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Diagnostic performance of RNA extraction-free dilution and heating method for the detection of severe acute respiratory syndrome coronavirus 2

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Abstract

Real-time quantitative polymerase chain reaction remains the gold standard for COVID-19 diagnosis, but RNA extraction is time-consuming, expensive, and associated with increased biosafety requirements. This study evaluated an extraction-free dilution and heating (EFDH) method as a simplified alternative to conventional extraction-based (EB) real-time quantitative polymerase chain reaction for severe acute respiratory syndrome coronavirus 2 detection. A total of 300 archived nasopharyngeal specimens, including 190 positives and 110 negatives, from the National Virology Reference Laboratory at the Ethiopian Public Health Institute, were analyzed. Samples were diluted 1:2 with RNase-free water, heated at 72°C for 15 min, and analyzed using an ABI 7500 Fast instrument. The EFDH method showed a sensitivity of 92%, a specificity of 100%, and an overall accuracy of 85.8%, producing 15 false-negative and 12 invalid results. Agreement with the EB method was high, with 95% concordance and a kappa coefficient of 0.89. Performance was strongest in samples with high viral loads (cycle threshold [Ct] < 20) and declined in those with low viral loads (Ct > 35). A significant correlation was observed between the two methods ($R^2 = 0.99$, p=0.001). These findings indicate that the EFDH approach reliably detects moderate-to-high viral loads and may serve as a practical testing option in resource-limited settings, especially during outbreaks when rapid and simplified workflows are needed.

Keywords: SARS-CoV-2; Extraction free; Heating; Dilution; Diagnostic performance

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1. Introduction

Coronaviruses belong to the *Coronaviridae* family. They are RNA viruses with an enveloped structure and a positive single-stranded genome. Their genome contains over 29,891 nucleotides, which encode for 9,890 amino acids. It also contains numerous

open-reading frames (ORFs) that encode both structural and non-structural proteins. These proteins play crucial roles in the viral life cycle and pathogenic processes.[1] The structural proteins consist of S (spike protein), E (envelope protein), M (membrane protein), and N (nucleocapsid protein). Furthermore, there are 16 non-structural proteins that assist in viral metabolism and interaction with the host immune system.[2] The genetic information of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent for Coronavirus Disease 2019 (COVID-19), plays a multifaceted role in diagnostic tool development, encompassing rapid assay development, enhanced detection sensitivity, variant identification, and informing public health responses.[3,4] Its significance underscores the critical intersection of genetics, diagnostics, and public health in effectively managing infectious disease threats.[4]

In viral outbreaks such as COVID-19, it is crucial to effectively manage and control the pandemics.[5] It is reported that up to 40% of individuals with SARS-CoV-2 infection may be asymptomatic or pre-symptomatic, yet have the potential to spread the virus.[6] Therefore, a dependable laboratory diagnosis is the key to preventing and controlling infections.[7]

COVID-19 can be diagnosed primarily by either immunological or molecular tests. Immunological tests include serological tests that detect antibodies (immunoglobulin [Ig]M and IgG) in blood or viral antigens in respiratory secretions obtained through nasal or throat swabs. These tests can be performed using point-of-care platforms, rapid testing systems that provide results within minutes and are particularly useful in non-laboratory settings. Serological tests are particularly important for diagnosing patients with mild to moderate disease when molecular diagnostics are not available. Serological tests play a crucial role in identifying individuals who have developed immunity against COVID-19.[8] On the other hand, molecular tests detect SARS-CoV-2 RNA in various specimens, primarily nasopharyngeal samples. Unlike serological tests, most molecular tests require specialized laboratory infrastructure, including advanced equipment and highly trained staff. This limitation restricts their widespread use compared to simpler immunological tests.[9] In addition to these primary diagnostic approaches, other laboratory parameters have been utilized to monitor patients with COVID-19.[10-12]

The specimen of choice for SARS-CoV-2 detection can be swabs from the nasopharynx, oropharynx, and anus, as well as saliva and sputum samples. In addition, the virus can be present in other bodily fluids, such as blood, urine, and feces.[13] The nasopharyngeal swab collection technique is among the most commonly used methods, with sensitivities reaching up to 98%.[14] Nasopharyngeal swabbing is regarded as the standard method for safely and conveniently collecting SARS-CoV-2 samples, as it minimizes direct exposure of the healthcare provider to respiratory secretions.[15]

The "gold standard" assay for diagnosing both symptomatic and asymptomatic cases is real-time quantitative polymerase chain reaction (RT-qPCR).[16] The first protocol, recommended by the World Health Organization (WHO), was published in 2020 by the Charité Institute at Berlin University in Germany.[4] This protocol utilizes TaqMan technology and specific primers and probes to detect the RNA-dependent RNA polymerase (*RdRp*), *E*, and *N* genes. In addition, Table 1 lists several in-house methods reported by WHO that are currently being validated in partner laboratories.[4]

Typically, three steps should be completed before conducting quantitative polymerase chain reaction (qPCR). These steps include: (i) purifying total RNA from the sample, (ii) eluting and concentrating the material, and (iii) synthesizing complementary DNA (cDNA) from the template RNA.[17] However, it is important to note that RNA extraction can be a labor-intensive, expensive, and time-consuming process that requires manual handling and carries a risk of exposure to infectious material, which may also introduce experimental errors.[17,18]

