Usefulness of the extraction-free RT-PCR methods for the SARS-CoV-2 diagnostics: An Italian experience

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Research Article

Keywords: SARS-CoV-2, COVID-19, RT-PCR, Heat inactivation, Italy

DOI: https://doi.org/10.21203/rs.3.rs-414564/v1

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Abstract

The extraction-based real-time reverse transcription polymerase chain reaction (RT-PCR) is currently the “gold standard” for the SARS-CoV-2 diagnostics. However, some extraction-free RT-PCR techniques have been recently developed. In this study we compared the performance of heated and unheated extraction-free methods with the traditional extraction-based SARS-CoV-2 RT-PCR. The unheated extraction-free showed a perfect agreement with the standard extraction-based RT-PCR. By contrast, the heat-treated technique was associated with an 8.2% false negativity rate. The unheated extraction-free RT-PCR for the SARS-CoV-2 molecular diagnostics is a valuable alternative to the traditional extraction-based methods and may accelerate turnaround times by about two hours.

Introduction

The ongoing pandemic of coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has implied the largest socioeconomic and health emergency worldwide. The availability of accurate COVID-19 diagnostic tests is a cornerstone measure to tackle the pandemic [1–5].

Nowadays, the real-time reverse transcription polymerase chain reaction (RT-PCR) is considered the “gold standard” assay for diagnosis of both symptomatic and asymptomatic cases [6] and included in the protocols issued by several international institutional bodies [7, 8]. These commonly used protocols have a ribonucleic acid (RNA) extraction step that may be seen as an important bottleneck in the routine laboratory testing process [9].

Since March 2020, some manufacturers reported supply shortages for RNA extraction kits driven by the sudden increase in their global demand. This situation called for alternative protocols with similarly high diagnostic accuracy in order to ensure the continuity of testing [10–13]. Consequently, different direct approaches that avoid RNA extraction have been suggested, including heat-processed methods [9, 14].

The objective of this study was to evaluate the diagnostic performance of two different extraction-free RT-PCR techniques and to compare their performance to the traditional extraction-based method in the real-world setting.

Methods

In this study a total of 98 confirmed nasopharyngeal swabs from COVID-19 symptomatic individuals were analysed. These samples were routinely collected in October 2020 and were eluted in the universal transport medium (UTM™, Copan Diagnostics Inc, US) and processed at the regional reference laboratory for COVID-19 diagnostic of the San Martino Policlinico Hospital, Hygiene Unit, Genoa, Liguria, Northwest Italy.
All samples were processed in parallel by using three methods: (i) standard extraction-based (EB) method performed with STARMag 96x4 Viral DNA/RNA 200C Kit (Seegene Inc., South Korea); (ii) RNA extraction-free (EF) method with a heating step (EFh+) and (iii) unheated RNA extraction-free technique (EFh–). For the EB method, total RNA was extracted and set up for RT-PCR by means of the Nimbus IVD Seegene platform using the Allplex™ 2019-nCoV Assay kit (Seegene Inc., South Korea), according to the manufacturer’s instructions. For the EF + method, thermolysis was first performed at 95°C for 5 min and at 55°C for 3 min on Biorad CFX96™ thermal cycler (Bio-Rad Laboratories, US). EF− method was identical to EFh+ unless the thermolysis step.

The material thus obtained was tested for the identification of SARS-CoV-2 by means of a one-step real-time multiplex RT-PCR quantitative assay on Biorad CFX96™ thermal cycler, targeting the nucleoprotein region (N), RNA-dependent RNA-polymerase (RdRp)/spike (S) proteins and envelop (E) region. Samples showing a cycle threshold (Ct) value < 40 for at least two genes were considered positive.

The standard EB technique was considered a reference method against both EFh+ and EFh−. A sensitivity with 95% confidence intervals (CIs) was calculated. As the observed Ct values were approximately normally distributed, the average target-specific difference in Ct values between the three techniques was computed and compared by applying the repeated measures analysis of variance (ANOVA) and follow-up Tukey honestly significant difference post-hoc test. Data were analysed in R stats packages, v. 4.0.3 [15].

