------|------------------|
|                          | N1 and N2       | N1      | N2           | N1           | N2               | N1               | N2               |
| Age (years)<sup>a</sup>  | 36.9 ±14.3      | 39.8±14.1| 39.7±14.1    | 38.0±14.4    | 39.1±14.9        | 36.8±14.1        | 35.7±13.5        | 36.6±15.6        | 39.4±18.8        |
| Sex (male)<sup>b</sup>   | 180 (38.6%)     | 132 (52.8%)| 133 (53.4%)  | 37 (52.1%)   | 30 (47.6%)        | 65 (47.4%)        | 65 (45.8%)        | 19 (42.2%)       | 25 (50.0%)       |
| Days of Symptoms<sup>a</sup> | 5.7±3.1         | 6.1±2.7  | 6.1±2.7      | 6.2±2.8      | 6.3±2.6          | 5.4±2.2          | 5.5±2.4          | 5.4±2.5          | 5.4±2.5          |

Abbreviation: Ct, cycle threshold

<sup>a</sup>Values represent media and standard deviation

<sup>b</sup>Values represent the absolute and relative frequencies
Figure A: Graph showing % Agreement between N1 and N2 replicates. The bars indicate whether the results were replicate or not replicate.

Figure B: Gel electrophoresis image with bands indicating N1 and N2 samples.

Figure C: Amplification plot showing Ct19, Ct29, and Ct35 with corresponding cycle numbers.

Figure D: Amplification plot similar to Figure C.
Supplemental Digital Content. The Supplemental Digital Content was not copyedited by Archives of Pathology & Laboratory Medicine.

Supplemental figure 1. Limit of detection (LoD) from N1 and N2 targets using commercial plasmids. Commercial plasmids provided by Integrated DNA Technologies (IDT) contained nucleocapsid phosphoprotein gene of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were used to determinates the LoD of N1 and N2 regions targets by primers sets and probes designed by United States’ Centers for Disease Control and Prevention. All samples’ Ct were determined using the same threshold value acquired from the standard curves. left panels show the linear regression and right panels show the amplification plots of plasmids dilutions detected by (A) N1 and (B) N2 primer/probe sets. (C) shows pairwise analyses of N1 and N2 Ct values of samples with 5 GC/μL of N gene. GC: genome copies.
>NC_045512.1:28274-29533 severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, Nucleocapsid Phosphoprotein gene

A) Sequence of the first case

B) Melting regions of nucleocapsid phosphoprotein (NP) gene in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants. (A) Sequence of the first case

Supplemental figure 2. Melting regions of nucleocapsid phosphoprotein (NP) gene in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants. (A) Sequence of the first case of SARS-CoV-2 infection was compared with disposable NP gene sequences from new variant isolates until September 2021. (B–C) shows a single mutation in the region of N1 and no mutations in region of N2 targets in the analyzed sequences.