ARTICLE / INVESTIGACIÓN

Cost and performance analysis of efficiency, efficacy, and effectiveness of viral RNA isolation with commercial kits and Heat Shock as an alternative method to detect SARS-CoV-2 by RT-PCR

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Abstract: In late 2019 a new virus reported in Wuhan, China, identified as SARS-CoV-2, rapidly challenging the healthcare system worldwide. The need for rapid, timely and accurate detection was critical to the prevention of community outbreaks of the virus. However, the high global demand for reagents during the years 2020 and 2021 generated a bottleneck in kits used for detection, significantly affecting developing countries and lagging their ability to diagnose and control the virus in the population. The difficulty in importing reagents, high costs and limited public access to the SARS-CoV-2 detection test led to the search for alternative methods. In this framework, different commercial nucleic acid extraction methodologies were evaluated and compared against heat shock as an alternative method for SARS-CoV-2 detection by RT-PCR to determine the diagnostic yield and its possible low cost compared to other methodologies. Nasopharyngeal samples were used where the diagnostic efficiency of the alternative method was 70 to 73%. The evaluation of the discriminatory efficacy of the technique took the sensitivity and specificity to establish its cut-off point, being 0.73 to 0.817, which allows discrimination between COVID-19 positives and negatives; as for the diagnostic effectiveness expressed as, the proportion of subjects correctly classified is between 80 and 84%. On the other hand, in terms of the costs necessary to carry out the detection, the alternative method is more economical and accessible compared to the commercial methods available in this comparison and evaluation, being possible its implementation in developing countries with high infection rates, allowing access to the diagnostic test with a reliable and low-cost method.

Key words: COVID-19, RT-PCR, Viral RNA.

Introduction

Coronaviruses (CoV) are part of Coronaviridae family with unsegmented single-stranded positive RNA genome belonging 26 to 36 kb length with wide host range, including humans[1-3]. In the history of humankinds have experienced previus infection, during the 1960s CoV-virus have been describe beta-coronavirus like OC43-CoV and HKU1-CoV, and alfa-coronavirus like 229E-CoV and NL63-CoV. Currently are endemic, causes of common colds and mild respiratory infections[4]. In the last two decades, two beta-coronavirus caused of respiratory illnesses have been monitored, between 2002/2003 the severe cute respiratory syndrome-related human coronavirus 1 (SARS-CoV), and 2012 the middle east respiratory syndrome-related coronavirus (MERS-CoV) both of them produced severe respiratory syndrome[3,5-7]. The novel coronavirus SARS-CoV-2 was reported in Wuhan, China, in December of 2019. SARS-CoV-2 cause COVID-19 challenged the health public system worldwide and genetic sequencing of the virus suggest that SARS-CoV-2 closely linked to SARS-CoV-1, affecting more than 180 countries[8-10]. The most widely used test for detection of SARS-CoV-2 fall into nucleic-acid test, as a multistep that involves, nasopharyngeal swab sample collection, isolation of viral genetic material and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)[10-13].

During the first few weeks of the COVID-19 pandemic, the global demand for nucleic acid extraction kits and required reagents had already in short supply, making them a limiting source for SARS-CoV-2 testing due to those kits are mainly produced in industrialized countries, which means a disadvantage in the access to COVID-19 testing. Consequently, being a challenge for middle and low-income countries in need of improving SARS-CoV-2 testing fueling the development of alternative SARS-CoV-2 RNA isolation methods and protocols[10,12-16]. Most European countries and the United States have to deal with the accelerated growth of infections and enormous pressure on their health systems, where cases started to grow exponentially[17].

In the case of Latin American countries, their first cases were registered between the end of February and the beginning of March 2020, with Brazil reporting the first cases in the region. COVID-19 poses a significant risk in Latin American countries because countries share many economic, political and health system similarities in controlling COVID-19 outbreaks and deaths[17-19], but the number of IUC beds,prepared medical workers and the robust or fragile public health system between each country created a framework of

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differences of how control the outbreaks of COVID-19[18-20]. During the implementation of COVID-19 prevention and control measures, the nature and stringency of the response varied from each country based on closed international borders and declaring a national health emergency to ordering a curfew. Despite measures taken in response to the first cases of COVID-19 in Latin America, widespread testing is a crucial strategy to control the spread of the pandemic[19,20]. The need for rapid and accurate detection of SARS-CoV-2 was critical for the prevention and control of communitarian outbreaks. For this reason, the immediate availability of the complete genome of SARS-CoV-2 allowed the development of diagnostic kits employing the Reverse Transcription Polymerase Chain Reaction (RT-PCR) for specific regions of the SARS-CoV-2 genome[1,8,21,22]. The standard molecular method was developed based on the US Center for Disease Control and Prevention (CDC), Charite and World Health Organization (WHO), based on the amplification of specific regions of viral gene N, E and RdRp and the purified RNA isolated from the nasopharyngeal sample[10,23-25].

Nevertheless, the increasing number of tests that were performed worldwide has created a high demand for reagents necessary for SARS-CoV-2 detection, mainly during March-July of 2020In addition, the high need for these reagents has caused a shortage of this product, forcing the public and private health sector in Latin America to prioritize test only for people who have symptoms and signs of COVID-19 increasing the bias, to be left behind in COVID-19 diagnosis and control[21,26-28].

Several commercial SARS-CoV-2 RT-PCR protocols employ manual extraction kits to isolate viral RNA from nasopharyngeal swabs[23,29,30], whereby an accurate extraction, recovery and quantification determine the efficacy of RT-PCR detection[8,31]. The more common methods for viral isolation are (1) silica-based membrane[13,32], also called solid-phase RNA extraction; (2) organic extraction using phenol-guanidineIsothiocyanate (GITC) and (3) magnetic beads[12,33]. All these methods allow cell and viral lysis using registered reagents by trademarks, which has made them a limiting resource for SARS-CoV-2 diagnosis, mainly in the peaks of contagious in the middle of 2020 and 2021[12,34].

The virus's rapid spread in Latin America and the high cost of COVID-19 tests due to shortage of supplies and reagents limits testing access. In March 2020, the cost of the RT-PCR test in Ecuador was between 80 to 120 USD. Later, in June 2021, the cost was reduced to 45 USD[35,36]. This value of 45 USD, according to Trudeau, represents 4.2% of the average monthly income of a middy-class person who would be willing to pay in Latin America in latent demand for COVID-19 tests, concerning other countries where the charges made by private's labs at the beginning of the pandemic scale of up to $70 in Brazil, $140 in Chile, $80 in Colombia and $137 in Uruguay[20,37].

