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Short Communication

Evaluation of alternative RNA extraction methods for detection of SARS-CoV-2 in nasopharyngeal samples using the recommended CDC primer-probe set

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ABSTRACT

Background: The efficiency of isolation and purification of the viral genome is a critical step to the accuracy and reliability of RT-qPCR to detect SARS-CoV-2. However, COVID-19 testing laboratories were overwhelmed by a surge in diagnostic demand that affected supply chains especially in low and middle-income facilities.

Objectives: Thus, this study compares the performance of alternative methods to extraction and purification of viral RNA in samples of patients diagnosed with COVID-19.

Study design: Nasopharyngeal swabs were submitted to three *in-house* protocols and three commercial methods; viral genome was detected using the primer-probe (N1 and N2) described by CDC and viral load of samples were determined.

Results: The *in-house* protocols resulted in detection of virus in 82.4 to 86.3% of samples and commercial methods in 94.1 to 98%. The disagreement results were observed in samples with low viral load or below the estimated limit of detection of RT-qPCR.

Conclusion: The simplified methods proposed might be less reliable for patients with low viral load and alternative commercial methods showed comparable performance.

1. Background

Diagnosis of infected individuals is the cornerstone to track transmission and guide strategies against the COVID-19 pandemic [6]. Therefore, the pandemic led to an unprecedented demand for diagnostic tests, overwhelming laboratories especially in low and middle-income countries.

2. Objectives

Here, we aimed to investigate the performance of three *in-house* simplified and three alternative commercial methods for viral RNA extraction and detection of SARS-CoV-2 in samples of patients with COVID-19.

3. Study design

Fifty-one nasopharyngeal swabs from individuals infected with SARS-CoV-2 were selected according to cycle threshold (CT) in three groups of seventeen samples (CT <20, between 20–30 and >30). For each extraction protocol 150 µL of PBS containing the nasopharyngeal samples were used. For simplified protocols three microtubes were used: first tube immediate incubated at 95 °C for 10 min; second tube incubation with 20 µL of proteinase K [20 mg/mL] (Promega, USA) at 56 °C for 10 min, followed by heating at 95 °C for 10 min; thirty tube 150 µL of Chelex100 [10%] (Sigma-Aldrich, USA) was added followed by vigorous vortexing, then incubation at 95 °C for 10 min. After heating, all tubes were centrifuged and placed in an ice-bath and then used for RT-qPCR assays. For purification using commercial kits three distinct

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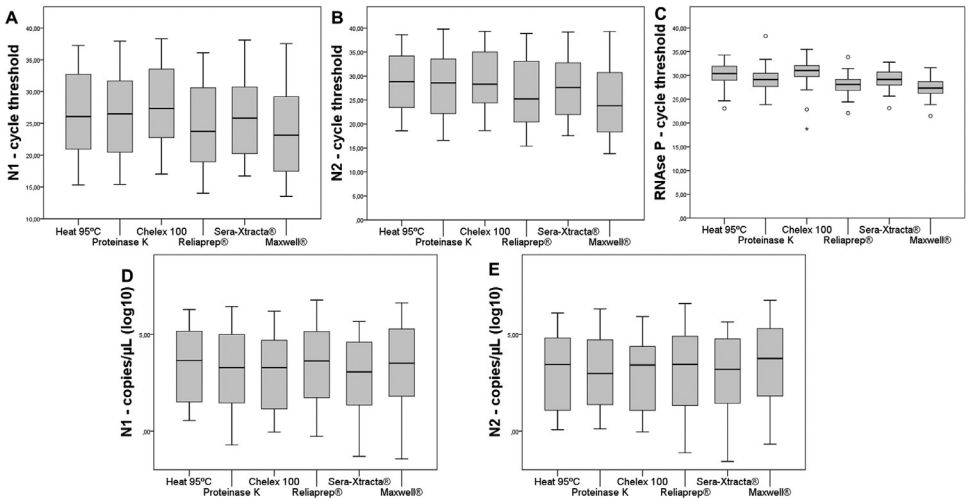

Fig. 1. Box plots of cycles thresholds and viral RNA load of clinical samples according with extraction methods; A. Cycles thresholds of SARS-CoV2 N1 target (Maxwell x Chelex100, P = 0.029); B. Cycles thresholds of SARS-CoV2 N2 target (Maxwell x Chelex100, P < 0.012); C. Cycles threshold of human RNAse P target (P < 0.05; Maxwell x proteinase K, P = 0.006; Maxwell x Heat, P = 0,000; Maxwell x Chelex100, P = 0,000; Reliaprep x Heat, P = 0,000; Reliaprep x Chelex, P = 0,000; Sera-Xtracta x Chelex100, P = 0,023; Proteinase K x Chelex100, P = 0,004); D. RNA copies of SARS-CoV2 N1 target; E. RNA copies of SARS-CoV2 N2 target.

