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RESEARCH ARTICLE

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Analysis by real-time PCR of five transport and conservation mediums of nasopharyngeal swab samples to COVID-19 diagnosis in Santiago of Chile

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Abstract

Due to the COVID-19 pandemic, many transport kits have been manufactured to preserve and transport nasopharyngeal swab samples (NPSs) from patients. However, there is no information on the performance of the different virus transport media (VTM) used in COVID-19 diagnosis in the population of Santiago de Chile. We compared the RT-qPCR amplification profile of five different viral transport kit mediums, including DNA/RNA Shield™, NAT, VTM-N, Ezmedlab™, and phosphate-buffered saline (PBS), for NPSs from Central Metropolitan Health Service, Santiago, Chile. The DNA/RNA Shield™ medium showed a better performance in terms of Cq and RFU values for the internal reference RNase P and viral ORF1ab probes. By contrast, the PBS transport medium registered higher Cq values for the viral and reference gene, compared to the other VTM. DNA/RNA Shield™ shows higher relative fluorescence units (RFUs) and lower Cq values for the reference gene. Collectively, our results suggest that the PBS medium could compromise the sample diagnosis because of its lower RT-qPCR performance. The NAT, Ezmedlab and VTM-N, and DNA/RNA Shield™ media show acceptable RT-qPCR parameters and, consequently, seem suitable for use in COVID-19 diagnosis.

Carlos Barrera-Avalos and Roberto Luraschi contributed equally to this study.

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KEYWORDS

false negative diagnostic, nasopharyngeal sample transport kits, RNA subunit of ribonuclease P (RNase P) amplification, SARS-CoV-2 diagnostics, SARS-CoV-2 ORF1ab gene, virus transport medium

1 | INTRODUCTION

The severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) has led to a wide spectrum of challenges in the process of viral detection in the control of the COVID-19 pandemic. The current massive worldwide molecular method to detect the SARS-CoV-2 genome in any sample is by RT-RT-qPCR, a molecular diagnostic technique that identifies the genetic material of the virus from the upper respiratory tract, including oropharyngeal, nasopharyngeal, or saliva samples.[1,2] One of the critical steps for virus detection is the pre-analytical stage that involves the collection, preserving, and transporting of the sample to the clinical laboratory.[3,4] The Centers for Disease Control and Prevention (CDC) indicates that the sample should be transported in a suitable VTM for an efficient diagnosis of the COVID-19.[5] On the other hand, the Food and Drug Administration (FDA) recommended the use of an alternative viral transport medium (VTM) to counteract the constantly increasing demand in the transport and preservation of the viral sample.[6] Owing to the stock-out suffered due to the constant increase in the demand for diagnosis of SARS-CoV-2, many transport kits have been manufactured and used to supply the minimum inputs necessary to maintain the traceability of the infection. However, it is necessary to determine the capacity of the different kits to preserve the SARS-CoV-2 virus for its correct diagnosis. In this study, we evaluated the performance of five commercial kits aimed for collection, preservation, and transport of nasopharyngeal swab samples (NPSs), for detection and diagnostic of SARS-CoV-2 by RT-RT-qPCR. The solutions included in the analysis were DNA/RNA Shield™, NAT medium (NAT™), VTM-N, Ezmedlab™, and phosphate-buffered saline (PBS) transport medium. These solutions were chosen because they were available and widely used in the Central Metropolitan Health Service in Chile. We observed differences in the amplification of the internal control (RNase P) and viral gene (ORF1ab), probably associated with the different medium and preservation characteristics.

