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

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AUTOR: Luis Enrique Calvo Chica

Tutor: PhD. Carolina Proaño-Bolaños

Co-Tutor: M.Sc Andrea Carrera-Gonzalez

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Firma:

Carolina del Carmen Proaño Bolaños

CI: 1714818760

Firmado electrónicamente por:
ANDREA GRETA CARRERA GONZALEZ

Andrea Greta Carrera González

CI: 1718343435

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La vida se encuentra plegada de retos, y uno de ellos es la universidad. Tras verme dentro de ella, me he dado cuenta que más allá de ser un reto, el acompañamiento de familiares, docentes, amigos, extraños y personas ajenas quienes son pilar fundamental en la universidad he podido desarrollar habilidades y descubrir destrezas que jamás pensé estuviesen en mí. Siendo la universidad una base no solo para mi entendimiento del campo en el que me he visto inmerso, sino para lo que concierne a la vida y mi futuro.

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RESUMEN

A finales de 2019 un nuevo virus reportado en Wuhan, China, identificado como SARS-CoV-2 se propagó rápidamente desafiando al sistema de salud alrededor del mundo. La necesidad de una detección rápida, oportuna y precisa era fundamental para la prevención de brotes comunitarios del virus. Sin embargo, la elevada demanda mundial de reactivos durante los años 2020 y 2021 generaron un cuello de botella en kits empleados para la detección, afectando en gran medida a países en vías de desarrollo, rezagando su capacidad de diagnóstico y control del virus en la población. La dificultad de importación de reactivos, elevados costos y la limitante capacidad de acceso público a la prueba de detección de SARS-CoV-2 direccionó a la búsqueda de métodos alternativos. En este marco, se evaluaron y compararon diferentes metodologías de extracción de ácidos nucleicos comerciales frente al choque térmico como método alternativo para detección de SARS-CoV-2 mediante RT-PCR, con el fin del determinar el rendimiento diagnóstico y su posible bajo costo frente a las otras metodologías. Se emplearon muestras nasofaríngeas donde la eficiencia diagnóstica del método alternativo fue del 70 a 73%. La evacuación de la eficacia discriminatoria del método se tomó la sensibilidad y especificidad para establecer su punto de corte, siendo 0.73 a 0.817 que permite discriminar entre positivos y negativos de COVID-19. En cuanto a la efectividad diagnóstica expresada como la proporción de sujetos correctamente clasificados es entre 80 y 84%. Por otro lado, en términos de costos necesarios para llevar a cabo la detección, el método alternativo es más económico y accesible frente a los métodos comerciales disponibles en esta comparación y evaluación, siendo posible su implementación en países en desarrollo con altas tasas de contagio, permitiendo el acceso a la prueba diagnóstica con un método fiable y de bajo costo.

Palabras clave: COVID-19, RT-PCR, ARN viral.

ABSTRACT

In late 2019 a new virus reported in Wuhan, China, identified as SARS-CoV-2 spread rapidly challenging the healthcare system around the world. 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, greatly affecting developing countries, 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, in order 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 method took the sensitivity and specificity to establish its cut-off point, being 0.73 to 0.817, which allows discriminating between COVID-19 positives and negatives. As for the diagnostic effectiveness expressed as the proportion of subjects correctly classified, it 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.

Keywords: COVID-19, RT-PCR, Viral RNA.

1. INTRODUCTION

Coronaviruses 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 infection by two betacoronaviruses, the severe acute respiratory syndrome-related human coronavirus 1 (SARS-CoV) and the middle east respiratory syndrome-related coronavirus (MERS-CoV) both of them produced severe respiratory syndrome (3–6). The novel coronavirus SARS-CoV-2 was reported in Wuhan, China, in December of 2019. SARS-CoV-2 cause COVID-19 challenged the 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 (7–9). 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) (9–12).

During the first few weeks of COVID-19 pandemic, the global demand of nucleic acid extraction kits and required reagents had already in short supply, made 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 to improve SARS-CoV-2 testing fueling the development of alternative SARS-CoV-2 RNA isolation methods and protocols (10,12–16).

Most European countries and United States have to deal with the accelerated growth of infections and enormous pressure over their health systems, where cases started to growth exponentially (17). In case of Latin American countries, their first cases were registered between the end of February and beginning of March 2020, being Brazil who registered the first cases in the region. COVID-19 poses a major risk in Latin American countries, due to countries share many economic, political and health system similarities to control COVID-19 outbreaks and deaths (17–19). During the implementation of COVID-19 prevention and control measures, the nature and stringency of the response varied each country based in closed international borders and declared 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 spread of a pandemic (20,21).