Laboratories across the globe face constraints on equipment and reagents during the COVID-19 pandemic. Here, we compare and evaluate a simple approach causing lysis to the cells by heat shock and using the solution directly to RT-PCR[9,21,38]. This methodology could be an alternative to perform a reliable and rapid diagnosis of SARS-CoV-2, compared with the CDC RT-PCR gold standard that takes about 3 hours to complete, particularly for developing countries where all needed reagents for diagnosis must be imported[10]. These approaches can help to access public or private COVID-19 tests at reasonable prices; however, these data reflected the problem of price variability over time due to high demand and importation paperwork for reagents and kits for testing in a developing country.

Nucleic acid extraction typically involves three general steps: cell lysis, separation of RNA/DNA from other macromolecules such as DNA/RNA, proteins, and lipids, followed by RNA/DNA elution[34]. Several commercial SARS-CoV-2 RT-PCR protocols employ manual extraction kits to isolate viral RNA from nasopharyngeal swabs[24,35,36], whereby an accurate extraction, recovery and quantification determine the efficacy of RT-PCR detection[9,37]. The more common methods for viral isolation are (1) silica-based membrane[14,38], also called solid-phase RNA extraction; (2) or-ganic extraction using phenol-guanidineIsothiocyanate (GITC) and, (3) magnetic beads[13,39]. All these methods allow cell and viral lysis using registered reagents by trademarks that has made them a limiting resource for SARS-CoV-2 diagnose mainly in the peaks of contagious in middle of 2020 and 2021[13,40].

The most common method for nucleic acid extraction uses Silica-based membrane technology, which relies on the ability of silica particles to adsorb DNA/RNA molecules under certain analytical conditions, and then eluted RNA precipitation using elution special buffers or nuclease-free water[11,34,38]. Another technique for RNA isolation requires the use of magnetic particles, that has several advantages based on (a) hydrogen-binding interaction with an underivatised hydrophilic matrix, typically silica, under chaotropic conditions, (b) ionic exchange under aqueous conditions by means of an anion exchanger, (c) affinity and (d) size exclusion[41]. Although there are numerous ways to extract and isolate RNA, most labs gravitate toward using organic extractions or com-mercially available kits. Acid guanidinium thiocyanate-phenol-chloroform is ongoing used to obtained nucleic acids, where the pH will determine the separation of nucleic acids and proteins. Polar RNA will remain in the upper polar phase, DNA will accumulate in the interphase and de-natured proteins will dissolve in the lower organic phase[34,42-44]. In the face of shortage of kits, re-agents and consumables; it is clear that a huge effort needs to be made to scale up current COVID-19 testing, thus is needed to evaluate alternative protocols, reagents, and approaches to allow a good nucleic-acid isolation for molecular detection of SARS-CoV-2. One of these ap-proaches used is heat-shock technique, that allows free-RNA extraction without purification that can be used directly in RT-PCR.

Considering the context of developing countries, high selling prices and access limitation to the public health system, our aim was to evaluate and compare the efficiency, efficacy and effec-tiveness of using commercial kits with the heat shock as method for extraction of genetic material for molecular detection of SARS-CoV-2 by RT-PCR, in order to propose a low-cost and reliable method.

Materials and methods

The samples were obtained from the project "Molecular diagnosis of SARS-CoV-2 in suspected COVID-19 samples from the Amazon region". In which the guidelines The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) and the Ecuadorian law of data protection were followed to carry out this observation, wherein no patient data have been included since it is a methodological analysis. The samples were employed after the diagnosis report was released to the MSP personnel.

Samples collection

A nasopharyngeal swab was the reference sampling method used to detect SARS-CoV-2, collected by health-care personnel using synthetic fibber swabs according to World Health Organization (WHO) general guidelines for respiratory sample collection. The samples were stored in 2 mL microtubes with 700 µL of Tris-EDTA buffer, pH 8.0[46]. Samples were received from Molecular Biology and Biochemistry laboratory at Universidad Regional Amazónica Ikiam, the inclusion/exclusion criteria for samples reception were: (1) transportation at 4 °C, (2) triple sealing for samples (collection tube with biofilm in caps, bio-safe bag and external box), (3) epidemiological information of patients, and (4) the samples should not be spilled.

Viral RNA extraction methods

Viral RNA extraction was performed using five different commercial kits, based on their four other technologies, following manufacturers' instructions with minor modifications. A total of 72 samples were selected (Figure 1). The five commercial kits were classified according to the purification method used to isolate viral RNA (Table 1).

The kits were named A, B, C, D, E and F. 35 samples were used with kits A, B and C, while 37 were analyzed with kits D, E, and F. One negative control (nuclease-free water) was included in each group.

Kit	Description of purification method	Required sample volume	RNA elution volume	Processing time	Number of isolated samples
Kit A	Lysis type: Manual lysis using virus binding buffer and proteinase K. Purification method: Silica membrane-based RNA resuspension: Elution Buffer	200 µL	50 µL	2-3 h	Set of 35 samples
Kit B	Lysis type: Manual lysis using guanidine salts Purification method: Silica membrane-based RNA resuspension: Elution buffer	140 µL	60 µL	2-3 h	Set of 35 samples
Kit C	Lysis type: Manual lysis using Viral RNA buffer Purification method: Silica membrane-based RNA resuspension: RNA – free water	200 µL	15 µL	1-2 h	Set of 35 samples
Kit D	Lysis type: Manual lysis by magnetic beads-based Purification method: complementary hybridization between nucleic acid and beads RNA resuspension: RNA-free water	200 µL	50 µL	3-4 h	Set of 37 samples
Kit E	Lysis type: Manual lysis using phenol and guanidine thiocyanate in a mono-phase separation. Purification method: organic phases using chloroform RNA resuspension: RNA – free water	200 µL	-	1-2 h	Set of 37 samples
Kit F	Lysis type: Heat Shock Purification method: N/A RNA resuspension: N/A	10 µL	1 µL	10 min	Set of 72 samples

Table 1. Description of commercial kits to isolate viral RNA according to manufacturers' instruction.