2 | MATERIALS AND METHODS

2.1 | Samples

NPSs of patients that belong to the Central Metropolitan Health Service (CMHS; Santiago of Chile) were included in the study. The swab samples were collected in patients who arrive at the Center with the suspicion of being infected with COVID-19. At the time of sampling collection, none of the patients analyzed was already admitted to the healthcare center for COVID-19 or any other condition. The nasopharyngeal sample was taken by a trained healthcare worker and authorized for this procedure by the CMHS, following the indications detailed on the Interim Guidelines for Collecting and Handling of Clinical Specimens for COVID-19 Testing (Centers for Disease Control and Prevention.[7] In particular, the procedure was completed when the swab was saturated with fluid. Then, the NPSs were preserved and transported using: (1) Genosur (catalog number: DM0001VR; Genosur LLC, NW) that contains an RNA stabilization buffer called DNA/RNA Shield™ (Zymo Research Corp) (*n* total = 136 samples). (2) IMPROVIRAL™ NAT medium (NAT) (catalog number: 550040; Improve Medical Instruments Co. Ltd), medium based on guanidine salts (*n* total = 25 samples). (3) Ezmedlab™ (catalog number: 04010206) medium based on guanidine salts (*n* total = 23 samples). (4) CITOSWAB® VTM-N medium based on guanidine salts (catalog number: 2118-0015) (*n* total = 90 samples). (5) Winkler LTDA. PBS sterile solution (catalog number 634280) (*n* total = 90 samples). All the samples arrived at the laboratory before the first 24 h after the sampling collection taking care of the cold chain temperature.

2.2 | Total RNA extraction

Total RNA extraction was carried out using the Total RNA purification Kit (96 deep well plate format; Norgen Biotek Corp). Briefly, 250 µl of NPS from each patient was collected in a 1.5-ml tube and vortexed with 500 µl of lysis buffer (buffer RL: absolute ethanol; 1:1) for 1 min. Then, the solution was centrifuged at 14 000*g* for 5 min at room temperature. Subsequently, 700 µl of the lysate was transferred to a 96-filter plate and centrifuged at 1690*g* for 6 min. The 96-filter plate was washed two times with 400 µl of wash solution A. After each wash, the plate was centrifuged at 1690*g* for 4 min. Then, the plate was centrifuged at 1690*g* for 10 min to any volume trace. Finally, the total RNA was eluted using 70 µl of Elution solution A and centrifuged at 1690*g* for 7 min. The purified RNA was evaluated immediately by quantitative reverse transcription PCR (RT-qPCR).

2.3 | SARS-CoV-2 detection by RT-RT-qPCR

The detection of viral SARS-CoV-2 genome sequence was carried out using the ORF1ab probe (TaqMan™ 2019nCoV Assay Kit v1, Thermo Fisher Scientific, Cat. no. A47532) using a one-step strategy. Positive internal control probes for ORF1ab and RNase P (TaqMan™ 2019-nCoV Control Kit v1; Thermo Fisher Scientific, Cat. no.