The need of rapid and accurate detection of SARS-CoV-2 was critical for the prevention and control of communitarian outbreaks. For this reason, the rapid 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 SARS-CoV-2 genome (2,8,22,23). Standard molecular method was developed based on the U.S Center for Disease Control and Prevention (CDC), Charite and World Health Organization (WHO), based in the amplification of specific regions of viral gene N, E and RdRp and the purified RNA isolated from nasopharyngeal samples (10,24–26). Nevertheless, the increasing number of tests that were preformed worldwide has created a high demand of reagents necessary for SARS-CoV-2 detection mainly during March-July of 2020In addition, high demand of 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 (20,22,27–29).

Several commercial SARS-CoV-2 RT-PCR protocols employ manual extraction kits to isolate viral RNA from nasopharyngeal swabs (24,30,31), whereby an accurate extraction, recovery and quantification determine the efficacy of RT-PCR detection (8,32). The more common methods for viral isolation are (1) silica-based membrane (13,33), also called solid-phase RNA extraction; (2) organic extraction using phenol-guanidineIsothiocyanate (GITC) and, (3) magnetic beads (12,34). 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 (12,35).

The rapid spread of virus in Latin America, 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 at 45 USD (36,37). These value of 45 USD according with Trudeau represents the 4.2% of the average monthly income of middy-class person would be willing to pay in Latin America in a latent demand for COVID-19 test, respect to 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 (21,38).

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 (9,22,39). 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 perform, 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 convenient 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 (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 (10,33,41). 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 (42). Although there are numerous ways to extract and isolate RNA, most labs gravitate toward using organic extractions or commercially 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 denatured proteins will dissolve in the lower organic phase (41,43–45). In the face of shortage of kits, reagents 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 approaches 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 effectiveness of using commercial kits with the heat shock method for molecular detection of SARS-CoV-2 by RT-PCR, in order to propose a low-cost and reliable method.

2. MATERIALS AND METHODS

The samples for this study 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, in which 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.

2.1 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, biosafe bag and external box), (3) epidemiological information of patients, and (4) the samples should not be spilled.

2.2 Viral RNA extraction methods

Viral RNA extraction was performed using five different commercial kits, based on their four different technologies, following manufactures' instruction 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).

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

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

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

2.3 Quantification of viral RNA by Spectrophotometry

The total RNA isolate 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 ratio of optical density (OD) at wavelengths of 260/280 and 260/230 (Figure 1). The values used for OD260/280 ranged

from 1.8 to 2.0 like an acceptable indicator of good-quality RNA, and for OD260/230 in the same range. If these values were out of the range were considered like an indicator of organic or chaotropic agents' contamination.

