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Original Article **Open Access**

Comparative evaluation of automated KingFisher Flex Purification System 96 (ThermoFisher Scientific) and manual QIAamp Viral RNA Mini Kit (Qiagen) extraction methods for SARS-CoV-2

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Abstract:

Background: The extraction step of the viral material of the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) influences the quality of reverse transcriptase-polymerase chain reaction (RT-PCR) results in diagnosis of coronavirus disease 2019 (COVID-19). The purpose of this cross-sectional study was to evaluate the diagnostic performance of the automated extraction system "KingFisher Flex Purification System 96 (ThermoFisher)" compared to the manual method with the "QIAamp Viral RNA Mini Kit (Qiagen)".
Methodology: From October to December 2020, comparative diagnostic evaluation of two methods of SARS-CoV-2 RNA extraction methods was conducted on 159 fresh and 120 frozen nasopharyngeal and oropharyngeal specimens collected from travellers and suspected cases or contacts of COVID-19 patients in Burkina Faso. The FastPlexTM Triplex 1-Step COVID 19 Detection Kit (RT-PCR, RNA extraction free) (Precigenome LLC) was used to amplify on the same PCR plate, RNA extracts from manual QIAamp Viral RNA Mini Kit and automated KingFisher Flex Purification System 96 (ThermoFisher) using the QuantStudio5 thermal cycler (Applied Biosystems). Analysis of the diagnostic performance of the SARS-CoV-2 RT-PCR assay following RNA extraction by the two methods was done using an online OpenEpi software.
Results: For fresh samples, the study found a slightly higher RT-PCR positivity rate following manual extraction (12.6%) than automated extraction (9.4%). For frozen samples, the positivity rate was far higher for manual (38.33%) than automated extraction method (20.83%). The results show that the performance of the automated extraction was inferior when compared to the manual extraction for both fresh samples (sensitivity 35%, specificity 94.2%) and frozen samples (sensitivity 43.5%, specificity 93.2%). However, using McNemar Chi-square with Yates correction, there was no significant difference in positivity rate of RT-PCR (x^2=0.76, p=0.38) between the two extraction methods for the fresh samples, but there was a significant difference (x^2=12.9, p= 0.0003) in the extraction of the frozen samples.
Conclusion: The results of this study showed that KingFisher Flex Purification System 96 (ThermoFisher) automatic extraction method was less sensitive and specific than QIAamp Viral RNA Mini Kit (Qiagen) manual extraction method. This information can serve as guide to laboratories in the choice of RNA extraction methods to use for RT-PCR detection of SARS-CoV-2.

Keywords: SARS-CoV-2; RNA extraction; Diagnostic; Performance; RT-PCR

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Évaluation comparative des méthodes d'extraction automatisée KingFisher Flex Purification System 96 (ThermoFisher Scientific) et manuelle QIAamp Viral RNA Mini Kit (Qiagen) pour le SRAS-CoV-2

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Résumé:

Contexte: L'étape d'extraction du matériel viral du syndrome respiratoire aigu sévère-coronavirus-2 (SRAS-CoV-2) influence la qualité des résultats de la réaction en chaîne de la transcriptase inverse-polymérase (RT-PCR) dans le diagnostic de la maladie à coronavirus 2019 (COVID-19). Le but de cette étude transversale était d'évaluer les performances diagnostiques du système d'extraction automatisé "KingFisher Flex Purification System 96 (Thermo- Fisher)" par rapport à la méthode manuelle avec le "QIAamp Viral RNA Mini Kit (Qiagen)"
Méthodologie: D'octobre à décembre 2020, une évaluation diagnostique comparative de deux méthodes d'extraction de l'ARN du SRAS-CoV-2 a été menée sur 159 échantillons nasopharyngés et oropharyngés frais et 120 échantillons congelés nasopharyngés et oropharyngés prélevés sur des voyageurs et des cas suspects ou des contacts de patients COVID-19 au Burkina Faso. Le kit de détection FastPlexTM Triplex COVID 19 (RT-PCR, sans extraction d'ARN) (Precigenome LLC) a été utilisé pour amplifier sur la même plaque PCR, des extraits d'ARN du kit manuel QIAamp Viral RNA Mini et du système automatisé KingFisher Flex Purification System 96 (ThermoFisher) à l'aide du thermocycleur QuantStudio5 (Applied Biosystems). L'analyse des performances diagnostiques du test SARS-CoV-2 RT-PCR après extraction de l'ARN par les deux méthodes a été effectuée à l'aide d'un logiciel OpenEpi en ligne.
Résultats: Pour les échantillons frais, l'étude a révélé un taux de positivité RT-PCR légèrement plus élevé après extraction manuelle (12,6%) qu'après extraction automatisée (9,4%). Pour les échantillons congelés, le taux de positivité était beaucoup plus élevé pour la méthode d'extraction manuelle (38,3%) que pour la méthode d'extraction automatisée (20,8%). Les résultats montrent que les performances de l'extraction automatisée étaient inférieures à celles de l'extraction manuelle pour les échantillons frais (sensibilité 35.0%, spécificité 94,2%) et les échantillons congelés (sensibilité 43,5%, spécificité 93,2%). Cependant, en utilisant McNemar Chi-carré avec correction de Yates, il n'y avait pas de différence significative dans le taux de positivité de la RT-PCR (x^2=0,76, p=0,38) entre les deux méthodes d'extraction pour les échantillons frais, mais il y avait une différence significative (x^2=12,9, p=0,0003) dans l'extraction des échantillons congelés.
Conclusion: Les résultats de cette étude ont montré que la méthode d'extraction automatique KingFisher Flex Purification System 96 (ThermoFisher) était moins sensible et spécifique que la méthode d'extraction manuelle QIAamp Viral RNA Mini Kit (Qiagen). Ces informations peuvent servir de guide aux laboratoires dans le choix des méthodes d'extraction d'ARN à utiliser pour la détection par RT-PCR du SRAS-CoV-2.

Mots-clés: SRAS-CoV-2; extraction d'ARN; diagnostique; performance; RT-PCR

Introduction:

Coronavirus disease 2019 (COVID-19) was declared a pandemic on March 11 2020 by the World Health Organization. The causative pathogen is the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (1,2). The molecular tests for detecting SARS-CoV-2 include reverse transcriptase-polymerase chain reaction (RT - PCR), transcription - mediated amplification (TMA), nicking enzyme-assisted reaction (NEAR), loop - mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and systems using clustered regularly interspaced short palindromic repeat (CRISPR-Cas) and next-generation sequencing (3). Also, antigen detection and serological tests are used for epidemiological study (3). RT-PCR is the 'gold standard' for COVID-19 diagnosis because it detects nucleic acid associated with genes such as spike (S), envelope (E), membrane (M), and nucleocapsid (N), open reading frame polyprotein (ORF1ab), and non-structural proteins such as NSP12 which encodes RNA-dependent RNA polymerase (RdRp) (4,5).

The quality of nucleic acid extraction and purification influences the sensitivity, reproducibility and accuracy of the RT-PCR test (6). In the last 10 years, several new manual, semi-automated and automated commercial nucleic acid extraction systems using magnetic beads or silica particles have been developed for DNA, RNA or total nucleic acid extraction (7). Thus, the magnetic separation extraction method uses a magnetic field to separate micrometer-sized paramagnetic particles from a suspension. The method is simple and reliable to purify several types of biomolecules, such as DNA, plasmids, RNA and proteins but requires more handling time (8,9). Furthermore, the extraction method with centrifugation columns uses purification materials such as glass fibre, silica and filter paper. The advantages of this method are ease of use, flexibility and automation capability (10,11). Manual nucleic acid extraction methods have limitations of contamination and inhibition. Contamination, in particular, is very possible in samples with high viral load in the early stages of SARS-CoV-2 infection (12,13)