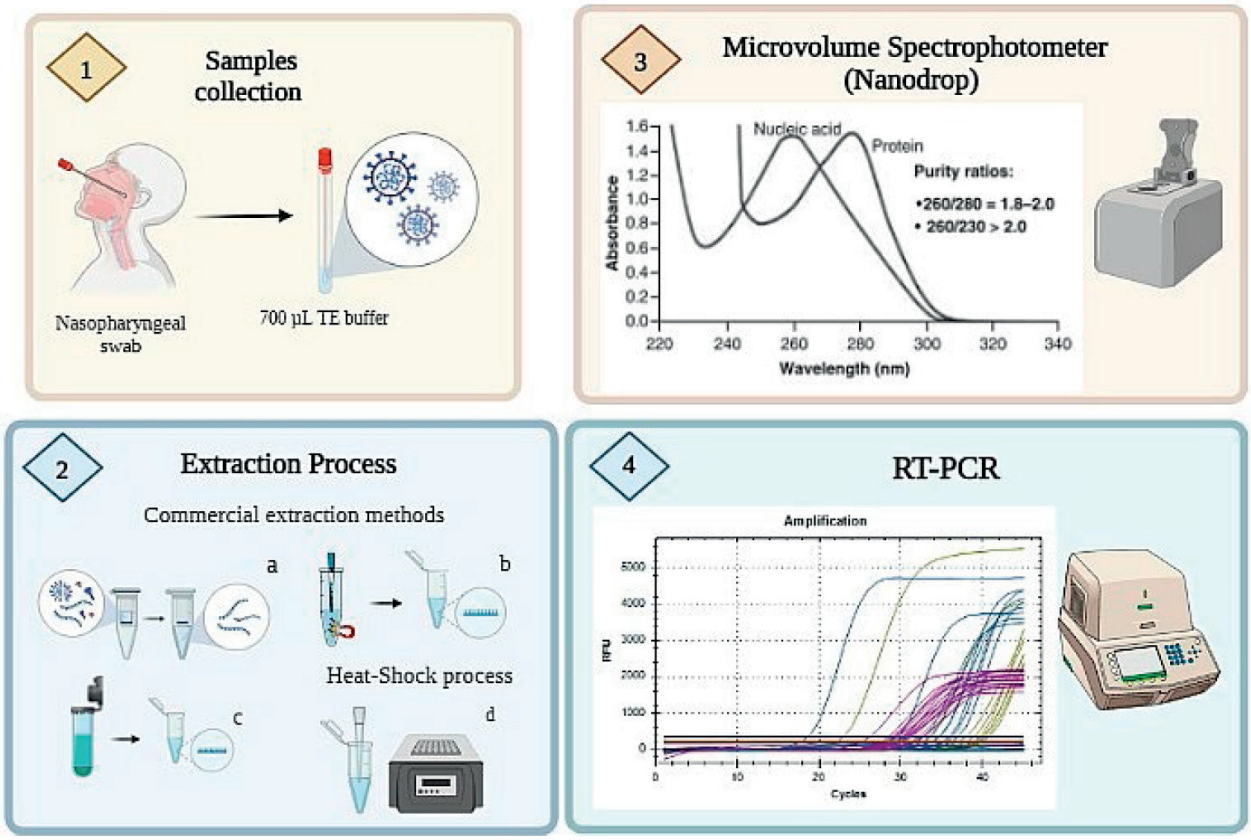

Figure 1. Schematic overview of SARS-CoV-2 RNA extraction and RT-PCR testing procedure. 1). Sample collection. 2). Extraction processes (a.) Silica-based membrane extraction. (b) Magnetic beads extraction. (c.) Mono—phasic organic extraction. (d.) Heat-Shock RNA process. 3). RNA quantification. 4). RNA Amplification by RT-PCR.

Quantification of viral RNA by Spectrophotometry

The total RNA isolated with the different methods was analyzed to determine the concentration and purity with NanoDrop™ One/Onec Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). The concentration was obtained in ng/µL of RNA, and purity was calculated using the optical density (OD) ratio at wavelengths of 260/280 and 260/230 (Figure 1). The values used for OD260/280 ranged from 1.8 to 2.0, a good indicator of good-quality RNA, and for OD260/230 in the same range. If these values were out of the range were considered an indicator of organic or chaotropic agents' contamination.

Heat-Shock of nasopharyngeal swabs samples (kit F)

An alternative extraction method was evaluated in this report, which consisted of an RNA ex-traction using Heat-Shock. The method was performed using stablish samples maintained in re-frigeration at -20 °C, thawed to 4 °C and homogenized, were taken 10 µL of nasopharyngeal swab sample which was heated 95°C for 10 min and then at 4 °C for 10 minutes, until the RT-PCR procedure[10,45-48]. To analyze the alternative method, Bayes' theorem was used to determine the likelihood of sample to be positive, and can be evaluated with Bayesian probability formalism for repeated sampling from same patien[11,49]. The samples were analyzed by duplicate and triplicate using probability odds conversion for positive likelihood ratio (LR+)[1].

$$Positive\ Likelihood\ Ratio = \frac{Sensitivity}{1-Specificity} \quad [1]$$

$$Positive\ Likelihood\ Ratio = \frac{0.700}{1-0.933} = 10.45$$

Real-time Retro-Transcriptase Polymerase Chain Reaction (RT-PCR) to detect of SARS-CoV-2

RT-PCR of 72 viral RNA samples was carried out using a commercial one-step detection kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probes) by Da An Gene© (Da An Gene Co., Ltda, of Yat-sen University, China) following manufactures' instructions on CFX96 BioRad Touch Real-Time PCR Detection System[2,49,50]. According to the approval of the Chinese Center for Disease Control and Prevention, ORF1ab and N genes were the amplification target regions for SARS-CoV-2 released by WHO to detect SARS-CoV-2 using the PCR kit[51-54]. In addition, this kit includes an endogenous internal standard detection system, which is used for monitoring RNA extraction and PCR amplification, thereby reducing false negative results. The analytical sensitivity of Da An Gene (2019-nCoV) RT-PCR, according to the manufacturer's instruction, was 500 copies/mL as the Limit of Detection(LoD). This kit does not have cross-reaction with other pathogens, including SARS and MERS coronavirus being Open Reading Frame 1ab (ORF1ab) and Nucleocapsid protein (N) target genes in SARS-CoV-2[1,55-57].

Cost analysis

For this report, Activity-Based Costing Model (ABC Model) was performed to analyze the cost of five commercial kits evaluated, including the Heat-Shock reaction[58,59]. This analysis was based on elemental material needed to conduct an RT-PCR response considering direct and indirect costs necessary for the process and their outcome interpretation.

The analysis of the total cost to detect SARS-CoV-2

was established considering (1) direct cost, the raw material (supplies and additional reagents), lab workforce, equipment depreciation, and Personal Protection Equipment (PPE). The cost was obtained through quotes and invoices requested during the years 2020 and 2021.

Statistical analysis

Data such as RNA concentration were represented through the median and interquartile range (IQR), while RNA purity was expressed through the Mean and Standard Deviation (SD) of the optical density (OD) ratio. A non-parametric ANOVA-like Friedman test was applied to analyze the RNA concentration and purity used to detect differences between each extraction methodology.