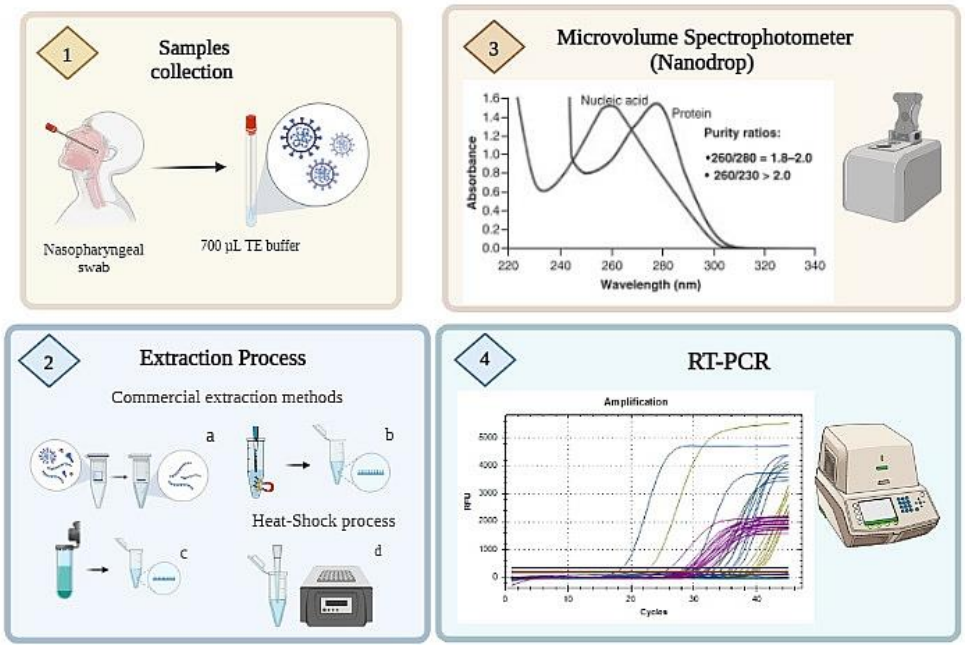

Figure 1. Schematic overview of SARS-CoV-2 RNA extraction and RT-PCR testing procedure.
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1). Sample collection. 2). Extraction processes (a.) Silica-based membrane extraction. (b) Magnetic beads extraction. (c.) Mono—phasic organic extraction. (d.)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

2.4 Heat-Shock of nasopharyngeal swabs samples (kit F)

An alternative extraction method was evaluated in this report, which consisted of an RNA extraction using Heat-Shock. The method was performed using stablish samples maintained in refrigeration 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 (7,9,47–51). To analyze the Heat-Shock process described as kit F, the Bayes' theorem was used to determine the likelihood of being positive if the samples were analyzed by duplicated and triplicate using probability odds conversion for positive likelihood ratio, which is calculated as Sensitivity over 1-Specificity [1], which values was obtained from confusion matrix approach. The

information obtained from diagnostic test (in this case Heat-Shock RNA amplification) can be evaluated with Bayesian probability formalism for repeated sampling from same patient, taking into account the probability of detecting a positive case of COVID-19 (10,52).

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

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

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

RT-PCR of 72 viral RNA samples was carried out using 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 (1,53,54). According to the approval of Chinese Center for Disease Control and Prevention, ORF1ab and N genes where the amplification target regions for SARS-CoV-2 released by WHO to detect SARS-CoV-2 using the RT-PCR kit (55–58). 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 manufactures' instruction was 500 copies/mL as 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 (2,59–61).

2.6 Cost analysis

For this report, Activity-Based Costing Model (ABC Model) was performed to analyze the cost of five commercial kits evaluated, including Heat-Shock (62,63). This analysis was based on reagents and consumables needed to conduct an RT-PCR reaction, 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 stablished considering (1) direct cost, 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.

2.7 Statistical analysis

Data such as RNA concentration were represented through median and interquartile range (IQR); while, RNA purity was represented through Mean and Standard Deviation (SD) of 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.

Accuracy [2], Sensitivity [3] and Specificity [4] were estimated for diagnostic efficiency, as indexes, using a confusion matrix approach (64–66). The confusion matrix and confidence 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 (65,67), 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.

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

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

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

To summarize and understand the overall discriminative efficacy of the test, the Area Under the Curve (AUC) was used as form to evaluate the discriminatory efficacy following the criteria: AUC ranges from 0 to 1, and an AUC of 0.5 suggest no discrimination ability (65). Although AUC is the most commonly used global index for

discriminative efficacy, the Youden Index with a range similar to AUC can provide a criterion for choosing the “optimal cut-off point” value for diagnostic test (65,68,69). Finally, a p-value < 0.05 is considered statistically significant in all statistical analysis, considering the effectiveness of kits to isolate SARS-CoV-2 nucleic acids.

For the alternative method to obtained viral RNA (Heat-Shock), Bayes’ theorem was used to calculated *a posteriori* probability based on the results of the confusion matrix approach. The idea of a good screening test is a high degree of true positives and high specificity, as well as a permissive number of false positives. Bayes’ theorem allows the provider to convert the results of a test to probability (64,70). 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 Multi-Dimensional Scaling was implemented to create a map, which displayed the relative position of variables, given a proximity matrix (71).

3. RESULTS

3.1. RNA Quantification

The RNA extraction yield was calculated based on the median. The latter was expressed on the mean ratio (OD260/280) as it is represented in Table 2. The concentration of the purified set of RNAs for Silica-based membrane (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 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. Magnetic beads (kit D) values between 16.02 and 615.13 ng/μL, while for unpurified set of RNAs no presented outcome, and finally for organic phases using chloroform (kit E) values between 14.95 and 160.66 ng/μL, while for unpurified set of RNAs no presented outcome. In case of alternative method Heat-Shock (kit F) was excluded because it is not purified and concentrated. In our study, the amount and purity were quantified in 72 samples which are used for all five kits considered for comparison.

