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TRIzol-based RNA extraction for detection protocol for SARS-CoV-2 of coronavirus disease 2019

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Abstract

Diagnostic testing is important for managing the 2019 novel coronavirus (SARS-CoV-2). We developed an optimized protocol for SARS-CoV-2 RNA extraction from the surface of the respiratory mucosa with nasopharyngeal swabs and compared the sensitivity of RNA extraction methods. RNA extraction was performed using three different procedures (TRIzol, QIAamp, VMT-TRIzol) from nine positive SARS-CoV-2 samples. SARS-CoV-2 was detected by real-time reverse transcriptase PCR (RT-PCR) using a detection kit for SARS-CoV-2 (Sun Yat-sen University). Compared to RT-PCR results, there were no discernible differences in detection rates when comparing the three different extraction procedures. On the basis of these results, the use of TRIzol as a transport medium and RNA extraction method for SARS-CoV-2 detection may be a helpful alternative for laboratories facing shortages of commercial testing kits.

Keywords: Diagnostic techniques, RT-PCR, SARS-CoV-2, throat swab, TRIzol
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Introduction

Coronavirus disease 2019 (COVID-19) is an acute infection of the respiratory tract that emerged in late 2019 in Wuhan, Hubei province, China. Phylogenetic analysis conducted by the China Novel Coronavirus Investigating and Research Team showed that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) falls into the genus *Betacoronavirus,* including coronaviruses discovered in humans and wild animals [1]. It is well established that most reported cases had similar clinical manifestations at the onset of illness, such as fever, cough, myalgia or fatigue, headache and conjunctivitis [2,3]. Most cases were of respiratory diseases such as acute respiratory distress syndrome and multiple organ failures [4,5].

In view of the rapid spread of SARS-CoV-2, real-time reverse transcriptase PCR (RT-PCR) remains the reference standard for diagnosing COVID-19 among the many diagnostic platforms available [6,7]. Converging evidence demonstrates that SARS-CoV-2 RNA may be identified by quantitative RT-PCR of respiratory tract samples 1 or 2 days before the appearance of the first symptoms; the SARS-CoV-2 RNA remains detectable for 7 to 12 days in moderate cases and up to 2 weeks in severe cases [8–10]. Corman *et al.* [6] were the first to validate diagnostic RT-PCR tests of SARS-related coronavirus. Two assays targeting the E gene (envelope) and RNA-dependent RNA polymerase (RdRp) genes were selected, where the E gene assay acts as a first-line screening tool and the RdRp gene assay as the confirmatory testing; however, the N gene assay is slightly less sensitive than RdRp and E gene assays [7].

We conducted RT-PCR for SARS-CoV-2 RNA with a kit (Sun Yat-sen University). The ORF1ab and N regions are highly conserved among sarbecoviruses and are chosen for probe and primer design [6]. These assays have been evaluated using a

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panel of positive and negative controls (ORF1ab/N) [6]. Since the start of the COVID-19 pandemic, RNA kits have been unavailable or even inaccessible in certain countries. Several previous studies have established that TRIzol is an applicable material for RNA extraction from various biological samples [1,11].

The aim of the current study was twofold. Firstly, we examined the possibility of replacing an RNA extraction kit with a TRIzol-based RNA extraction method and using surrogate viral transport medium by TRIzol reagent for SARS-CoV-2 detection. Our protocol is based on purification of TRIzol-based COVID-19 RNA and comparison of RT-PCR results of patient samples collected by pharyngeal swab immersed in viral transport medium or directly immersed in TRIzol reagent.

Materials and methods

A total of nine patients positive for SARS-CoV-2 were included in this study after providing informed consent. The samples were collected from the surface of the respiratory mucosa with nasopharyngeal swabs during the clinical course in the infectious diseases department of the Bejaia hospital centre. SARS-CoV-2 was confirmed by positive nasopharyngeal swab at the time of hospital admission. For each volunteer, at the same time point, two nasopharyngeal swabs were collected. The first was immediately placed in a 15 mL cone with 2 mL of TRIzol reagent. The second was immersed in viral transport medium (Fig. 1).