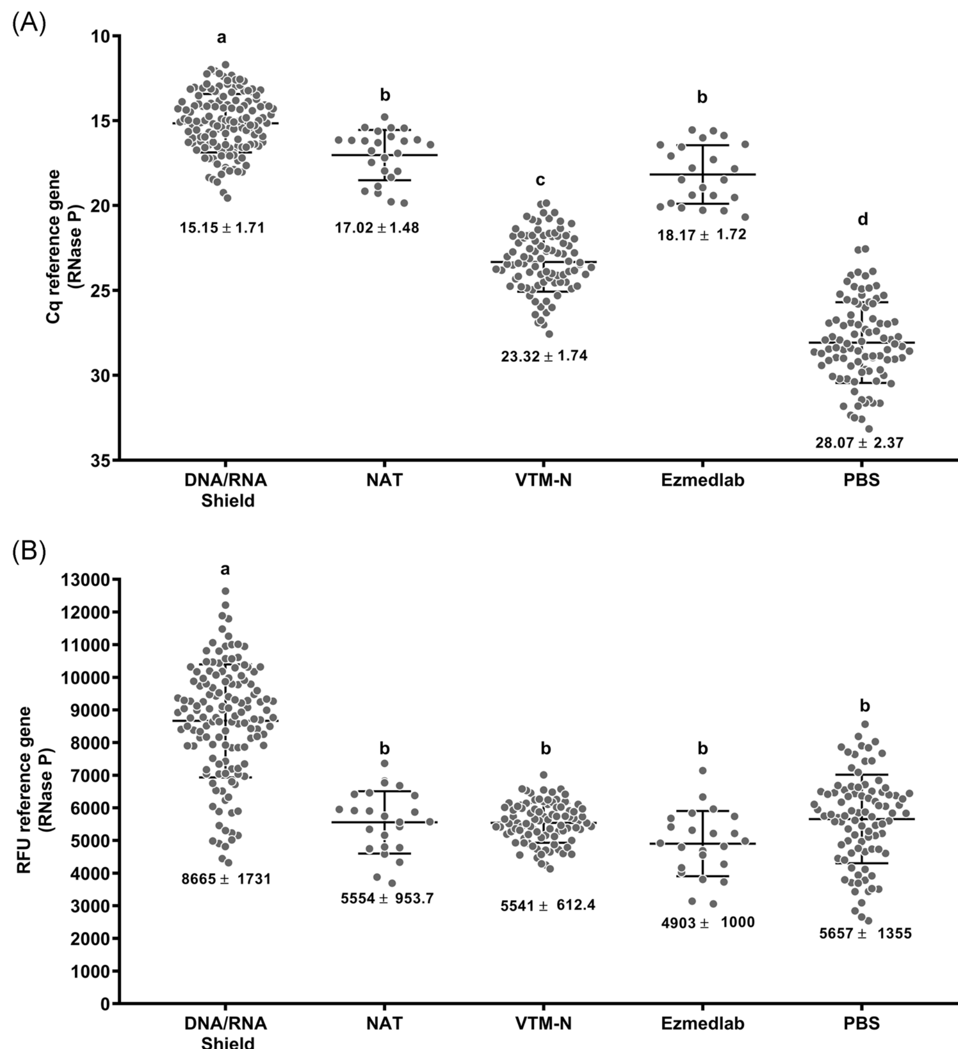

FIGURE 1 RT-qPCR detection parameters for internal reference RNase P probe from nasopharyngeal swab samples (NPSs) preserved and transported in different viral transport media kits. (A) Cycle of quantification (Cq) and (B) relative fluorescent units (RFUs) value comparison for NPSs preserved and transported in DNA/RNA Shield™ (n = 136), NAT (n = 25), VTM-N (n = 90), Ezmedlab (n = 23) transport kits and PBS solution (n = 90), all followed by an RNA extraction process. For statistical analysis, one-way ANOVA-test with multiple comparison test and descriptive statistics analysis was applied. Lowercase letters above spot columns denote transport kits with no significant differences between them ($p < 0.05$). Below spots, the mean ± standard deviation (mean ± SD) is indicated from sample amplification parameters obtained for all kits evaluated

A47533) were included and assessed individually in the 96-well PCR plate. The/polymerase from TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems™, Cat. no. 44-444-36) was included in each reaction. The reaction contained 5 µl of TaqMan™ Fast Virus 1-Step Master Mix 4X, 1 µl of ORF1ab assay 20X (FAM detector channel), 1 µl of RNase P assay 20X (HEX detector channel), 11 µl of nuclease-free water, and 2 µl of extracted RNA sample. The amplification thermal conditions included the reverse transcription at 50°C for 5 min, predenaturation at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. All the RT-RT-qPCR reactions were performed on the Agilent AriaMx Real-Time PCR System (Agilent Technologies, Part no. G8830A). Quantification cycle (Cq) and relative fluorescence units (RFUs) data were extracted from each NPS using the Agilent AriaMx software.

2.4 | Data representation and statistical analysis

The sample size needed to achieve significant differences was calculated using G-Power Software (version 3.1.9.2). To do it, we applied the strategy of comparison of population means to determine significant differences between transport media kits. Thus, a medium effect size was set up (f = 0.25), with a α value = 0.05, and a power (1−β) = 0.8. Taking into consideration that there were five different experimental groups, we determined that an n total = 200 was required to find significant differences between different transport media. In our manuscript, the n total of samples analyzed was equal to 364. Thus, with this number of samples, we ensure the chance to find a significant effect. GraphPad Prism 8 statistical software was used to analyze and plot the data obtained. From the total received