The Friedman test was used to analyze concentration and purity, Friedman compares differences of an independent variable for experimental designs that involve repeated/related measures. Between different kits the observed difference was analyzed by Friedman test, and p-value < 0.001 was considered statistically significant. The concentration and purity of each kit were significantly different from those of the others (Figure 2 and 3) in terms of each technology applied to obtained RNA purified. However, kits C, D and E presents a mean of RNA purity very closed to each other with minimum SD differences. Silica-based membrane extraction (kits A, B and C) shows the higher values of obtained of nucleic acids with max-values of: 3.09; 3.43 and 2.23. Magnetic beads (kit D) show values of 2.07; meanwhile organic phase extraction (kit E) shows values of 2.24.

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

	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	-	-	-	-

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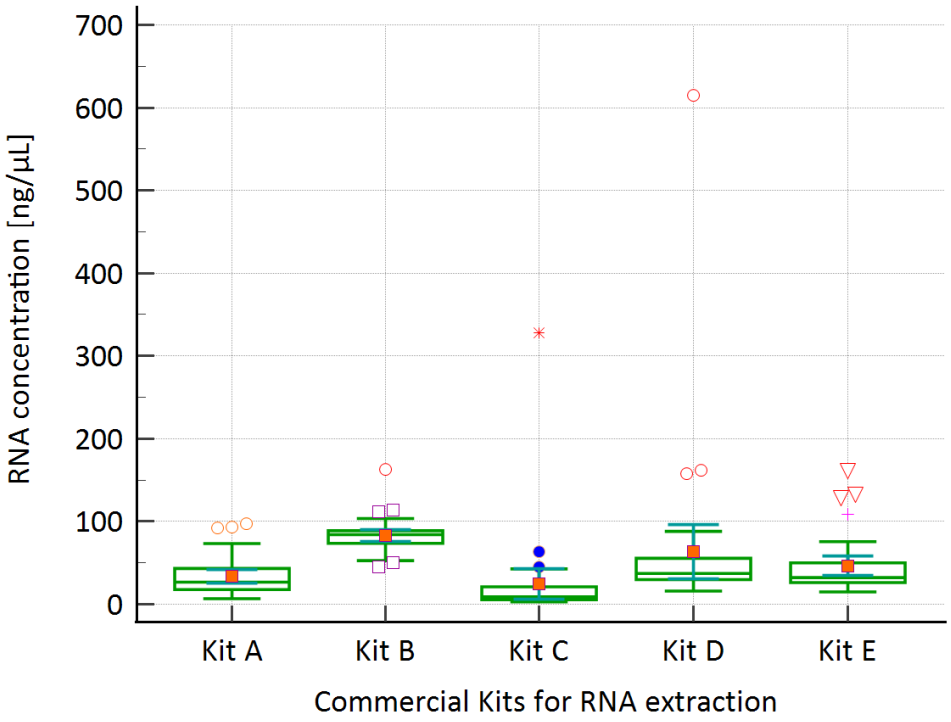

Figure 2. Box-plot of RNA concentration.
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The use of Friedman's test for concentration was based on the fact that the data failed the ANOVA-MR test. Comparison with each kit shows data with low-dispersion, obtained values that not exceeded in general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