RNA extraction

RNA extraction from samples was performed using three different procedures. In the first and second procedures, samples comprised nasopharyngeal swabs immersed in viral transport medium, and RNA was extracted by using TRIzol reagent (Invitrogen) or the QIAamp Viral RNA Mini Kit (Qiagen) as recommended by the manufacturer. Regarding the third procedure, the nasopharyngeal swabs were immersed directly in TRIzol RNA isolation reagent, and RNA was extracted by using the TRIzol method. Thus, we performed three RNA extractions for each patient. Following the manufacturer's instructions, the sample was incubated in 1 mL TRIzol for samples immersed directly in TRIzol or 750 µL TRIzol and 250 µL of viral transport medium for samples immersed in viral transport medium, followed by an addition of 200 µL chloroform and shaking for 15 seconds. Then the tube was centrifuged for 15 minutes at 20<thinsp>000*g* at 4°C. The clear upper aqueous layer, which contains RNA, was transferred to a new 1.5 mL tube, and 0.5 mL of isopropanol per millilitre of initial TRIzol was added. Gentle mixing by inverting 5 times was performed before incubating for 10 minutes at room temperature. The sample was centrifuged for 15 minutes at 20<thinsp>000*g* at 4°C. The supernatant was discarded, and the remaining pellet was resuspended in 1000 µL of 80% ethanol. The sample was again centrifuged for 5 minutes at 15<thinsp>000*g* at 4°C. The extra ethanol was removed, first with a 200 µL pipette and then with a 2 µL pipette. (The pellet should look glassy.) Total RNA isolated was eluted by 20 µL of nuclease-free water (Ambion). The RNA concentration and purity were determined with BioDrop (Biochrom) by calculating the ratio of optical density at wavelengths of 260/280 nm and 260/230 nm.

Real-time PCR

Quantitative RT-PCR was performed on the StepOne Plus real-time PCR system (Applied Biosystems) using a detection kit for SARS-CoV-2 RNA (Sun Yat-sen University, catalog no. DA-930). This kit is based on one-step RT-PCR technique with the following cycle parameters: 15 minutes at 50°C for reverse transcription, 15 minutes' inactivation at 95°C followed by 45 cycles of 15 seconds at 94°C and 45 seconds at 55°C. ORF1ab and N regions that are highly conserved among sarbecoviruses were selected for probe and primer designs [7]. Primers and fluorescent probes (the N gene probe is labeled with FAM and the ORF1ab probe with VIC) were specific for the detection of SARS-CoV-2. For the determination of results, the sample was considered positive for the novel 2019 coronavirus if it had an amplification curve in the FAM and if the VIC value had a $\leq$40 cycle threshold (Ct) value (Sun Yat-sen University, catalog no. DA-930).

Statistical analysis

Statistical comparisons using one-way analysis of variance followed by Bonferroni tests were performed and were further used to determine whether specific group mean differences were significant. The minimum alpha level of significance was set at 0.05. Data are presented as mean ± standard error.

Results and discussion

RNA concentration

Over the last two decades, the laboratory diagnostic methods for human coronavirus infections have developed considerably [5]. The primary (and preferred) method for diagnosis is the collection of upper respiratory samples via nasopharyngeal and oropharyngeal swabs [8,12]. TRIzol reagent (a phenol–guanidine isothiocyanate solution) is commonly used to isolate RNA from cells and tissues [13]. Moreover, phenol and guanidinium isothiocyanate are effective at inactivating