Studies have compared some of these new extraction methods and reported that they differ in their ability to recover viral RNA, indicating that no single RNA extraction method is optimal for all viruses (14,15). Comparative studies of manual and automated nucleic acid extraction methods have been carried

on viruses such as rotavirus (16) and New Castle disease virus (17). A comparison study of six automated nucleic acid extraction systems (KingFisher ML, Biorobot EZ1, easyMAG, KingFisher Flex MagNA Pure Compact, Biorobot MDX) and one manual kit (Allprep DNA/ RNA Mini Kit) for respiratory pathogens reported that the systems differed in nucleic acid recovery, reproducibility, and linearity in a pathogen-specific manner (18).

In Burkina Faso, several extraction methods are used for SARS-CoV-2 RNA extraction but have not been formally compared to determine which is the most efficient. From the beginning of the pandemic in March 2020 in Burkina Faso, the "QIAamp Viral RNA Mini Kit (Qiagen)" was the first kit used by most laboratories involved in COVID-19 diagnosis. Then other manual kits such as MGIEasy Nucleic Acid Extraction Kit (MGI Tech Co., Ltd), MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Applied Biosystems™), NUCLISENS® MINIMAG® (BioMérieux), Abbott Sample Preparation and automated kits such as MagNA PURE 96 and 24 (Roche), NucliSENS®easy MAG® 24 (BioMerieux), KingFisher Flex Purification System 24 and 96 (ThermoFisher), Arrow 12 (NorDiag/ DiaSorin), Abbott m2000 sp instrument 96 (Abbott) and abGenix™ 32 Nucleic acid extractor (AIT Biotech) (19). The QIAamp Viral RNA Mini Kit (Qiagen) produced more detectable RNA than the aforementioned kits (20).

After the acquisition of the automatic extractor (KingFisher) im our laboratory, we wanted to compare its performance to those of "QIAamp Viral RNA Mini Kit (Qiagen)", which prompted its choice as a reference. The main objective of this study therefore was to evaluate the performance of the automated extraction system "KingFisher Flex Purification System 96 (ThermoFisher)" in comparison to the manual method with the "QIAamp Viral RNA Mini Kit (Qiagen) in the perspective of its routine use.

Materials and method:

Study setting and design:

The study is a comparative evaluation of two RNA extraction methods for the *in vitro* detection of SARS-CoV-2, conducted between April and August 2021 in the Biomedical Research Laboratory (LaReBio) at the Institute for Research in Health Sciences (IRSS/CNRST), Ouagadougou. This is one of the laboratories involved in the COVID 19 diagnosis in Burkina Faso.

Ethics approval:

The Ministry of Health/Burkina Faso approved the evaluation of COVID-19 tests with the letter number N°2020/00004382/MS/ SG/DGAP/DLBM/sc dated 28 December 2020. It was carried at LaReBio, as recommended by the quality management system to any new method. This technical validation study is a contribution to the improvement of COVID-19 diagnosis in the laboratory. All the samples used under anonymous and confidential.

Nature and origin of the samples:

Nasopharyngeal and/or oropharyngeal specimens, including 159 fresh specimens [collected into viral transport medium (VTM) less than 24 hours and stored at 4-8°C] and 120 frozen specimens (collected into VTM from October to December 2020, stored at -80°C). All the samples were from the travellers' sites (airport, CMA Kossodo and IRSS) and suspected cases or contacts of COVID-19 patients.

Sample analysis methods:

All samples were extracted in duplicate using the two extraction methods; manual "QIAamp Viral RNA Mini Kit (Qiagen, Germany) and automated "KingFisher Flex" (ThermoFisher Scientific, USA). The amplification of both RNA extracts was done on the same PCR plate as shown in the flow chart (Fig 1).