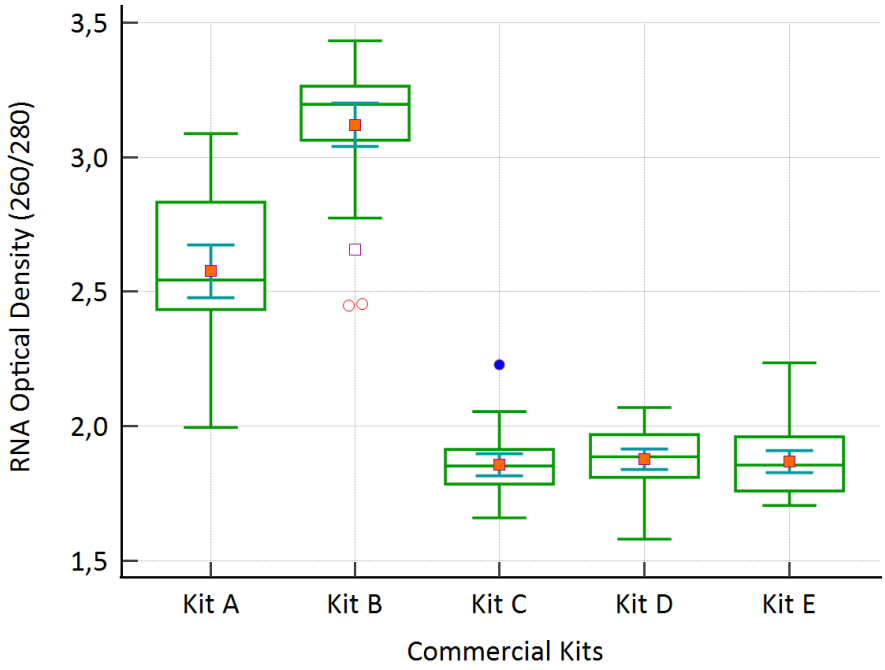

Figure 3. Box-plot of Optical Density.
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For OD data analyzes, based on the data analysis of concentration, Friedman's test was chosen to visualize the differences between the purity of RNA obtained during the extraction process. Kit A and B shows highest 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 present a low-outlier compared with kit D and E.

3.2 Heat-Shock method (kit F) analysis

As mention 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 interpretation of a test for a given individual regardless of prevalence by assigning prior probabilities/odds in order to determine post probabilities/odds for a given data point, in this case the LR+.

The reach of Bayes' theorem was set in three prevalence sceneries: low, moderate, and high pre-test probability of COVID-19 infection according to the grade of exposure. 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 80–90% of COVID-19 may include all known symptoms, with a known close contact with confirmed COVID-19 and, additionally add an estimated probability pre-test of 22.9% based on data of 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 obtained the pre-test probabilities, LR+ needs to be converted into odds (because LR+ is a ratio of odds) and then to be reverted back to probabilities, 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.

Table 3. Bayesian probabilistic formalism of positive likelihood ratio (LR+) post-test probabilities for low, moderate and high prevalence of COVID-19.
Note: $odds = \frac{Probability}{1-Probability}$; $Probability = \frac{odds}{odds+1}$;
$Post\ test\ odds = pre\ test\ odd \times LR +$

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

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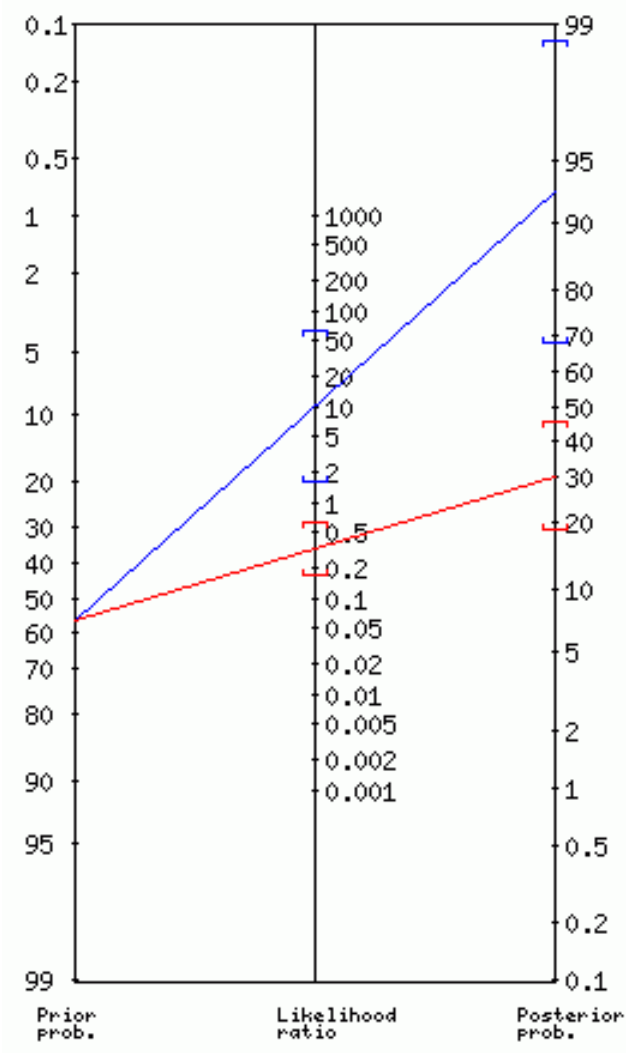

Figure 4. SARS-CoV-2 Fagan Nomogram.
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The Fagan nomogram was used to provide a visual estimate of post-test probabilities based on SARS-CoV-2 prevalence, and the capacity of evaluate for duplicate and triplicate the samples using heat shock, to improve 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 positive test (blue line), the LR+ was approximately 11 (CI: 1.55 -71) and for 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), post-test probability was 30% (odds: 0.4) with CI: 18% - 46%.