Accuracy [2], Sensitivity [3] and Specificity [4] were estimated for diagnostic efficiency as indexes using a confusion matrix approach[60-62]. The confusion matrix and confidence

$$Accuracy = \frac{True\ Positives + True\ Negatives}{True\ Positives + True\ Negatives + False\ Positives + False\ Negatives} \quad [2]$$

$$Sensitivity = \frac{True\ Positive}{True\ Positive + False\ Negative} \quad [3]$$

$$Specificity = \frac{True\ Negatives}{True\ Negatives + False\ Positive} \quad [4]$$

interval (95%) were calculated using a diagnostic test evaluation software MedCalc version 20.027 (MedCalc Software Ltd, Ostend, Belgium).). The classification accuracy for SARS-CoV-2 was assessed by the ROC (Receiver Operating Characteristic) curve, which is a useful graphical tool to evaluate the performance of a binary classifier as its discrimination threshold is varied, analysis based on sensitivity as a function of 1-specificity of a diagnostic test, to evaluate the performance of a binary classifier as its discrimination threshold is varied examining the biomarker's discriminative efficacy[61,63], based on how True Positive Rate (TPR) and False Positive Rate (FPR) changes in the classification threshold is varied between infected and non-infected groups.

To summarize and understand the overall discriminative efficacy of the test, the Area Under the Curve (AUC) was used to evaluate the discriminatory effectiveness following the criteria: AUC ranges from 0 to 1, and an AUC of 0.5 suggests no discrimination ability[61]. Although AUC is the most commonly used global index for diagnostic accuracy, the Youden Index, with a range similar to AUC, can provide a criterion for choosing the "optimal cut-off" value for diagnostic tests[61,64,65]. Finally, a p-value < 0.05 is considered statistically significant in all statistical analyses assessing the kits' effectiveness in isolating SARS-CoV-2 nucleic acids.

For the alternative method to obtain viral RNA (Heat-Shock), Bayes' theorem was used to calculate a posteriori probability based on the confusion matrix results. The idea of a good screening test is a high degree of true positives, high specificity, and a permissive number of false positives. Bayes' theorem allows the provider to convert the results of a test to probability[60,66]. The prevalence, in this calculation, would act as the pre-test or prior probability of disease and combined with the Positive Predictive Value (PPV) would generate a post-test probability for any patient (all-comers) regardless of the individual's risk. Finally, to study the cost necessary to perform a RT-PCR a Multidimensional Scaling was implemented to create a map, which displayed the relative position of variables, given a proximity matrix[67].

Results

RNA Quantification

The RNA extraction yield was calculated based on the median, and visually comparable in Figure 2. The latter was expressed on the mean ratio (OD260/280) as it is represented in Table 2 and Figure 3, where the Friedman test was used to analyze concentration and purity comparing differences of independent but repeated and related variables measure. The average concentration of the straight set of RNAs for kit A shows values between 10.91 and 96.87 ng/µL and a low-value range of 6.75 to 6.91 ng/µL; kit B values between 45.09 and 162.57 ng/µL, while for an unpurified set of RNAs no presented outcome; kit C values between 10.914 and 327.56 ng/µL, while the unpurified set of RNAs presented with a range between 2.988 and 8.945 ng/µL; kit D values between 16.02 and 615.13 ng/µL, while for an unpurified set of RNAs no presented outcome, and finally for kit E values between 14.95 and 160.66 ng/µL, while for an unpurified set of RNAs no given outcome. In the case of kit, F was excluded because it was not purified and concentrated. In our study, the quantity and purity were estimated in 72 samples which were used for all five kits considered for comparison.

The Friedman test was used to analyze concentration and purity; Friedman compares independent variable differences for experimental designs involving repeated/related measures. The Friedman test analyzed the observed difference between different kits, and a p-value < 0.001 was considered statistically significant. The concentration and

	RNA concentration (ng/µL)		RNA purity ($OD_{260/280}$)	
Name of Kit	Median (IQR)	p-value (Friedman test)	Mean ± SD	p-value (Friedman test)
Kit A	26.71 (18.26 – 42.94)	<0.001	2.54 ± 0.51	<0.001
Kit B	84.12 (73.69 – 88.33)	<0.001	3.12 ± 0.57	<0.001
Kit C	8.97 (5.64 – 19.68)	<0.001	1.85 ± 0.33	<0.001
Kit D	37.30 (30.00 -53.34)	<0.001	1.87 ± 0.11	<0.001
Kit E	32.26 (26.33 – 48.26)	<0.001	1.86 ± 0.12	<0.001
*Kit F	-	-	-	-

Note IQR: Interquartile Range; SD: Standard Deviation; * Quantification of kit F not assessment by the interference of inside cellular substances produced by heat-shock lysis.

Table 2. The median yield of viral RNA concentration and mean A260/280 OD ratio purity of extracted RNA by six extraction kits.

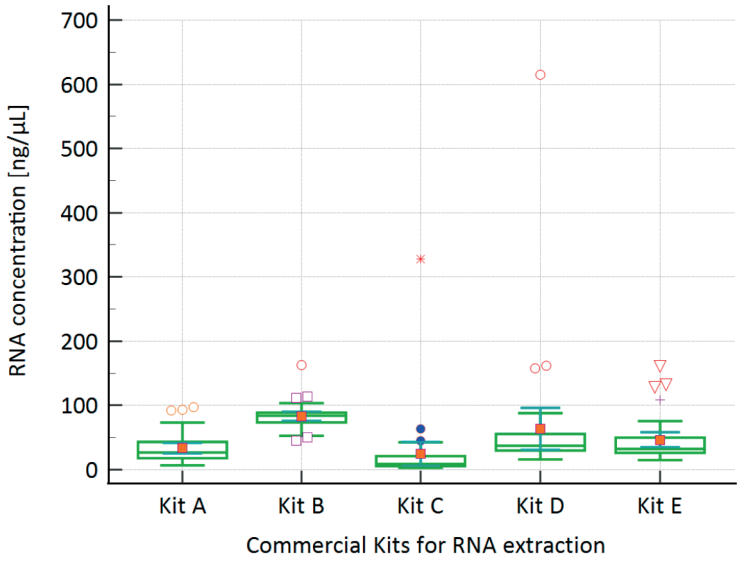

Figure 2. Box-plot of RNA concentration. The use of Friedman's test for concentration was based on the fact that the data failed the ANOVA-MR test. Comparison with each kit shows data with low-dispersion, obtained values that not exceeded in general 100 ng/µL of nucleic acid concentration. Atypical data are seen in all kits; however, kits C (*) and D (ᵒ) show extreme outliers compared to each other.