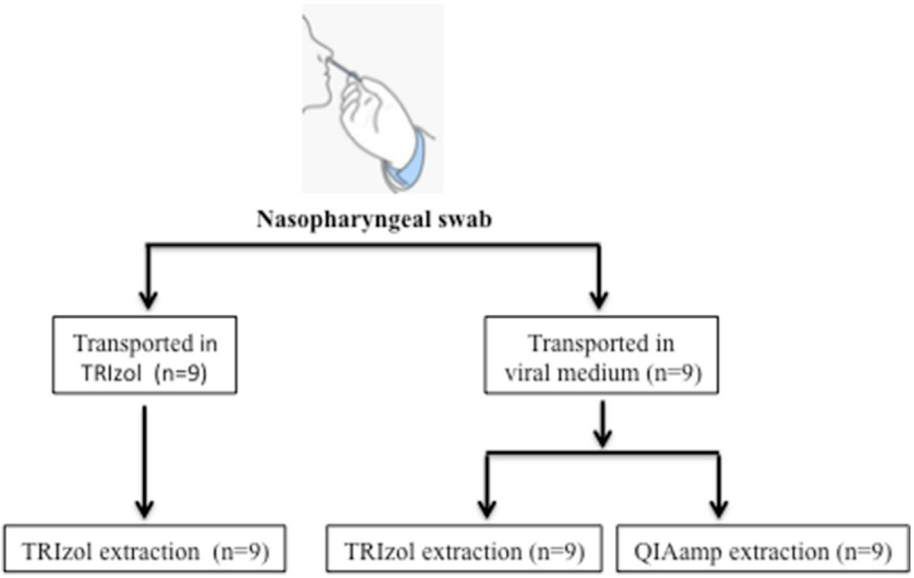

FIG. 1. Experimental design of different RNA extraction methods from human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) samples. SARS-CoV-2 was confirmed by positive nasopharyngeal swab specimen at hospital admission. For each volunteer, at the same time point, two nasopharyngeal swabs were collected. The first was immediately placed in a 15 mL cone with 2 mL of TRIzol reagent. The second was immersed in viral transport medium.

endogenous ribonucleases [13,14]. As expected, we found significant differences in yields of total RNA extracted using different methods (Table 1). Among the three RNA extraction methods, TRIzol reagent yielded the highest concentration of RNA (105 ± 20 ng/μL), followed by QIAamp Viral RNA Mini Kit (83 ± 11 ng/μL) and VMT-TRIzol (viral transport medium and TRIzol RNA extraction) (49 ± 10 ng/μL). Previous studies have clearly shown that TRIzol reagent yields a higher RNA concentration compared to a spin column–based method (RNeasy Mini Kit, Qiagen) [15–17].

Spectrophotometric evaluation of RNA extraction

We then proceeded to determine whether these differences in yields were without compromised RNA quality. Interestingly, the 260/280 absorbance ratio for samples prepared using the TRIzol method (average ratio, 1.77) was comparable with that of samples isolated using the QIAamp Viral RNA Mini Kit (average ratio, 1.86). Nonetheless, we did observe that the ratio 260/280 was significantly lower, at 1.7, using the VMT-TRIzol procedure, suggesting that this RNA is impure. It should be noted, however, that the use of the TRIzol method for purification increases sample handling time (45 minutes per ten samples) compared to the QIAamp method (30 minutes per ten samples). Our results complement several studies which previously demonstrated that TRIzol is considerably simpler, more convenient and more cost effective than commercially available kits [11]. A number of studies have shown that the use of TRIzol reagent has already been described for viral RNA extraction such as *Trichomonas vaginalis* [18], enterovirus A71 (EV-A71), coxsackievirus A16 (CA16) [19] and most recently SARS-CoV-2 [11].

RT-PCR and sensitivity detection

To assess virus replication, we performed quantitative RT-PCR to detect viral RNA in clinical samples. SARS-CoV-2 was detected by RT-PCR assay using a detection kit for 2019 novel RNA according to the manufacturer's protocol (Sun Yat-sen University). A SARS-CoV-2–positive clinical sample was used to evaluate the sensitivity of SARS-CoV-2 detection assays. All samples were distinguished by a corresponding Ct value. Negative controls gave no Ct value, and positive controls gave

TABLE 1. **RNA yield, purity and time to handle different RNA extraction methods from human sample severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)**

Method	RNA yield (ng/μL)	RNA purity (260/280)	Time per ten samples (minutes)
TRIzol (n = 9)	104.50 ± 19.51	1.77 ± 0.21	45
VMT-QIAamp (n = 9)	82.67 ± 11.12	1.86 ± 0.17	30
VMT-TRIzol (n = 9)	49.83 ± 10.76	1.68 ± 0.22	45

Abbreviations: TRIzol, TRIzol reagent as transport medium and for RNA extraction; VMT-QIAamp, viral transport medium and QIAamp kit for RNA extraction; VMT-TRIzol, viral transport medium and TRIzol for RNA extraction.