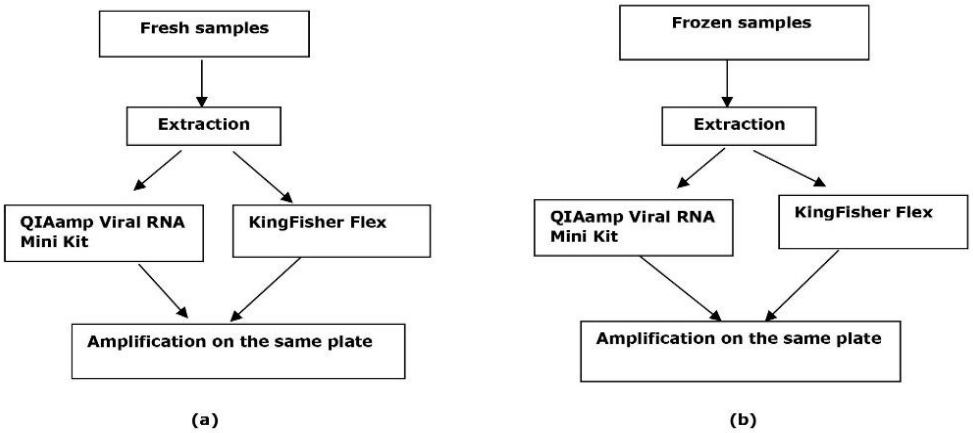

Fig 1: Flowchart of the analysis steps for fresh (a) and frozen (b) samples

The manual method "QIAamp Viral RNA Mini Kit" uses centrifugation columns on which a silica matrix is fixed. The principle is to lyse the cells to isolate the nucleic acid, attach it to a silica matrix, remove all contaminants by washing and elute the purified nucleic acid in a buffer. The KingFisher Flex Purification System 96 (ThermoFisher) is an automated extraction instrument. It provides consistent high-throughput extraction and purification of DNA, RNA, proteins and cells. It uses the MagMAX™ Viral/Pathogen Kit (ThermoFisher Scientific, USA) based on magnetic bead technology, designed to isolate and purify viral RNA and DNA.

RNA RT-PCR assay:

The FastPlexTM Triplex 1-Step COVID-19 Detection Kit (RT-PCR, RNA extraction free) (Precigenome LLC) was used to amplify on the same PCR plate the RNA extracts from the QIAamp Viral RNA Mini Kit and the KingFisher Flex Purification System 96 (ThermoFisher) using the QuantStudio5 thermal cycler (Applied Biosystems). The targets by the amplification kits are; ORF1ab (FAM fluorochrome) and N (HEX fluorochrome) and the internal control (CY5 fluorochrome) (Table 1).

Table 1: Interpretation of RT-PCR results

ORF1ab (FAM)	N (HEX)	IC (CY5)	Results
+	+	Not considered	SARS-CoV-2 positive
+	-		
-	+	+	SARS-CoV-2 Negative
-	-	-	Invalid

Negative result: Ct value > 39; Positive result: Ct value ≤ 39; Invalid: Ct >39 or no Ct detected for internal control (all samples' internal control)

Statistical analysis:

Data were entered into Excel 2016 and analyzed on R software. The mean Ct values of SARS-CoV-2 RT-PCR following manual and automated extraction methods were compared using the student's '*t*' test and the significance level was set at $p<0.05$. The sensitivity, specificity, positive predictive and negative predictive values of RT-PCR following automated KingFisher Flex extraction was calculated using the QIAamp manual method as the 'gold standard' with Open Epi. (http://www.openepi.com/Menu/OE_Menu.htm).

Results:

Characteristics of patients with fresh samples:

The mean age of the patients was 40.26 ± 12.89 years (age range 5 - 74 years). The age group 30-40 years was in the majority (32.07%), followed by age group 40-50 years with 25.79%. Most patients (66.67%) were male with gender ratio of 2.95. The most frequent reason for sample collection was travel at 50.94%, followed by contact cases of SARS-CoV-2 infected patients at 19.49%. The majority (95.60%) of the patients resided in Ouagadougou and the rest were undefined.