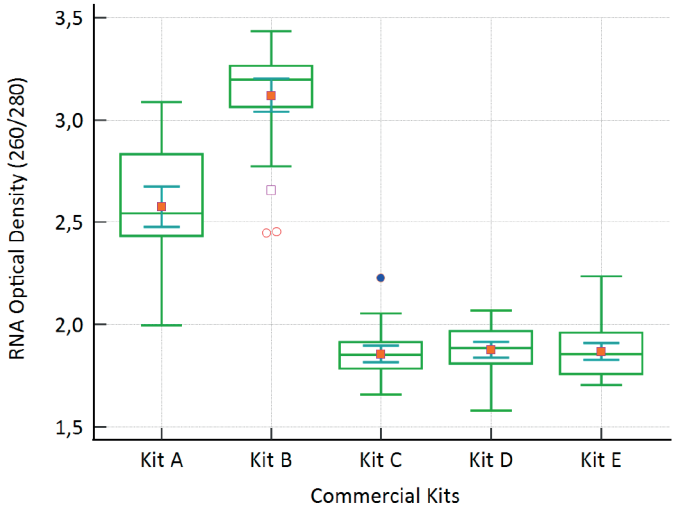

Figure 3. Box-plot of Optical Density. For OD data analyzes, based on the data analysis of concentration, Friedman's test was chosen to visualize the differences between the purity of RNA obtained during the extraction process. Kit A and B shows highest disperse in the interquartile range of the values of each group compared with kit C, D and E, which shows a similar box, low-disperse data and similar mean. In addition, kit B shows extreme outliers, but the nucleic acid purity ratio is better. On the other hand, kit C (•) shows stable values of purity but presents a low outlier compared with kit D and E.

purity of each kit were significantly different from those of the others (Figures 3 and 4).

Heat-Shock inactivation (kit F) analysis

As mentioned above in methodology to evaluate the obtention of nucleic acid using an alternative method called kit F (Heat shock) and use in RT-PCR amplification. Positive Likelihood ratio (LR+) was calculated (LR+= 10.45), and Bayes' theorem uses the LR+ to facilitate the interpretation of a test for a given individual regardless of prevalence by assigning prior probabilities/odds to determine post probabilities/odds for a given data point, in this case, the LR+.

The reach of Bayes' theorem was set in three sceneries: low, moderate, and high pre-test probability of COVID-19 infection according to the exposure grade. To understand the Bayes' theorem, statistical approaches were used, where Individuals in a presumed low prevalence environment would constitute a low pre-test probability between 10–20% of COVID-19 infection, whereas an individual with cough and fever with known cases of COVID-19 may be assigned a moderate pre-test probability 40–60% of disease. A high pre-test probability of 80–90% of COVID-19 may include all known symptoms, with known close contact with confirmed COVID-19 and an estimated probability pre-test of 22.9% based on data on the prevalence of COVID-19 in the population of Ecuador. For each of these individuals, a positive RT-PCR test result will have different implications, namely post-test odds (which can be converted to a probability for ease of interpretation).

To obtain the pre-test probabilities, LR+ needs to be converted into odds (because LR+ is a ratio of odds) and then to be reverted to possibilities; table 3 and Figure 4 provide a visual gauge of how a LR+ (10.45) changes post-test probabilities based on disease prevalence and a priori probabilities.

RT-PCR analysis

The Da An Gene© kit detects the open reading frame 1a and 1b gene from the region ORF (ORF1ab) and the nucleocapsid protein (N-gene). To validate the results for RT-PCR, the negative control NC (ORF1ab/N) did not show curve for ORF1ab and N genes, but showed an amplification curve for RNAse P gene as internal RT-PCR control, and Ct value under 35 cycles. Positive control PC (ORF1ab/N) showed amplification curves for ORF1ab and N genes, as well as for RNAse P gene as internal control.

To test positive for SARS-CoV-2 in a sample, the result of RT-PCR amplification for ORF1ab gene, N genes, and Ct values need to be under 40 cycles. If the Ct values are up 40 cycles for ORF1ab and N genes, a negative result was considered. In addition, in both cases the internal control (RNAse P gene) must be presented in amplification curves in RT-PCR. The hole detection time was ap-proximately 90 minutes.

Double sample test				Triplicate sample test			
Pre-test probability	Pre-test odds	Post-test odds	Post-test probability	Pre-test probability	Pre-test odds	Post-test odds	Post-test probability
0.10	0.04	0.42	0.29	0.29	0.41	4.28	0.81
0.20	0.25	2.61	0.72	0.72	2.57	28.85	0.96
0.229	0.30	3.14	0.76	0.76	3.16	33.02	0.97
0.30	0.43	4.49	0.82	0.82	4.55	47.55	0.98
0.40	0.67	7.00	0.88	0.88	7.33	76.60	0.99
0.50	1.00	10.45	0.91	0.91	10.11	105.65	0.99
0.60	1.50	15.68	0.94	0.94	15.66	163.54	0.99
0.70	2.33	24.35	0.96	0.96	24	250.8	0.996
0.80	4.00	41.8	0.98	0.98	49	512.05	0.998
0.90	9.00	94.05	0.99	0.99	99	1034.55	0.999

Note: $odds = \frac{Probability}{1-Probability}$; $Probability = \frac{odds}{odds+1}$; $Post\ test\ odds = pre\ test\ odd \times LR +$

Table 3. Bayesian probabilistic formalism of positive likelihood ratio (LR+) post-test probabilities for low, moderate and high prevalence of COVID-19.