approaches were evaluated according to manufacturer instructions and final elution in 50 µL of molecular biology grade water: Reliaprep® viral TNA System (Promega, USA); Sera-Xtracta® virus/pathogen kit (GE Healthcare, USA); Maxwell® RSC 48 viral TNA (Promega, USA). After extraction/purification the eluted samples were stored at −70 ˚C before RT-qPCR amplification. Amplifications were conducted according to CDC 2019-nCoV diagnostic panel [4] in a QuantStudio3 Real Time PCR System (Thermo Fisher Scientific, USA), with the following modifications: reactions were performed in a final reaction volume of 15 µL using GoTaq Probe 1-Step RT-qPCR System (Promega, USA). For commercial methods 4 µL of eluted nucleic acids were used and for simplified extraction methods 2 µL of nucleic acids were used with addition of Dimethyl sulfoxide (DMSO) to a final concentration of 2% in reaction (Supplementary file 1). Six-fold dilution standard curve was developed using known concentrations of a plasmid containing the complete nucleocapsid gene from SARS-CoV-2 (Integrated DNA Technologies, USA), ranging from 10^5 to 1 copies/µL. The amplification data and quantification assays were analyzed by QuantStudio Design & Analysis Software (Thermo Fisher Scientific, USA). Quantifications and CT of targets were statistically analyzed using Kruskal–Wallis and post hoc using Dunn-Bonferroni tests. Values of $P \leq 0.05$ were considered statistically significant. All analyses were performed using IBM SPSS version 20.0 for macOS (IBM Corporation, USA).

4. Results

The lowest limit detection for the diagnostic panel RT-qPCR assay was 10 genome copies per microliter (Supplementary file 2). From fifty-one samples used to evaluate the methods performance, detection of SARS-CoV-2 RNA was confirmed by at least one extraction/purification method. The simplified *in-house* methods resulted in detectable results in 82.4% (CI 71.9%–92.8%) to 86.3% (CI 76.9%–95.7%) samples, and commercial purification kits in 94.1% (CI 87.6%–100%) to 98% (CI 94.2.5%–100%) samples. The observed rate of inconclusive results was 0% to 7.8% (CI 0%–15.2%) (Table 1).

The Chelex 100 protocol resulted in increased CT for all assays. For RNAse P assay the commercial kits showed lower CTs compared to *in-house* protocols. However, there were no significant differences in the CT values or viral quantification (N1 assay and N2 assay) among the different protocols (Fig. 1).

Comparing each sample result according to the RNA extraction/purification protocol, eleven samples (21.6%) showed disagreement results in N1 or N2 assay. Those inconclusive results and disagreement between different protocols were observed in elutes with low yield. Since all elutes of clinical samples obtained from Sera-Xtracta®virus kit resulted in detectable N1 or N2 assays, showing the best performance among protocols evaluated, we used the data of Sera-Xtracta®virus protocol to analyze disagreement samples. The obtained CTs and quantifications of agreement and disagreement samples showed that agreement among protocols is related to the viral load in clinical samples (Fig. 2).

Table 1
Comparison results summary between alternative and commercial methods of nucleic acid extraction for detection of SARS-Cov2.

Method	Results		
	Detectable	Not detectable	Inconclusive
Heat 95 ˚C	82.4% (42/51)	9.8% (5/51)	7.8% (4/51)
Proteinase K	86.3% (44/51)	9.8% (5/51)	3.9% (2/51)
Chelex 100	84.3% (43/51)	7.8% (4/51)	7.8% (4/51)
Reliaprep® viral TNA	98% (50/51)	2.0% (1/51)	0
Sera-Xtracta® virus	98% (50/51)	0	2.0% (1/51)
Maxwell® RSC 48	94.1% (48/51)	2.0% (1/51)	3.9% (2/51)

5. Discussion

The RT-qPCR performance is affected by the efficiency of the nucleic acids extractions methods. Comparison of the available commercial kits in our laboratory showed not significant differences in assays CT or viral load quantification. The automated Maxwell® RSC 48 is recommended by CDC, but surprisingly showed lowest detection rate, 94.1%, while the silica column-based method Reliaprep® viral TNA and beads-based method Sera-Xtracta® virus detected 98%. Indeed, detailed analysis showed that concordance between commercial kits in samples with viral load above assays LOD is 100%. Accordingly, there was no significant difference between the manual and automatized commercial extraction methods performance evaluated.