Characteristics of patients with frozen samples

The mean age of patients was 34.87± 14.51 years (age range 11-78 years). The age group 20-30 years was the most represented (26.67%), followed by age group 30-40 years with 22.5%. Most of the patients were male (60%). The most frequent reason for testing was "contact" of SARS-CoV-2 cases (49.16%), followed by travelers (45%) and others (suspected cases and controls). Almost all (96.7%) of the patients resided in Ouagadougou and the samples were collected from the Ouagadougou sampling sites.

Evaluation of the automated and manual extraction methods for fresh samples:

The study found a slightly higher RT-PCR positivity rate of fresh samples following manual extraction (12.6%, 20/159) than automated extraction (9.4%, 15/159). Seven (4.4%) samples were positive following both manual and automated extraction while 131 (82.4%) were negative. Compared to manual extraction, automated extraction had a specificity of 94.2%, sensitivity of 35.0%, PPV of 46.7% and NPV of 90.9% for fresh samples but using McNemar Chi-square with Yates correction, there was no significant difference between both methods of extraction ($x^2=0.76$, $p=0.38$) with the fresh samples (Table 2a).

Evaluation of the automatic and manual extraction method for frozen samples:

For the frozen samples, there was a significant difference in the RT-PCR positivity rate following manual extraction (38.33%, 46/120) and automated extraction (20.83%, 25/120) ($x^2=12.9$, $p=0.0003$). Twenty (16.7%) samples were positive following both manual and automated extraction and 69 (57.5%) samples were negative following both extraction methods. Compared to manual extraction, automated extraction had a specificity of 93.24%, sensitivity of 43.48%, PPV of 80.0% and NPV of 72.63% (Table 2b).

Table 2a: Comparative evaluation of RT-PCR results following automatic and manual extraction methods from fresh samples

RNA extraction results/samples		**Nasopharyngeal samples**		**Oropharyngeal samples**		**Total**
		Positive	**Negative**	**Positive**	**Negative**	
Results of the automatic extraction	Positive	7	7	0	1	15
	Negative	13	128	0	3	144
	Total	20	135	0	4	159
	Sensitivity (95% CI)	35.00% (18.12 - 56.71)				
	Specificity (95% CI)	94.24% (89.05 - 97.06)				
	PPV (95% CI)	46.67% (24.81 - 69.88)				
	NPV (95% CI)	90.97% (85.17 - 94.65)				
	Kappa de Cohen coefficient (95% CI)	0.33 (0.17 - 0.48)				
	McNemar with Yeates correction	x^2= 0.76, p=0.38				

PPV: Positive predictive value, NPV: negative predictive value, 95% CI: 95% confidence interval

Table 2b: Comparative evaluation of RT-PCR results following automatic and manual extraction methods from frozen samples

RNA extraction results/samples		**Nasopharyngeal samples**		**Oropharyngeal samples**		**Total**
		Positive	**Negative**	**Positive**	**Negative**	
Results of the automatic extraction	Positive	13	4	7	1	25
	Negative	19	54	7	15	95
	Total	32	58	14	16	120
	Sensitivity (95%CI)	43.48% (30.21 - 57.75)				
	Specificity(95%CI)	93.24% (85.14 - 97.08)				
	PPV (95% CI)	80.0% (60.87 - 91.14)				
	NPV (95% CI)	72.63% (62.92 - 80.59)				
	Kappa de Cohen Coefficient (95%CI)	0.40 (0.23 - 0.56)				
	McNemar with Yeates correction	x^2= 12.90, p=0.0003				

PPV: Positive predictive value, NPV: negative predictive value, 95% CI: 95% confidence interval

Table 3: Mean cycle threshold (C_t) values in RT-PCR of SARS-COV-2 genes following RNA extractions from fresh and frozen samples