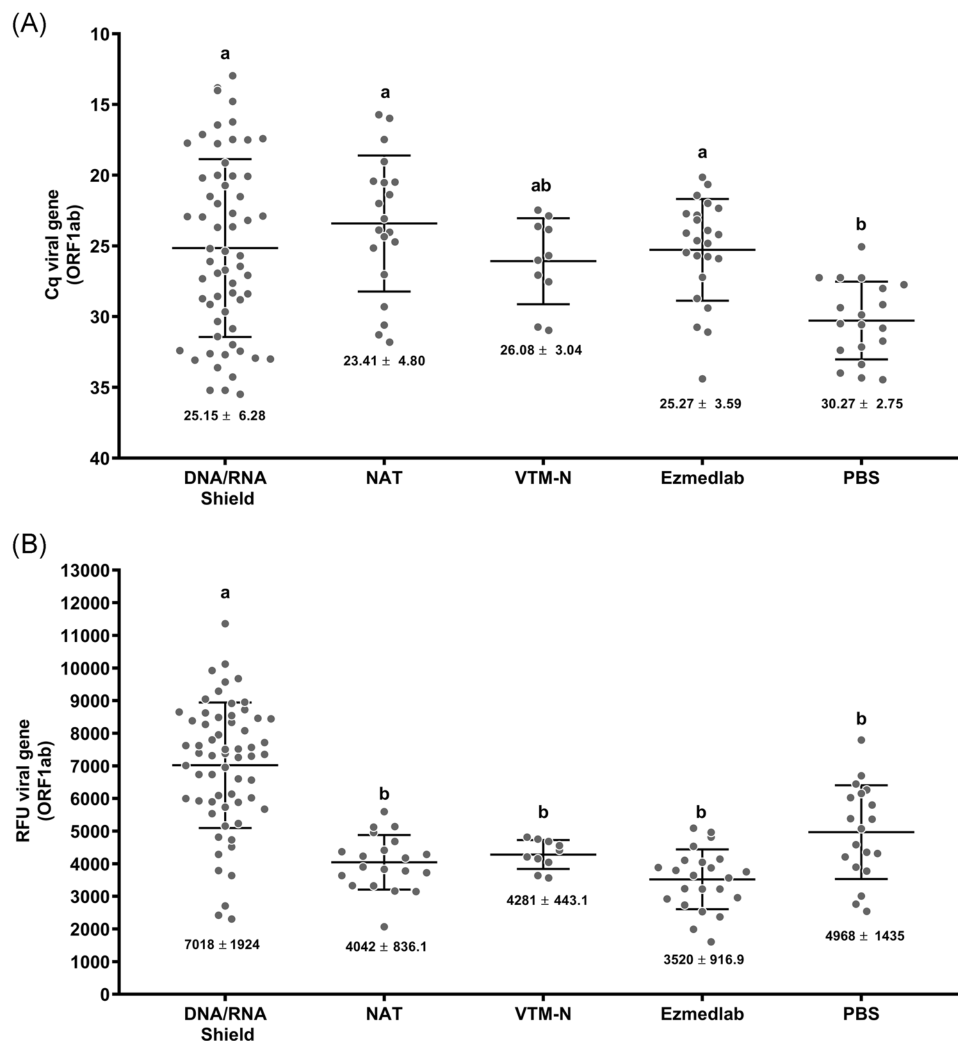

FIGURE 2 RT-qPCR detection parameters for viral ORF1ab probe from nasopharyngeal swab samples (NPSs) preserved and transported in different viral transport media kits. (A) Cycle of quantification (Cq) and (B) relative fluorescent units (RFUs) value comparison for NPSs preserved and transported in DNA/RNA Shield™ (n = 61), NAT (n = 20), VTM-N (n = 10), Ezmedlab (n = 23) transport kits and PBS solution (n = 19), all followed by an RNA extraction process. For statistical analysis, one-way ANOVA test with multiple comparison test and descriptive statistics analysis was applied. Lowercase letters above spot columns denote transport kits with no significant differences between them ($p < 0.05$). Below spots, the mean ± standard deviation (mean ± *SD*) is indicated from sample amplification parameters obtained for all kits evaluated

samples from April to September 2020, the cumulative number of samples, frequency of age, and gender of the patient (by month in total and positive samples) were graphed. For comparisons between different transport media, the Cq and RFU values were analyzed. A one-way ANOVA was used to determine differences between the cycle of quantification (Cq) value and RFUs from samples preserved and transported in the mediums evaluated in this study. A p-value of <0.05 was considered statistically significant.