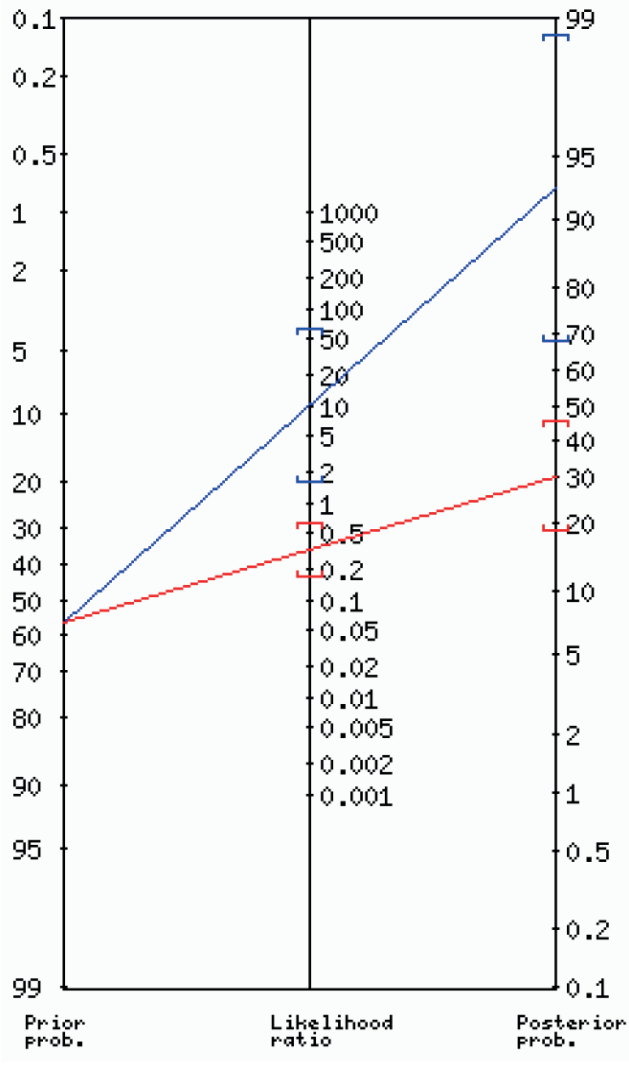

Figure 4. The Fagan nomogram was used to provide a visual estimate of post-test probabilities based on SARS-CoV-2 prevalence and the capacity of evaluate for duplicate and triplicate the samples using heat shock to improve the estimation of a patient's risk of having or contracting the disease when testing positive based on disease prevalence and a priori probabilities. Prevalence, in this graphic and calculation, act as pre-test odd (1.3) or prior pre-test probability (57.14%). For the positive test (blue line), the LR+ was approximately 11 (CI: 1.55 -71), and for the post-test, probability was 94% (odds: 14.7) with CI: 67% -99%. On the other hand, for the negative test (red line), the LR- was 0.32 (CI: 0.16 – 0.64), the post-test probability was 30% (odds: 0.4) with CI: 18% - 46%.

For diagnostic test validation, confirmation of the presence of a disease is important but along with that ruling out the presence of disease in healthy patients, being necessary to care aboid cross contamination of sample and add a control the extraction prior to amplification to reduce false positive an false negatives. Common metrics like accuracy, sensitivity and specificity was calculated using a confusion matrix based on results of True Positives, True Negatives, False Positives and False Negatives. Terms to quantify the diagnostic efficiency and diagnostic effectiveness expressed as a proportion of correctly classified samples of any diagnostic test. Table 4 shows the data obtained and used to build a receiver operating characteristic curve (ROC), calculate the area under curve (AUC), and Youden index based on approach as the classification threshold (optimal cut-off point) between the infected and non-infected groups represented in Figure 5.

Cost Analysis for SARS-CoV-2 diagnostic test

An excellent way to analyze the different kits that have been assessed is multidimensional scaling (MDS), a statistical method that provides a graphical representation between objects in multifaceted spaces using distances between them. In cases where the relations between objects are unknown, distances between each other can be calculated. MDS is a technique of interdependence used when any or all of the variables are not dependent and cannot be explained by another when they are involved in the mutual relationship among all variables.

In Figure 6, MDS represents 6 variables (indicators) used in the study of cost analysis between six different methodologies of extraction; the indicators were sensitivity, specificity, direct and indirect cost (for 2020 and 2021), concentration [ng/µL] and Optical Density (A260/280). MDS stress (Goodness of Fit) has been found as 0.9999804 for coordinate 1, and 0.9999804 for coordinate 2, which indicates the correct adjustment of latent coordinates created since the original data (indicators), where the grouping and distance adjustment of data concerning coordinate 1 and coordinate 2 indicates a well similarity between each kit, mainly for A, C, D and E by 2020 and considerable similarity by 2021. However, in kits B and F, for 2020 and 2021, there were significant differences between indicators.

In terms of cost, the evaluation of supplements necessary for a reaction was divided into direct and indirect costs for the years 2020 and 2021. For kits A and C (Silica-based), D (magnetic beads) and E (organic extraction), globe cost for the reaction was similar during 2020; meanwhile, for kit B (silica-based), the cost was highest than all methods, values obtained for this evaluation are presented in Table 5. Finally, for kit F, the reaction cost was cheaper than all methods. On the other hand, for 2021, an evident cost reduction for all kits is appreciable, where the cost of kits A, C, D and E have a clear separation, diverging from each other. However, the economic reduction for kit B is irrelevant since it is still the most expensive at the commercial level. Meanwhile, for kit F the cost for the reaction is more economical compared to 2020, a method that can be applied for developing countries since its cost allows public access.

Discussion

Around the world, several efforts are being focused on the fast development of novel and reliable diagnostic tests based on nucleic acid kits. However, a severe shortage of nucleic acid extraction kits due to the sudden surge in demand, the reduced production capacity, and delays in ship-

	Positive samples	Negative Samples	Mean Ct-positive samples	Standard Deviation	Accuracy	Specificity	Sensitivity
Kit A	20	15	ORF1ab: 31.89 N: 28.16	ORF1ab: 5.25 N: 5.24	100%	100%	100%
Kit B	18	14	ORF1ab: 35.01 N:31.49	ORF1ab: 6.02 N: 5.66	91%	90%	93%
Kit C	20	14	ORF1ab: 31.24 N:28.83	ORF1ab: 5.71 N: 5.63	97%	93%	100%
Kit D	22	13	ORF1ab: 33.25 N:31.24	ORF1ab: 16.97 N: 16.15	95%	87%	92%
Kit E	18	15	ORF1ab: 32.84 N:27.57	ORF1ab: 10.14 N: 12.62	89%	100%	82%
Kit F	16	15	ORF1ab: 35.39 N:33.04	ORF1ab: 2.23 N: 2.68	82%	93%	70%

Note: Ct: Cycles threshold; **SD:** Standard deviation

Table 4. Comparison of accuracy, specificity and sensitivity for different RNA extraction kits.