Fresh samples				
Targets	**QIAamp C_t (mean± SD)**	**KingFisher Flex C_t (mean± SD)**	**C_t difference (C_t Kingfisher - C_t QIAamp)**	**p value**
ORF1ab	31.32 ± 3.41	33.68 ± 2.76	+2.36	<0.0001*
N	33.74 ± 4.69	34.55 ± 4.87	+ 0.81	0.1319
IC	26.88 ± 3.69	26.6 ± 3.60	-0.28	0.4939
Frozen samples				
Targets	**QIAamp C_t (mean± SD)**	**KingFisher Flex C_t (mean± SD)**	**C_t difference (C_t Kingfisher - C_t QIAamp)**	**p value**
ORF1ab	30.74± 5.98	30.73± 4.65	-0.01	0.9885
N	33.04± 4.36	32.04± 3.80	-1	0.0594
IC	27.18± 4.00	27.96± 3.39	+0.78	0.1045

ORF1ab: Open reading frame1ab, N: nucleocapsid protein, IC: internal control; *: statistically significant difference

Comparison of mean C_t values of different SARS-CoV-2 genes following manual and automated extraction of fresh samples:

There was a gain of 2.36 and 0.81 cycles of amplification for the ORF1ab and N gene respectively for KingFisher Flex automated over the QIAamp extraction method from the fresh samples with a statistically significant difference between the mean C_t values for the ORF1ab gene (p<0.0001), but there was no significant difference between the mean C_t values for the N gene (p=0.1319) between the manual and automated extraction methods. Also, there was no significant difference between the mean C_t values of the internal control between the manual and automated extraction (p=0.4939), although there was a loss of threshold cycle (C_t) of -0.28 cycles for the internal control (Cy5) with the KingFisher Flex automated method compared to QIAamp manual extraction method (Table 3).

Comparison of mean C_t values of different SARS-CoV-2 genes following manual and automated extraction of frozen samples:

There was no statistically significant difference in the mean C_t value of the ORF1ab gene between the manual and automated extraction methods (p=0.9885) although there was a loss of -0.01 amplification cycle by the automated method. Similarly, there was no significant difference between the mean C_t values of the N gene between the manual and automated extraction (p=0.0594) although there was a loss of one amplification cycle (-1 cycle) by the automated extraction method. Also, there was no significant difference between the mean C_t of the internal control following manual and automated extraction (p= 0.1045) although there was a gain of +0.78 cycles by the automated KingFisher Flex over manual QIAamp extraction method (Table 3).

Discussion:

This study compared an automated extraction method for SARS-CoV-2 RNA with a manual method, used as reference. It shows that compared to the "QIAamp Viral RNA Mini Kit (Qiagen)", the "KingFisher Flex Purification System 96 (ThermoFisher)" automated extraction method loses some performance. Indeed, the study found a slightly higher SARS-CoV-2 RT-PCR positivity rate for fresh samples for manual extraction (12.6%) than for automatic extraction (9.4%). For frozen samples, there was also difference in the positivity rate following manual extraction (38.3%) than automatic extraction (20.8%). A previous study (21) showed that there was no statistically significant difference (p=0.629) in the RT-PCR positivity rate (92.5% vs 90%) between manual and automated methods. This may be explained by the fact that the methods used are different. In this previous study, automated liquid-based high-throughput RNA extraction platform (PHASIFY™) was compared with the widely used magnetic bead-based total nucleic acid extraction (MBTE) platform (NucliSENS ® easyMAG ®).