3.3 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 NC (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 and N genes, the 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 approximately 90 minutes.

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 is also necessary. A 2x2 confusion matrix approach was used to obtained common metrics like accuracy defined as the overall proportion of true test results, both true positives and negatives; sensitivity, which is the proportion of true positives; meanwhile, specificity is the proportion of true negatives. To obtained the values for Accuracy, Sensitivity and Specificity a derivation formula from a confusion matrix was used. Terms to quantify the diagnostic efficiency of any diagnostic test. Also, diagnostic effectiveness expressed as a proportion of correctly classified samples. Table 4 shows the data obtained to build a ROC curve based on confusion matrix approach as the classification threshold (optimal cut-off point) is varied between the infected and non-infected groups represented in Figure 5, and constructed introducing the values of confusion matrix approach to MedCalc. ROC curve and Youden index can help to distinguish between two populations to examine the continuous diagnostic markers to find the best cut-off point of discrimination of diagnostic test, while Area Under Curve can help to define the discriminatory efficacy. Table 5 shows the positives and negatives samples and mean cycle threshold of positive samples.

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

Note: Ct: Cycles threshold.

	True Positive	True Negative	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%

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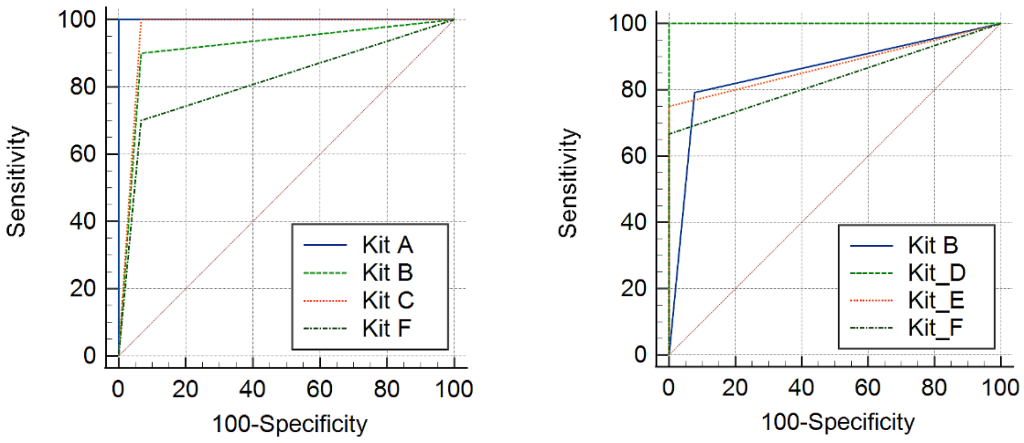
For silica-based membrane and heat shock as show Figure 5a, the Area Under Curve values were for kit A: 1.000 (CI 95%: 0.900-1.000); kit B: 0.917 (CI 95%: 0.773-0.983); kit C: 0.967 (CI 95%: 0.843-0.999) and finally for heat shock, kit F: 0.817 (CI 95%: 0.650-0.927). Statistically difference ($p < 0.05$) for kits A and F where p-value was 0.0032, for kits C and F p-value was 0.035, and for the rest of kits there weren't significant statistical differences. To know the optimal cut-off value the Youden index (J) was calculated for all kits based on their technology. For kit A J-value was 1 indicate that there were not false positives or false negatives. For kit B J-value was 0.83, kit C J-value was 0.93, kit D J-value was 0.79, kit E J-value was 0.82, and finally kit F J-value was 0.73.