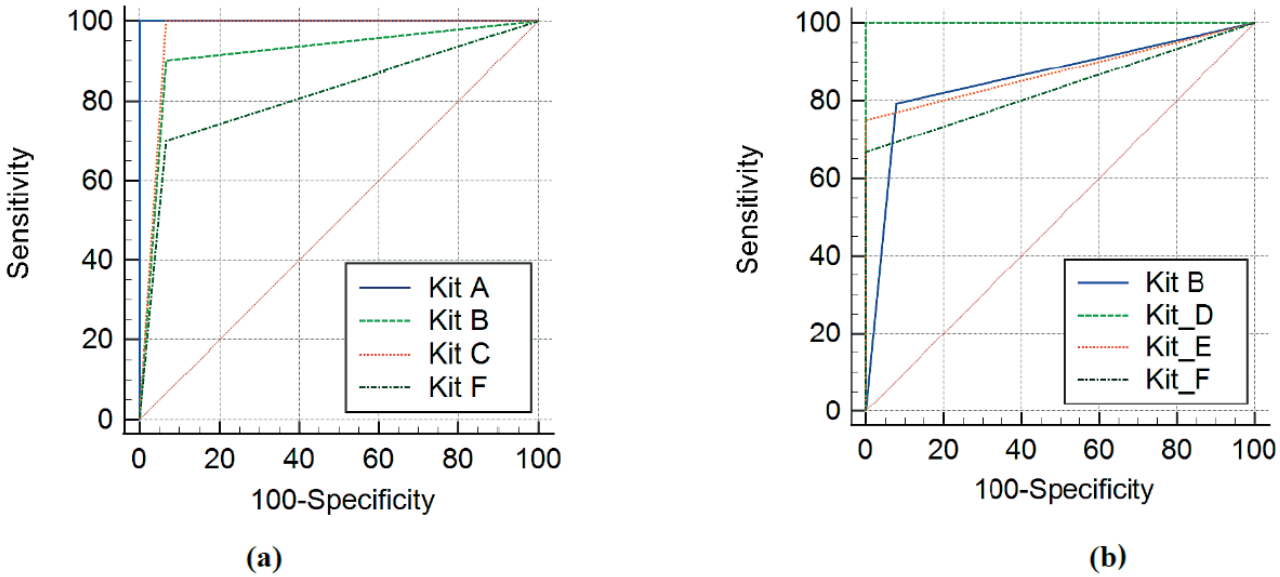

Figure 5. Receiver Operating Characteristic (ROC) curve for different RNA extraction kits. a.) It shows the ROC curve for silica-based extraction and heat shock treatment to obtain the cut-off point for the kit. b.) It shows the ROC curve for non-column extraction and heat shock treatment to get the cut-off point for kits.

Kit	Sensitivity	Specificity	Direct Cost USD (2020)	Indirect Cost USD (2020)	Direct Cost USD (2021)	Indirect Cost USD (2021)	Concentration (ng/µL)	OD 260/280
A	100%	100%	55,00	51,99	62,56	37,11	34,12	2,58
B	90%	93%	64,25	51,97	70,85	37,09	83,27	3,12
C	100%	93%	53,96	51,97	61,37	37,09	24,65	1,86
D	92%	87%	53,12	51,99	60,15	37,11	63,09	1,88
E	82%	100%	52,78	52,05	58,26	37,17	46,05	1,87
F	70%	93%	47,01	50,35	49,16	35,47	N/A	N/A

Table 5. Indicators to cost analysis for six different extraction methodologies.

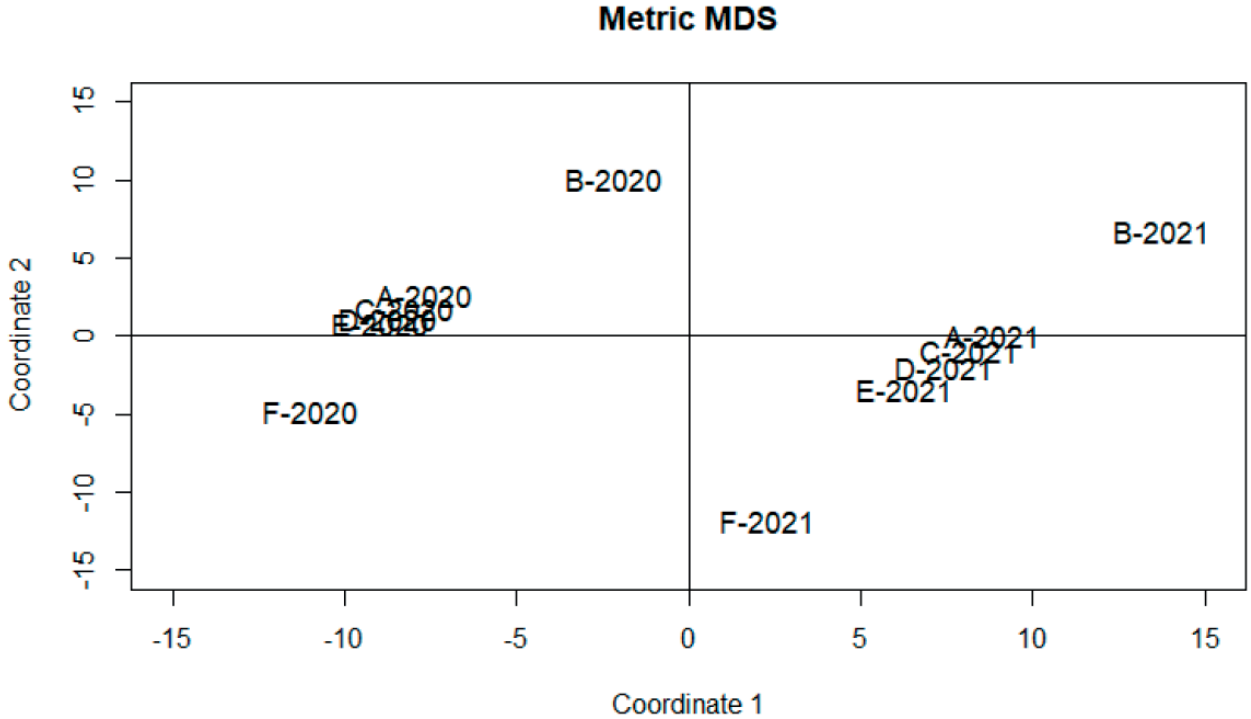

Figure 6. Multidimensional Scaling for different viral RNA extraction kits for 2020 and 2021.

ment challenge the global health system, mainly for developing countries during the first months and the rapid spread of COVID-19 in 2020 and 2021. Management of COVID-19 requires widespread and accessible testing, where the primary step to be diagnosed is to obtain a purified and concentrated viral RNA to be used in the RT-PCR technique to detect SARS-CoV-2. US CDC considers this a "gold standard" technique to its high sensitivity and specificity, significantly faster than other molecular available viral detection techniques[13,68].

Thus, the method used for RNA extraction is the most crucial variable, where the extraction efficiency influence significantly the yield and quality of RNA; thereby, it represents an essential variable in detecting the presence of the SARS-CoV-2 genome by RT-PCR[13]. In this way, many commercial kits use different methods to allow fast, sensitive and reproducible detection of viral RNA. Along this line, reliable protocols are crucial for those molecular laboratories without automated nucleic acid extraction, where the extraction process significantly influences the yield of RNA.

The results obtained from each different kit tested showed that the quantification of RNA is an essential step before RNA-based essays, where the diagnosis requires an accurate RNA quantification to estimate the success of the extraction to determine the appropriate amount of extract for downstream medical applications like RT-PCR for the diagnosis of SARS-CoV-2[31]. Preliminary studies report that direct-to-test addition of unpurified samples allows for SARS-CoV-2 detection of low copy load samples, but may decrease test sensitivity, amplification cycle later and delayed detection of viral RNA[9,10].