Some studies evaluated alternative or in-house simplified methods to override the step of purification in RT-qPCR for SARS-CoV-2 [1–3,5,7–10,11,12], however comparing the available studies, different protocols lead to different results, which are not reproducible and the multiple variables involved could affect the performance of each assay. In our experiments, to inactivate viruses and reduce the risk of contamination for simplified methods, the samples were heated for 10 min at 95 ˚C prior to RT-qPCR. That simplified protocol of heat-inactivation showed that viral RNA could be detected from samples stored in PBS with agreement rates of 84% to 87.5% compared to commercial extraction methods. To evaluate if the simplified protocol of heat-inactivation could be further improved, we added a previous step of proteinase K treatment, which could enhance nucleic acids yield by inactivating RNAses present in samples as well as other potential PCR inhibitors [5], and the incorporation of Chelex-100, a chelating agent that binds to cellular components and stabilizes the RNA during heat [12]. However, unlike a previous study

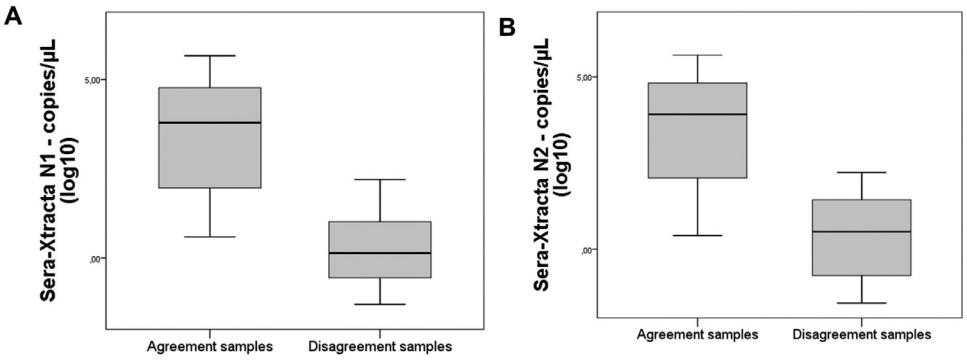

Fig. 2. Box plots of viral RNA load by Sera-Xtracta® according with result agreement of clinical samples by alternative methods (Heat 95°C, Proteinase K and Chelex 100); A. RNA copies of SARS-CoV2 N1 target, P = 0.000; B. RNA copies of SARS-CoV2 N2 target, P = 0.000.

that observed increased detection when pre-treatment with proteinase K was employed [5], we were unable to observe such improvement in simplified protocol of heat-inactivation.

The limitations of our study include, for example, the lack of a gold standard for SARS-CoV-2 detection, which lead to the calculation of agreement percentage, and the fact that samples were collected and stored in PBS, and thus we could not observe the performance of our simplified protocols in a distinct matrix of transport medium. Further, we could not spike samples with known concentrations of synthetic genomic RNA, which could be relevant to evaluate the efficiency of extractions/purifications methods.

Although we have used PBS for sample transport, we performed assays with reduced amplicons sizes (71 bp and 67 bp for N1 and N2), samples were stored at −70 °C, and RT-PCR was immediately performed after RNA extraction to avoid RNA degradation or kept for short periods at −70 °C and then analyzed by RT-PCR. For simplified extraction methods discrepant results were observed in samples with low viral load and some below the LOD of assays. These findings might be explained in part by sample dilution and smaller RNA input compared to commercial methods that concentrate RNA during elution, resulting in an increase for RNAse P CTs. Indeed, different studies observed that direct RT-qPCR approach resulted in a drop of sensitivity, as CT values increased above 35 [8,9,11].

The comparisons of commercial kits performance in this study support the interchangeability of these methods and other factors such supply chain availability, cost, and hands-on time should be evaluated to appoint the best RNA extraction/purification method in each facility. The simplified extractions approach might be less robust and affected by several conditions and should only be conducted under emergency use and following proper validation. In conclusion, the data demonstrate that distinct extraction methods have comparable results in samples with viral load above the LOD or assay using the CDC proposed primer-probe set.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcvp.2021.100032.

References

[1] J. Alcoba-Florez, R. González-Montelongo, A. Íñigo-Campos, D.G. de Artola, H. Gil-Campesino, L. Ciuffreda, A. Valenzuela-Fernández, C. Flores, The Microbiology Technical Support Team, Fast SARS-CoV-2 detection by RT-qPCR in preheated nasopharyngeal swab samples, Int. J. Infect. Dis. 97 (2020) 66–68 AugEpub 2020 May 31, doi:10.1016/j.ijid.2020.05.099.