2.5 | Ethics statement

All the experimental procedures included in this study was authorized by the Ethical Committee of the University of Santiago of Chile (No. 226/2021) and the Scientific Ethical Committee of the Central Metropolitan Health Service, Ministry of Health, Government of Chile (No. 370/2021), and following the Chilean law in force. Data analysis used for this study was conducted only using the internal sample code numbers assigned at the moment to receive them for diagnostic purpose. Accordingly, the samples have been irreversible anonymized.

3 | RESULTS

3.1 | RT-qPCR amplification profile of five different commercial transport media kits

We analyzed NPSs collected in different viral transport mediums. Most of the NPSs coming from the Central Metropolitan Health

Service zone were preserved and transported routinely using the DNA/RNA Shield™ medium. However, because the stock-out suffered due to the constant increase in the demand for diagnosis of SARS-CoV-2 in Chile, we received NPSs in other four different transport mediums, including NAT and VTM-N, Ezmedlab transport medium, and PBS solution. The evaluation of these NPSs kits was made with the internal reference RNase P probe (that works as control of total RNA extraction) and the viral ORF1ab probe.

When the internal reference RNase P probe Cq value was compared between all transport mediums, they showed significant differences between them, except between NAT and Ezmedlab transport mediums (Figure 1A). Samples preserved and transported in DNA/RNA Shield™ medium showed a Cq value 15.15 ± 1.71 (Figure 1A). Samples preserved and transported in NAT and Ezmedlab mediums showed no significant differences between them for the internal reference RNase P probe. These samples showed a mean Cq value of 17.02 ± 1.48 and 18.17 ± 1.72, respectively (Figure 1A). For the samples preserved and transported in VTM-N medium and PBS solution, there was a marked shift toward high Cq values, with the mean Cq value of 23.32 ± 1.74 and 28.07 ± 2.37, respectively (Figure 1A).

Concerning the RFUs value for the internal reference RNase P probe, samples preserved and transported in DNA/RNA Shield™ medium showed the highest mean RFU value, suggesting an improved quality of RNA extracted from NPSs. This transport medium showed a reference RNase P probe mean RFU value of 8665 ± 1731 (Figure 1B). On the other hand, samples preserved and transported in NAT, VTM-N, Ezmedlab mediums, and PBS solution showed no significant differences between the RFU from internal reference RNase P probe, with mean RFU values of 5554 ± 953.7, 5541 ± 612.4, 4903 ± 1000, and 5657 ± 1355, respectively (Figure 1B).

When the Cq value of the viral ORF1ab probe was compared between all mediums, these showed no significant differences between them, except for samples preserved and transported in PBS solution, where it shows higher Cq values compared to the other means of transport (Figure 2A). Samples preserved and transported in DNA/RNA Shield™ medium showed viral ORF1ab probe mean Cq value of 25.15 ± 6.28 (Figure 2A). This kit showed a wide range of viral Cq values as can be inferred from the standard deviation of its value, with positive results from Cq 12.96 till 35.49, close to the limit of detection of the ORF1ab probe (Cq = 37.14) from Thermo Fisher RT-qPCR Kit. In addition, the Cq values observed for those samples transported in DNA/RNA Shield™ medium registered a greater number of samples with lower Cq value and closer to the limit of detection compared to the other transport medium kits.

The samples preserved and transported in PBS solution showed the highest viral ORF1ab mean Cq value of 30.27 ± 2.75 (Figure 2A), suggesting the transport medium could affect the quality of the RNA extracted.

Regarding the viral ORF1ab RFU value showed by these different mediums, samples preserved and transported in DNA/RNA Shield™ medium showed the highest mean RFU value, suggesting an improved quality of RNA extracted from NPSs. This medium showed a viral ORF1ab probe mean RFU value of 7018 ± 1924 (Figure 2B). By contrast, samples preserved and transported in NAT, VTM-N, Ezmedlab mediums, and PBS solution showed no significant differences between them.