(a) **(b)**

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

On the other hand, for non-column-based extraction and heat shock as show Figure 5b, the AUC values for kit B (gold standard): 0.857 (CI 95%: 0.703-0.950), kit D: 1.000 (CI 95%: 0.905-1.000), kit E: 0.875 (CI 95%: 0.725-0.960), and finally for heat shock, kit F: 0.833 (CI 95%: 0.675-0.935). In pairwise comparison of ROC curves, statistically difference ($p < 0.05$) for kit B and D the p-value was 0.0127, for kit D, and E p-value was 0.00056 and finally for kits D, and F the p-value was 0.0007. For the rest of kits there were not significant statistical differences.

3.4 Cost Analysis for SARS-CoV-2 diagnostic test

To analyze the different kits that have been assessed, a good way is multidimensional scaling (MDS) which is a statical method that provides a graphical representation between objects in multidimensional space using distances between them. In cases where the relations between objects are not known, but distances between each other can be calculated. MDS is a technique of interdependence used when any or all of the variables are noy dependent and cannot be explained by another, when they are involved in the mutual relationship among all variables.

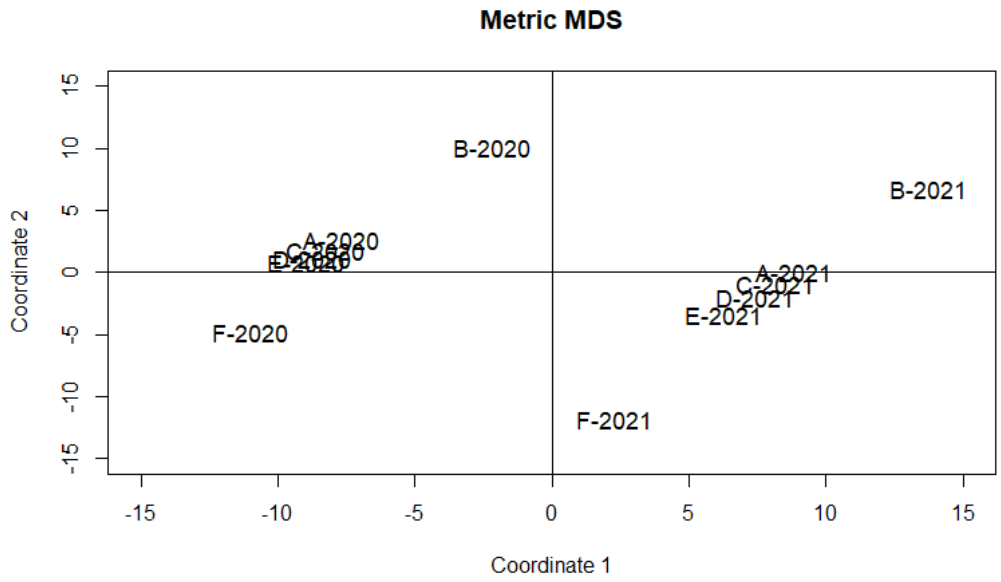

Figure 6. Multidimensional Scaling for different viral RNA extraction kits for 2020 and 2021.

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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 indicate the correct adjustment of latent coordinates created since the original data (indicators), where the grouping and distance adjustment of data respect to 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 kit B and F, for both, 2020 and 2021 there were significant differences between indicators.

In terms of cost, the evaluation of supplements necessary for a reaction was divided for years 2020 and 2021 as direct and indirect cost mainly. For kits A and C (Silica-based), D (magnetic beads) and E (organic extraction) globe cost for reaction were 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 cost for reaction was cheapest than all methods. On the other hand, for 2021 an evident reduction of cost for all kits is appreciable, where the cost of kit A, C, D and E have a clear separation,

diverging from each other's. However, for kit B the economic reduction is not relevant, since it is still the most expensive at commercial level. Meanwhile, for kit F the cost for the reaction is more economical compared to 2020 being a method that can be applied for developing countries since its cost allows public access.

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

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

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4. DISCUSSION

Around the world several efforts are being focused on fast development of novel and reliable diagnostic tests based on nucleic acid kits. However, severe shortage of nucleic acid extraction kits due the sudden surge in demand, the reduced production capacity, and delays in shipment challenge the global health system, mainly for developing countries during the first months and rapid spread of COVID-19 in 2020 and 2021. Management of COVID-19 requires widespread and accessible testing, where the main step to be diagnose it is obtained a purify and concentrated viral RNA to be used in RT-PCR technique to detect SARS-CoV-2. Which is considered as "gold standard" technique by U.S CDC due to high sensitivity and specificity, significantly faster compared to other molecular available viral detection technique (13,72).