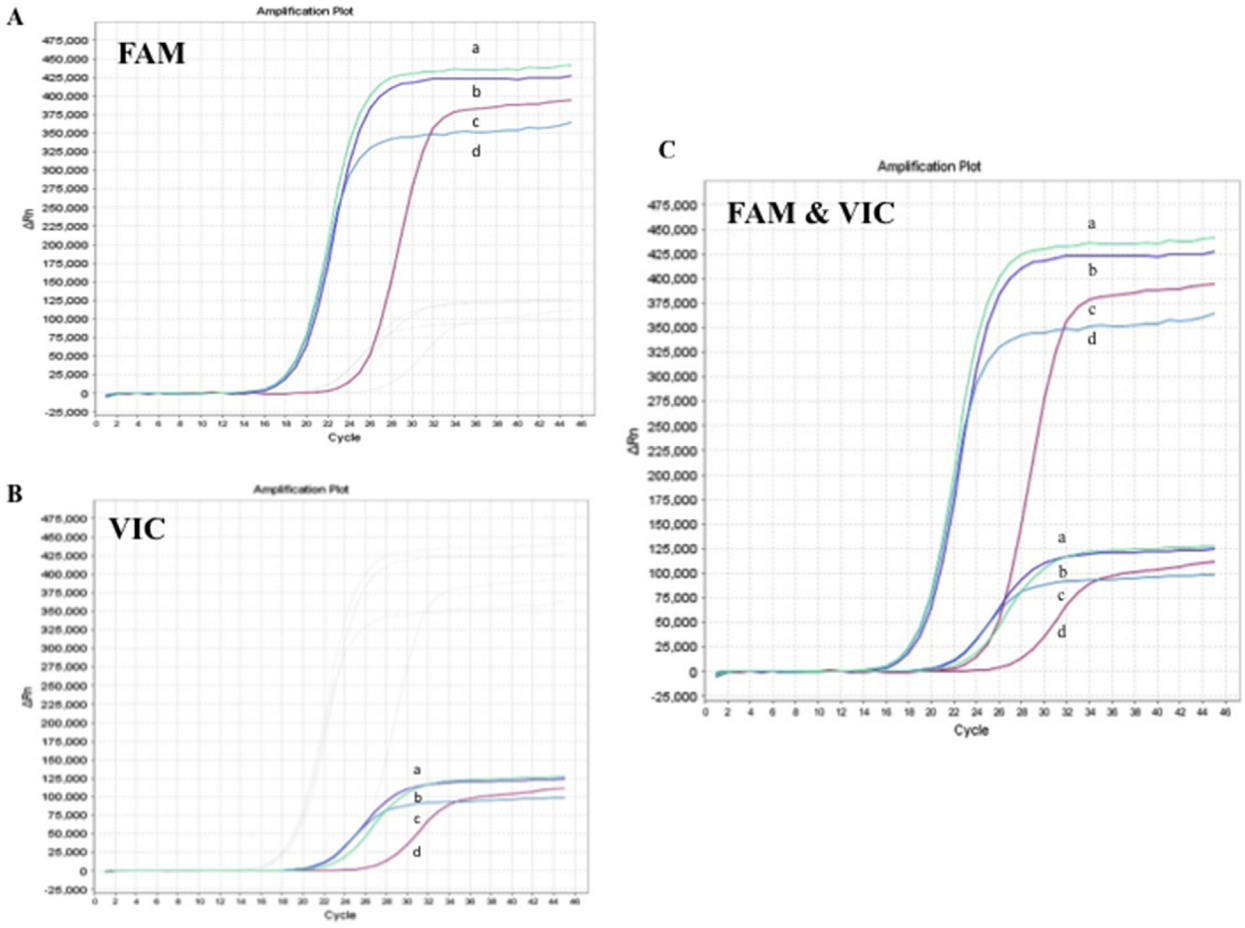

FIG. 2. Real-time reverse transcriptase PCR (RT-PCR) results for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing coronavirus disease 2019 (COVID-19). (A) FAM (N gene) quantitation. (a) RNA extraction and transport medium used TRIzol. (b) QIAamp kit for RNA extraction. (c) Viral transport medium with TRIzol RNA extraction. (d) Positive control. (B) VIC (ORF1ab) quantitation. (a) RNA extraction and medium transport were performed with TRIzol. (b) QIAamp RNA extraction with viral transport medium. (c) TRIzol extraction with viral transport medium. (d) Positive control. (C) FAM and VIC quantitation. (a) RNA extraction and medium transport were performed with TRIzol. (b) QIAamp RNA extraction with viral transport medium. (c) TRIzol extraction with viral transport medium. (d) Positive control. Three RNA extractions were performed for each patient.

Ct values of $\leq$32 for both assays, FAM (N gene) and VIC (ORF1ab), as per the manufacturer's recommendations (Sun Yat-sen University). As expected, the Ct values of the positive controls were 25.70 and 26.48 respectively for the FAM and VIC assays (Figs. 2(A) and (B)). Our result was in perfect agreement with the manufacturer's recommendations. We then tested the effectiveness of the three different procedures (TRIzol, QIAamp, VMT-TRIzol) for SARS-CoV-2 RNA extraction. We found no significant difference between Ct values of samples extracted with the three procedures (Fig. 3). Our findings are consistent with those observed by other approaches, such as computed tomographic scan and rapid diagnostic test [20]. Overall, we compared three RNA extraction methods from human SARS-CoV-2 samples for the N and ORF1ab genes, reporting nine positive results for the two marker genes, with positivity confirmed by all three RNA extraction methods.