[2] C. Ambrosi, C. Prezioso, P. Checconi, D. Scribano, M. Sarshar, M. Capannari, C. Tomino, M. Fini, E. Garaci, A.T. Palamara, G. De Chiara, D. Limongi, SARS-CoV-2: comparative analysis of different RNA extraction methods, J. Virol. Methods 287 (2021) 114008 JanEpub 2020 Nov 4. PMID: 33160015; PMCID: PMC7640895, doi:10.1016/j.jviromet.2020.114008.

[3] E.A. Bruce, M.L. Huang, G.A. Perchetti, S. Tighe, P. Laaguiby, J.J. Hoffman, D.L. Gerrard, A.K. Nalla, Y. Wei, A.L. Greninger, S.A. Diehl, D.J. Shirley, D.G.B. Leonard, C.D. Huston, B.D. Kirkpatrick, J.A. Dragon, J.W. Crothers, K.R. Jerome, J.W. Botten, Direct RT-qPCR detection of SARS-CoV-2 RNA from patient nasopharyngeal swabs without an RNA extraction step, PLoS Biol. 18 (10) (2020) e3000896 Oct 2PMID: 33006983, doi:10.1371/journal.pbio.3000896.

[4] CDC. Division of viral diseases. Center for disease control and prevention. (2020) CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. [December 1, 2020]. https://www.fda.gov/media/134922/download

[5] A.W. Chu, W.M. Chan, J.D. Ip, C.C. Yip, J.F. Chan, K.Y. Yuen, K.K. To, Evaluation of simple nucleic acid extraction methods for the detection of SARS-CoV-2 in nasopharyngeal and saliva specimens during global shortage of extraction kits, J. Clin. Virol. 129 (2020) 104519 AugEpub 2020 Jun 23. PMID: 32629187, doi:10.1016/j.jcv.2020.104519.

[6] V.M. Corman, O. Landt, M. Kaiser, R. Molenkamp, A. Meijer, D.K.W. Chu, et al., Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR, Euro Surveill. 25 (2020) 2000045.

[7] A.S. Fomsgaard, M.W. Rosenstierne, An alternative workflow for molecular detection of SARS-CoV-2 - escape from the NA extraction kit-shortage, Copenhagen, Denmark, Euro Surveill. 25 (14) (2020) 2000398 2020 AprPMID: 32290902, doi:10.2807/1560-7917.ES.2020.25.14.2000398.

[8] O. Israeli, A. Beth-Din, N. Paran, D. Stein, S. Lazar, S. Weiss, E. Milrot, Y. Atiya-Nasagi, S. Yitzhaki, O. Laskar, O. Schuster, Evaluating the efficacy of RT-qPCR SARS-CoV-2 direct approaches in comparison to RNA extraction, Int. J. Infect. Dis. 99 (2020) 352–354 OctEpub 2020 Aug 10. PMID: 32791207, doi:10.1016/j.ijid.2020.08.015.

[9] N. Lübke, T. Senff, S. Scherger, S. Hauka, M. Andrée, O. Adams, J. Timm, A. Walker, Extraction-free SARS-CoV-2 detection by rapid RT-qPCR universal for all primary respiratory materials, J. Clin. Virol. 130 (2020) 104579 SepEpub 2020 Aug 5. PMID: 32795959, doi:10.1016/j.jcv.2020.104579.

[10] N. Merindol, G. Pépin, C. Marchand, M. Rheault, C. Peterson, A. Poirier, C. Houle, H. Germain, A. Danylo, SARS-CoV-2 detection by direct rRT-PCR without RNA extraction, J. Clin. Virol. 128 (2020) 104423 JulEpub 2020 May 7. PMID: 32416598, doi:10.1016/j.jcv.2020.104423.

[11] I. Smyrlaki, M. Ekman, A. Lentini, N. Rufino de Sousa, N. Papanicolaou, M. Vondracek, J. Aarum, H. Safari, S. Muradrasoli, A.G. Rothfuchs, J. Albert, B. Högberg, B. Reinius, Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR, Nat. Commun. 11 (1) (2020) 4812 Sep 23PMID: 32968075, doi:10.1038/s41467-020-18611-5.

[12] S. Ulloa, C. Bravo, B. Parra, E. Ramirez, A. Acevedo, R. Fasce, J. Fernandez, A simple method for SARS-CoV-2 detection by rRT-PCR without the use of a commercial RNA extraction kit, J. Virol. Methods 285 (2020) 113960 NovEpub 2020 Aug 22. PMID: 32835738, doi:10.1016/j.jviromet.2020.113960.