The purpose of many diagnostic processes of SARS-CoV-2 after nucleic acid extraction is the efficient detection and successful amplification of the target region in the viral RNA using RT-PCR, where an entire, high amount and good quality of nucleic acid template to be used fundamentally for the downstream molecular process[32]. In this study, a comparison between the six different methodologies for RNA extraction showed variations in the overall performance based on their other technology, where kits B, D and E outcomes obtained show a considerable amount of nucleic acid due to the use of similar required sample volume. However, kits A and C presented results of RNA yields decreased to kits B, D and C, which show extraction efficiency and methodology significantly influence the yield of RNA; despite using similar sample volumes, kit C has the most variable yield and concentration with significant differences in terms of IQR.

In the case of kit F, not having quantified the RNA concentration leaves it out of the comparison with the other commercial kits, given that being a raw genetic material, the generation of interference discriminates the quality of genetic material obtained by the heat shock, which would be used for amplification. However, doing so could have indicated an approximate concentration of RNA, thus evaluating qualitatively if the heat shock is favorable to obtaining quality genetic material.

The commercial kit's wavelength absorbance (OD260/280) shows acceptable purity, so values are proximate to 1.7 – 2.00 and upper 2.2. In this way, kit A and B silica-based membrane extraction present the best purity ratios indicating that the composition of the eluent was RNA. In contrast, kits C (same technology as A and B), D and E show an acceptable purity ratio but lower for optimal density ratios. Although spectroscopy can be used to determine the concentration and purity of RNA it lacks the power to determine the integrity of the RNA, which can affect the RT-PCR to detect nucleic acids for SARS-CoV-2 if the viral load and yield is not highest, being a considerable variable for COVID-19 diagnosis, and make an agarose electrophoresis to view the integrity of extraction would involve an additional cost. So, there are clinical and public health implications for the detection of samples with low levels of SARS-CoV-2 viral RNA. Even though, detection of viral RNA by PCR may not correlate with live transmissible virus for patients presenting early infection[70].

Due to the rapid spread of SARS-CoV-2, studies have tested the use of direct nasopharyngeal samples, indicating that the RNA isolation step could be omitted[12,38]. However, this approach results in reduced sensitivity and specificity of the downstream RT-PCR process. It may require an additional 3 to 7 PCR cycles to reach the detection threshold compared to that of reactions with purified RNA[2,12], compromising the detection of low viral loads. Still, studies reported sensitivity values ranging from 51%[38] to 91.4%[48] as commonly used measures of validity, including specificity. Sensitivity refers to its ability to detect a high proportion of confirmed cases while yielding few false negative results. Meanwhile, specificity, on the other hand, means that a specific test correctly identifies the actual negative and hence yields few false positive cases. Still, this result allowed a gap to increase the presence of False positive and false adverse claims, which can affect the control of spreading COVID-19.

The implementation of alternative methodologies like heat shock to obtain free RNA without concentration and purification, due to the limited supply chains, could be a good way to detect positive cases of SARS-CoV-2. Herein we report this approach as direct RT-PCR, which correctly identified 80 to 84% (diagnostic effectiveness) of samples previously shown to be positive for SARS-CoV-2 by RT-PCR featuring an RNA extraction. Studies that used a similar technique reported approach diagnostic effectiveness of 77.1, 92 and 95% of total positive samples[26,49,69] being the direct detection without RNA extraction, a reliable alternative for commercial kits, especially for kits that based on extraction technology is silica membrane. The advantage to put of sample to thermal treatment is the exposure of viral genome and denatures inhibitors of the PCR; however, the exposure sample to high temperatures above 95^{o}C for direct RT-PCR (without RNA extraction) may result in the dismissal of diagnostic efficiency in comparison to moderate temperatures 65-70 ^{o}C used in commercial kits which did not affe00ct RT-PCR[38,70]. Also, the use of mild temperatures allows a low capacity to affect their ability to discriminate to classify the healthy as healthy and the sick as sick, in comparison with the use of high temperatures. The Area Under the Curve, called AUC is one of the parameters to evaluate the discriminatory efficacy, obtaining values of 0.73; however, the Youden index can help to determine the highest cut-off, which determines the sensitivity and specificity together, getting a value of 0.817. However, this cut-off point does not necessarily determine the most heightened sensitivity or specificity that the test could achieve[71].

On the other hand, compared to mono-phasic extraction, where the typical extraction involves three general steps: cell lysis, separation of RNA from DNA, proteins, and lipids, followed by RNA concentration which presents a high yield than heat shock treatment that can be observed in the sensitivity and specificity by RT-PCR[39,42]. Finally, viral RNA

extraction using magnetic beads showed similar results with single-stage extraction and silica columns; however, when RT-PCR is performed, sensitivity and specificity vary considerably despite the beads having a certain affinity for RNA and the reagents used being specific.

As for the cost analysis using a multidimensional analysis, a clear difference in prices, concentration and purity of viral RNA obtained for the years 2020 and 2021 can be seen, where the distance between the variables analyzed reflects an increase in direct and indirect costs necessary to perform the RT-PCR process.

Conclusions

In conclusion, for the study presented, the use of alternative techniques such as an extraction RNA method prior to detection of SARS-CoV-2 can improve laboratory workflow. Considering the data, the technique has an acceptable diagnostic capacity for patients with a high viral load but a poor capacity for patients with low viral loads, we considered that the most significant limitation was associated with our inability to evaluate a greater number of samples, which could have made it possible to develop a more robust and extensible protocol. Presenting a clear disadvantage in this process as to diagnostic efficiency and discriminatory efficacy. Although this protocol allows the clinician to significantly reduce processing time, we believe it should only be used in clinical la-boratories where the lack of reagents for RNA extraction is a limiting factor, the main objective being to ensure the quality of the analysis during patient diagnosis. On the other hand, in terms of costs required to perform it, there is a clear advantage, mainly for developing countries where the costs of important inputs and reagents limit the ability to detect SARS-CoV-2 genetic material, and the use of the direct sample with RNase inhibitors can also increase the number of samples that can be processed per day. In terms of other alternatives technologies for extraction of nucleic acid, in this case viral RNA to SARS-CoV-2, low-tech solutions for COVID-19 supply chain crisis can be the implementing self-collected saliva, superficial nasal swabs including dry oral swabs without viral transport medium, being a prospectal technologies with low-invasive for patients that can be applicable for develop countries which use manual extraction methods. Consequently, dedicated biosafety practices need to be implemented to ensure the safety of laboratory personnel and reduce the risk of contamination. So that, heat shock technique could be implemented in cases where the expected positivity rates are high (symptomatic patients) representing an efficient alternative, to subsequently perform the kit extraction technique only in negative samples, which would reduce time and save costs considerably in the diagnosis.

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Institutional Review Board Statement

Ethical review and approval were waived for this study due to is an observational study, in which no patient data have been included since it is a methodological analysis.

Informed Consent Statement

Patient consent was waived due to is an observational study, in which no patient data have been included since it is a methodological analysis.

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Conflicts of Interest

The authors declare no conflict of interest.

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