More specifically, samples were detected as positive with a Ct value around 25.13 ± 5.17 for the FAM assay and 27.09 ± 4.98 for the VIC assay (Figs. 2(C), Fig. 3). In line with previous observations, the N gene assay is more sensitive than the ORF1ab gene assay in detecting positive clinical specimens [7]. Moreover, Chu *et al.* [7] reported that the N gene assay is about 10 times more sensitive than the ORF1ab gene assay in detecting positive clinical specimens. In their model, the ORF1ab assay is recommended as a confirmatory test, and the N gene RT-PCR is recommended as a screening assay [7,21]. Won *et al.* [11] established a RT-PCR–based assay protocol

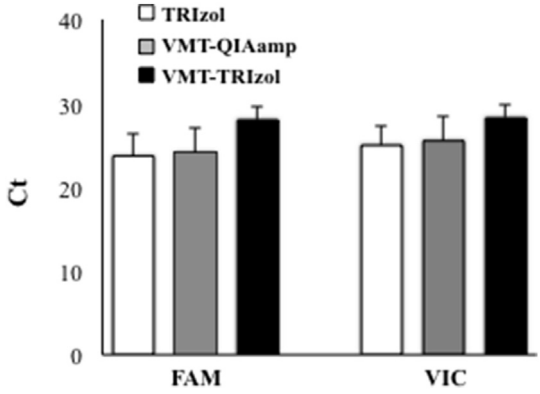

FIG. 3. Determination of detection efficiency of real-time reverse transcriptase PCR (RT-PCR). Average cycle threshold (Ct) values were compared between different procedures. Abbreviations: TRIzol, TRIzol reagent as transport medium and for RNA extraction; VMT-QIAamp, viral transport medium and QIAamp kit for RNA extraction; VMT-TRIzol, viral transport medium and TRIzol for RNA extraction. Values are expressed as mean ± standard error.

composed of easy specimen self-collection from a subject via pharyngeal swab, with TRIzol-based RNA purification and SYBR Green–based RT-PCR. In agreement with our data, this recent study demonstrated that TRIzol is equally sensitive as the commercially available kit [11].

Conclusion

This study contributes to the knowledge of the extraction of SARS-CoV-2 RNA by a TRIzol-based method, the results of which support an expansion of the potential application of diagnostic modalities. These results combine biosafety with a high-sensitivity detection protocol for SARS-CoV-2. Our protocol permits easy, safe handling of SARS-CoV-2. The use of TRIzol should be considered whenever extraction kits are unavailable.

Conflict of interest

None declared.

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