**Results**

On considering the traditional EB technique as a “gold standard”, EFh− method displayed a perfect accordance with no false negative results [sensitivity of 100% (95% CI: 95.3–100%)]. By contrast, a total of eight (8.2%) swabs treated as per EFh+ methodology were deemed false negative, giving a sensitivity of 91.8% (95% CI: 84.1–96.2%).

Figure 1 and Table 1 report the summary distributions and mean differences of Ct values according to the technique used and gene target. The omnibus ANOVA test rejected ($P < 0.001$) the null hypothesis that Ct values figured out by the three methods used had identical means; moreover, all the pairwise post-hoc comparisons were also statistically significant ($P < 0.01$). Indeed, the average Ct value for any gene was the lowest in EB group and the highest in the EFh+. Moreover, the highest between-method difference concerned RdRp/S gene, while the lowest one N gene (Table 1). Of note, all the eight false negatives determined by EFh+ method had a Ct value > 30, independently from gene target.
Table 1
Mean absolute difference in cycle threshold values, by method used and gene target*

| Method       | Genet target |         |         |         |
|--------------|--------------|---------|---------|---------|
|              | E            | RdRp/S  | N       |
| EFh + vs EB  | 4.92 (4.44–5.40) | 5.29 (4.74–5.85) | 4.10 (3.71–4.48) |
| Ef− vs EB    | 2.26 (1.89–2.63) | 2.87 (2.48–3.26) | 2.53 (2.13–2.93) |
| Ef+ vs Ef−   | 2.66 (2.34–2.98) | 2.42 (1.99–2.86) | 1.56 (1.23–1.90) |

*Results are reported as mean difference (95% CIs for paired samples)

Discussion

In the COVID-19 pandemic context it is crucial to obtain rapid and reliable results to diagnose patients in order to take timely public health decisions. Here, we demonstrated that the EF methods may be valuable alternatives to the traditional EB techniques. This study adds to the growing body of evidence on the utility of extraction-free methods to detect SARS-CoV-2 and to the best of our knowledge it is among the first European studies on the purpose.

In our study the RT-PCR readout obtained by the direct EFh– method had a full agreement with the traditional EB technique. Although there were significant differences between Ct values provided by these two methods, no disagreement in the overall output interpretation was found. On the other hand, we found some negative effect of the heat inactivation on virus detection; this, however, affected only samples with low viral loads (Ct > 30). Zou et al. [16] have similarly established that following the heat treatment about 13.0% of positive swabs turned negative and all these false negatives had a Ct value ≥ 37. Analogously, Grant et al. [17] have observed lower Ct values in unheated samples. Finally, our results are also in line with a recent study by Burton et al. [18] who observed that the heat treatment at 95°C for 5 min (like in our study) has increased an average Ct by 4.5–6 points. The application of lower heating regimens may be therefore useful in order to increase the sensitivity of EFh + method and should be further investigated. For instance, Burton et al. [18] have not registered any significant differences at heating regimens of 56/60°C.

To conclude, our data suggest that EF methods (especially unheated) are a useful tool in providing RT-PCR results in a shorter time, speeding up clinical diagnostics and the subsequent burden on molecular labs and hospitals. Indeed, the average processing time in our study was 270, 163 and 156 min for the standard EB, EFh + and EFh−, respectively. These EF methods therefore urge an internationally recognized protocolization.

Declarations
Availability of data and materials

The whole raw dataset used may be available from the corresponding author on reasonable request and following approval from the San Martino Policlinico Hospital

Funding

This study received no external funding

Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki. Ethical review and approval were waived for this retrospective analysis because it was based on the routine COVID-19 testing

Acknowledgements

The authors would like to thank Chessa Valerio, Qosjia Rexhina, Randazzo Nadia, Theimer Matteo for performing RT-PCR tests.

Conflict of interest

The authors declare no conflict of interest regarding this publication.

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Figure 1

Distribution of the cycle threshold values by method used and gene target