Our study shows that automatic extraction on fresh and frozen samples was more specific (94.24%, 93.24%) but less sensitive (35%, 43.48%) in detecting SARS-CoV-2 on RT-PCR. Using the 2x2 contingency table analysis, manual extraction was more sensitive than automated extraction, and although this

was not statistically significant for fresh samples on McNemar Chi-square with Yates correction (x^2=0.76, p=0.38), it was statistically significant for frozen samples (x^2=12.9, p= 0.0003). Contrariwise, researchers in Brazil (22) found that automated extraction (Loccus, Extracta Kit FAST) was the most sensitive (100%) compared to manual extraction (Bio-Gene Kit, Bioclin, Quibasa) and rapid extraction methods (Lucigen, Quick DNA Extract Kit). This discordant finding could be explained by the different extraction techniques used in these studies.

The mean cycle threshold (C_t) value of the RT-PCR for SARS-CoV-2 ORF1ab gene for fresh samples was significantly higher with the KingFisher Flex extraction (p<0.0001) than manual QIAamp Viral RNA Mini Kit extracts, indicating that manual extraction was more sensitive in detecting the ORF1ab gene from fresh samples, but there was no significant difference in the C_t for the N gene (p=0.1319) between the two methods. For the frozen samples, the mean C_t values of SARS-CoV-2 ORF1 ab (p=0.9885) and N genes (p=0.0594) were not significantly different for both manual QIAamp Viral RNA Kit and automated KingFisher Flex extraction methods.

The results obtained for the fresh samples in our study agrees with those of Esona et al., (16) who in comparing two automated methods found that the mean C_t values for the KingFisher Flex extracts were significantly higher (p=0.001) than those of the other methods (MagNA Pure Compact or RNaid kit). However, our results of the frozen samples contrast those of Ransom et al., (23) who in their study found that the mean C_t values of RT-PCR with the KingFisher Flex were significantly lower (p=0.05) than those of the EZ1 and easyMAG, and although there was a loss of 0.01 and 1 cycle for ORF1ab and N genes respectively with the KingFisher Flex extraction method for the frozen samples, which may indicate higher sensitivity of SARS-CoV-2 detection, these losses did not reach statistical significance (p>0.05). Nevertheless, our results showed that manual QIAamp Viral RNA Mini Kit, compared to KingFisher Flex Purification System 96, was more sensitive for the extraction of ORF1ab RNA from fresh samples than from frozen samples.

Furthermore, it has been extrapolated in a study (24) that each 3.3 increase in C_t value corresponds to approximately 1 log (i. e. 10-fold) less target in the primary clinical specimen subjected to PCR reaction. Similarly, some researchers have attempted to correlate C_t values with SARS-CoV-2 detection (25), thus gains (e. g. gain of 2.36 cycles from the manual over automatic for the ORF1ab gene in the fresh samples in our study) or losses (e. g. loss of -0.01 cycles from manual to automatic for the ORF1ab gene in the frozen samples) of C_t can influence "positivity" or "negativity" of RT-PCR test. It appears that automated extraction may be better suited for frozen than fresh samples, as the target genes are detected earlier. But our findings remain preliminary and requires to be validated in a more comprehensive study comparing the two methods.

Our study is limited by the non-repeatability of the extraction and RT-PCR assays on the tested samples, lack of information and data on the symptoms of the tested subjects and possible contamination with manual extractions. Nonetheless, the results of our study showed that automated RNA extraction with KingFisher Flex was less sensitive for RT-PCR detection of SARS-CoV-2 from fresh samples than manual extraction with QIAamp kit. This finding could serve as guide for laboratories in selecting extraction methods based on periods of COVID-19 sampling and target individuals to be diagnosed.

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Contributions of authors:

AAZ conceptualized the study and was involved in data curation, formal analysis, software, and writing original manuscript draft; HGO was involved in study conceptualization, laboratory analysis, supervision, review and manuscript editing; TRC was involved in data curation, review and manuscript editing; JAB was involved in data curation, review and manuscript editing; TS was involved in laboratory analysis, validation, review and manuscript ed iting; STS was involved in laboratory analysis, review and manuscript editing; CD was involved in data curation; ARN was involved in data curation; and NB was involved in supervision and data validation.

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Conflicts of interest:

No conflict of interest is declared.

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