4 | DISCUSSION

The real-time PCR (RT-RT-qPCR) technique has been the gold standard and the most recommended method for the diagnosis for Coronavirus SARS-CoV-2.[8] One of the critical stages for its diagnosis is the collection, preservation, and effective transport of the sample to the laboratory of diagnostics due to the nature of the RNA-genome of the virus. However, the high demand for supplies during the COVID-19 pandemic has generated several stock breaks of material and essential reagents. As consequence, currently, there is a wide repertoire of kits for preserving and transporting the NPSs for the control of the COVID-19 pandemic. However, the variation in the detection signal and Cq value observed in our study suggests that the transport and preservation medium should be carefully considered for the detection of SARS-CoV-2 and its implications in diagnosis. In this line, previous studies have documented the efficacy of the use of different transport mediums for NPSs preservation, either commercials[9–12] or in-house production[13–15] for the detection and diagnosis of COVID-19. Accordingly, Garnett et al.[10] compared the performance of at least six swabs commonly found in primary healthcare settings for disease diagnosis, showing no significant differences in viral detection. Thus, the results indicate that several alternatives for sample transport kits can be used if supplies run out. However, there are several other specimen transport and preservation mediums used extensively for the diagnosis of the disease in Santiago of Chile. To date, they have not been studied or compared with each other. That is the case of the DNA/RNA Shield™, NAT, VTM-N, Ezmedlab mediums, and PBS solution. In this study, the effect of different preservation and transport mediums for NPSs was analyzed and compared for the massive diagnosis of SARS-CoV-2 by RT-qPCR. We detected variations between these mediums in the Cq value for the amplification of the internal reference RNase P probe. In the case of the viral ORF1ab probe, we detected no variations in the Cq value for the amplification, except for samples preserved in PBS solution. Regarding the RFU values for internal reference RNase P and viral ORF1ab probes, DNA/RNA Shield™ medium showed the highest RFU values when compared to the other mediums.

These differences between mediums may be associated with its composition in which the NPSs are preserved and transported. In the first place, in the case of the DNA/RNA Shield™ medium its composition stabilizes nucleic acids and biological samples at room temperature, in addition to completely inactivating viruses, bacteria, and fungi. This agent has been widely used for sample collection and transport of various infectious agents.[16–18] Indeed, the group of Hamilton et al.[19] reported this reagent improves the detection of SARS-CoV-2 in saliva samples, by detecting the Spike protein at lower Cq values compared to samples preserved without DNA/RNA

Shield™. Similarly, the group of Coryell et al.[20] reported that DNA/RNA Shield™ medium gave the best results in terms of viral detection. Importantly, it also showed better stability compared to PBS solution after seven days of preservation.

On the other hand, the NAT transport medium showed viral ORF1ab Cq values similar to those observed with DNA/RNA Shield™ Kit. However, NAT showed for the internal reference RNase P RFU value about 3000 RFU less than samples preserved in DNA/RNA Shield™ medium. The NAT medium consists of an RNA stabilization solution with guanidine salts. This is a strong chaotropic agent with the ability to deactivate viruses and preserve nucleic acids by disabling the action of nucleases and protein structures.[21] The use of this viral stabilizing and inactivating agent has been widely used in the study of several viruses[22,23] including SARS-CoV-2 for their detection and diagnosis during the pandemic.[12,24,25] For example, Carvalho et al.[26] analyzed NPSs kept in the medium with guanidine salts, obtaining Cq values like those reported in our current study. On the other hand, the PBS-preserved NPSs showed higher Cq values for internal reference RNase P probe than DNA/RNA Shield™, NAT, VTM-N, and Ezmedlab mediums. This difference in the Cq values for the internal control could be associated with the absence of a stabilizing solution capable of preserving the quality of the viral RNA in transport kits containing only PBS medium. However, the use has been supported of PBS solution as alternatives to VTM medium for SARS-CoV-2 testing[27,28] even preserving the sample at room temperature for 72 h[10] or preserving the sample for a month at 4°C.[29] The logistic difficulties for the primary healthcare centers to preserve the samples in PBS solution represent a serious disadvantage compared to other transport mediums because it is mandatory maintaining a cold chain temperature range from the sampling collection until processing. Furthermore, in the PBS-based transport kits, there is a possibility for the potential presence of any remaining still active viral particle. Accordingly, prior heat inactivation is imperative for its safe handling in laboratory conditions with a class 2 safety level. The handling of active SARS-CoV-2 samples of respiratory viruses is recommended under strict protocols of type 3 biological safety laboratories.[30,31] Therefore, in our study, the PBS-preserved samples were necessarily thermally inactivated as described in the methodology section, where this process could affect the RNA integrity. In this way, the information about the RFU value is essential for the diagnosis, because samples with a low RFU are indicative of problems associated to sample degradation