Thus, the method uses for RNA extraction is the most important variable, where the extraction efficiency influence significantly the yield and quality of RNA, thereby it represents important variable to detect the presence of SARS-CoV-2 genome by RT-PCR (13). In this way, many commercial kits use different methods to allow a fast,

sensitive and reproducible detection of viral RNA, and along this line, reliable protocols are crucial for those molecular laboratories without automated nucleic acid extraction, where the extraction process influences significantly the yield of RNA.

The results obtained from each different kits tested showed that the quantification of RNA is an essential step prior to RNA-based essays, where the diagnosis require an accurate RNA quantification so as to estimate the success of the extraction to determine the appropriate amount of extract to downstream medical applications like RT-PCR for the diagnosis of SARS-CoV-2 (32). 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 target region in the viral RNA using RT-PCR, where an intact, high amount and good quality of nucleic acid template to be used are fundamental for downstream molecular process (33). In this study, comparison between the six different methodologies for RNA extraction showed variations in the overall performance based on their different technology's where kits B, D and E outcomes obtained show a considerable amount of nucleic acid, due to use similar required sample volume. However, kits A and C presented results of RNA yields decreased to kits B, D and C that show extraction efficiency and methodology influence significantly in the yield of RNA, in spite of using similar sample volumes, being kit C the most variable yield and concentration with significant differences in term 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 discriminating the quality of genetic material obtained by the heat shock which would be used for amplification. However, having done so could have indicated an approximate concentration of RNA, thus evaluating qualitatively if the heat shock is favorable to obtain quality genetic material.

Wavelength absorbance (OD260/280) for commercial kit showed acceptable purity so values were proximately to 1.7 – 2.00 and upper 2.2. In this way, kit A and B silica-based membrane extraction presented the best purity ratios indicating that the composition of

the eluent was RNA, while kits C (same technology like A and B), D and E shows an acceptable purity ratio, but lower 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.

Due to the rapid spread of SARS-CoV-2, studies have tested the use of direct nasopharyngeal samples indicated that RNA isolation step could be omitted (12,39). However, this approach results in reduced sensitivity and specificity of downstream RT-PCR process, and may require an additional 3 to 7 PCR cycles to reach the detection threshold compared to that of reactions with purified RNA (1,12) compromising the detection of low viral loads, but studies reported values ranging from 51% (39) to 91.4% (50) as commonly used measure of validity of diagnostic effectiveness. In this way, the values obtained for heat-shock diagnostic efficiency around 70 to 73%, which suggest to its ability of amplification unpurified RNA and detect a high proportion of the true cases, while yielding few false negative results. Meanwhile, specificity of test identifies the true negative, and hence yield few false positive cases; but this result allowed a gap to increase the presence of False positive and false negative cases, which can affect the control of spreading the COVID-19.

The implementation of alternatives methodologies like heat shock to obtain free-RNA without concentration and purification, due the limited supply chains, could be a good way to detect positive cases of SARS-CoV-2, and herein we report this approach as direct RT-PCR which correctly identified of 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 similar technique reported approach diagnostic effectiveness of 77.1, 92 and 95% of total positive samples (27,53,73) being the direct detection without RNA extraction a reliable alternative for commercial kits, especially for kits that based extraction technology is silica membrane. 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 dismiss of diagnostic efficiency in comparison to moderate temperatures 65-70 ^{o}C used in commercial kits which did not affect RT-PCR (39,74). Also, the use of moderate temperatures allows a low capacity to affect their

ability of discriminatory to classify the healthy as healthy and the sick as sick, in comparison with use of high temperatures. The Area Under Curve called AUC is one of the parameters to evaluate the discriminatory efficacy, obtained values of 0.73; however, the Youden index can help to determine the highest cut off which determine the sensitivity and specificity together, obtained a value of 0.817. However, this cut-off point does not necessarily determine the highest sensitivity or specificity that the test could achieve (75).

On the other hand, compare 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 presented a high yield than heat shock treatment that can be observed in the sensitivity and specificity by RT-PCR (40,44). 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 fact that the beads have a certain affinity for RNA and the reagents used are 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, reflecting an increase in direct and indirect costs necessary to perform the RT-PCR process.

5. CONCLUSIONS

In conclusion, for the study presented, the use of alternative techniques such as heat shock from crude samples to direct detection of SARS-CoV-2 can improve laboratory workflow. Considering the data, 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 laboratories 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.

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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