This study expands the knowledge about the performance of NPS transport solutions in pandemic circumstances and indicates the kits available in the market for collection, maintaining, and transporting samples and the modulation of Cq value range obtained for the internal control and SARS-CoV-2 detection. In this way, the displacement of Cq toward higher values may compromise the sensitivity of the PCR for the diagnosis of SARS-CoV-2. This could increase the possibility to get potential false-negative results. On the other hand, our results convincingly indicate that media containing viral inactivators such as DNA/RNA Shield™ and guanidine salts give better parameters, also considering that it avoids viral inactivation by heat and a potential lower RNA integrity for the sample, being easier its handling and processing in type 2 biosafety laboratories. This study broadens the knowledge regarding the data obtained from NPSs transport kits available in the market facing a stock-out event in SARS-CoV-2 pandemic circumstances. These results also serve as evidence for the application of these transport media in the massive diagnosis of new emerging infectious diseases, which facilitates the choice of the most viable sample transport kit. This is particularly relevant in the context of respiratory viruses spreading to countries where the laboratory's available massive infrastructure allows the handling and processing of samples only in a class 2 biosafety condition.

5 | CONCLUSION

Collectively, our results suggest that the DNA/RNA Shield™ medium shows a better performance in terms of Cq and RFU values for the internal reference RNase P and viral ORF1ab probes. Therefore, its use improves molecular diagnosis in relation to less ambiguity in the results obtained. Samples kept and transported in PBS show worse RT-qPCR parameters than the other media tested. PBS does not contain any viral inactivator, so its prior thermal inactivation is mandatory for handling in a type 2 biosafety laboratory. The NAT, Ezmedlab, and VTM-N media show correct RT-qPCR parameters and are suitable for use in COVID-19 diagnosis.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Conceptualization of the study was made by Mónica Imarai, Claudio Acuña-Castillo, Felipe E. Reyes-López, and Ana M. Sandino; Daniel Valdés, Claudio Acuña-Castillo, Felipe E. Reyes-López, and Ana M. Sandino supervised the study; Gaby Gutiérrez, Javiera Quiroz, Ailen Inostroza-Molina, Deborah Vargas, Daniel Valdés, and Claudio Acuña-Castillo were in charge of all the logistics of coordination and reception of samples from the Central Metropolitan Health Service; The logistics of sampling processing and RNA extractions were in

charge of Carlos Barrera-Avalos, Esteban Arenillas, Daniela Barría, Maximiliano Figueroa, Felipe Hernández, Carlos Mateluna, Javier Mena, Claudia Rioseco, Claudia Torrent, Claudio Vergara, and Valentina Wong; Roberto Luraschi, Javiera Alarcón, Julio Cartagena, Javiera Cayunao, Andrea Mella-Torres, Álvaro Santibañez, Sebastián Tapia, and Alejandro Undurraga received the RNA extracted and performed the RT-qPCR reactions; Felipe E. Reyes-López and Ana M. Sandino analyzed the data; Data representation was made by Roberto Luraschi, Eva Vallejos-Vidal, Claudio Acuña-Castillo, and Felipe E. Reyes-López; Carlos Barrera-Avalos, Roberto Luraschi, Eva Vallejos-Vidal, Claudio Acuña-Castillo, Felipe E. Reyes-López and Ana M. Sandino carried out the data processing and interpretation; Carlos Barrera-Avalos, Roberto Luraschi, Eva Vallejos-Vidal, Maximiliano Figueroa, Mónica Imarai, Claudio Acuña-Castillo and Felipe E. Reyes-López co-wrote the original draft. All the authors provided critical feedback and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

All the information about the results is showed and described in this manuscript. The data used to support the findings of this study (S1 Data set) are available in Supporting Information.

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