The process begins with the isolation of viral RNA from collected specimens, typically performed using commercially available RNA extraction kits. Proper handling during this step is essential to ensure accuracy and to prevent contamination. Once isolated, the RNA undergoes reverse transcription to synthesize cDNA. This

Table 1. List of real-time polymerase chain reaction protocols indicated by the World Health Organization

Institute	Gene targets
China CDC, China	*ORF1ab* and *N*
Institute Pasteur, France	Two targets in *RdRP*
United States CDC, USA	Three targets in the *N* gene
National Institute of Infectious Diseases, Japan	Multiple targets in coronavirus panel
Charité, Germany	*RdRP*, *E*, *N*
Hong Kong University, Hong Kong SAR	*ORF1b-NSP14*, *N*
National Institute of Health, Thailand	*N*

Abbreviations: CDC: Centers for Disease Control and Prevention; SAR: Special administrative region.
Source: https://www.who.int/who-documents-detail/molecular-assays-to-diagnose-COVID-19-summary-table-of-available-protocols.

step uses a master mix containing reverse transcriptase enzyme and is carried out in a thermal cycler to initiate the reaction. The resulting cDNA is then prepared for qPCR by mixing it with primers, distilled water, and either a DNA probe or SYBR Green dye. This mixture is then pipetted into a 96-well plate, which is subsequently placed in an RT-qPCR instrument programmed with the appropriate thermal cycling settings. To ensure reliable results, a negative control is included to confirm the absence of contaminants, and a positive control is used to validate the assay accuracy.[19]

The qPCR amplification process involves three key steps: denaturation, annealing, and extension. During denaturation, the temperature is raised to denature double-stranded DNA (dsDNA) into single strands. Annealing follows, during which the temperature is lowered to allow primers to bind to their specific target sequences on the DNA. In the extension step, DNA polymerase attaches nucleotides to synthesize the cDNA strand, completing the replication process. These three steps are repeated approximately 40 times, doubling the DNA in each cycle. A fluorescent signal is generated when either DNA probes are cleaved or SYBR Green binds to newly formed dsDNA. The intensity of this fluorescence increases with the number of DNA copies, enabling real-time monitoring of the amplification process. The cycle threshold (Ct) value is determined by the number of cycles needed for fluorescence to surpass a predetermined threshold and provides quantitative insights into the amount of target nucleic acid present. Lower Ct values indicate higher viral loads, while higher Ct values indicate lower viral loads.[19,20]

Real-time qPCR was the first standard molecular technique for SARS-CoV-2 detection since the emergence of COVID-19. This molecular diagnostic technique identifies nucleic acids by targeting and amplifying specific genes, producing millions of copies from a small initial sample. This amplification is monitored in real time, enabling precise and timely analysis.[19,21,22] Primers play a critical role in this process by binding specifically to the target gene. To ensure accuracy, primers must be well-designed with high specificity to avoid non-specific amplification or the formation of unwanted structures, such as primer dimers, which can lead to false-positive results.

Despite its effectiveness, RT-qPCR often exhibits variability in performance, largely influenced by the primers and probes used.[23,24] False results in viral polymerase chain reaction (PCR) testing often stem from a mix of biological, technical, and procedural issues. False negatives are especially common when sample quality is poor, viral load is low, or RNA extraction is inefficient. Problems during collection, handling, storage, or transport can further degrade the sample. Contaminants in clinical specimens may inhibit PCR, while cross-contamination during processing can lead to false positives. Mutations in primer or probe binding sites, as well as faulty equipment or reagents, also contribute to inaccurate results.[25,26] On the other hand, false positives may occur due to contamination, cross-reactivity with non-target organisms, or detection of residual viral material post-infection.[27] RNA extraction is a major bottleneck in the workflow due to its labor-intensive and specialized nature, particularly during high-demand periods such as the COVID-19 pandemic. The global shortage of RNA extraction kits has further strained testing capacities. However, numerous countries have adopted in-house RT-qPCR protocols as their standard diagnosis tool. This highlights the need for new diagnostic platforms that are fast, sensitive, and accessible to address this problem.[28]

To address the challenges in conventional RT-qPCR, it is crucial to develop novel testing methods that do not require advanced resources or extended processing time. Ideally, a test that delivers results within 2–3 h would support timely preventive measures, as patients could immediately receive the necessary support and care.[29] Globally, various direct approaches that avoid RNA extraction have been suggested, including heat-processed methods.[30,31] Our method is novel in that it does not use chelating agents, proteinase K, or other additional chemicals. We use heat and dilution to reduce the cost of the RNA extraction step. The cost reduction is particularly important in low-income countries such as Ethiopia when testing large numbers of samples. This consideration provided the main rationale for the present study. The objective of this study is to compare the diagnostic performance of our extraction-free dilution and heating (EFDH) method with that of extraction-based methods for SARS-CoV-2 detection.

2. Materials and methods

2.1. Study population, sample size calculation, and selection of stored specimens

Individuals tested for SARS-CoV-2 during the fifth wave of the pandemic (June–August 2020) were considered the study population. The study involved random selection and retrieval of stored nasopharyngeal specimens. The sample size was calculated using the double-population proportion formula, yielding 300 samples. Accordingly, a total of 300 samples (190 positive and 110 negative) were retrospectively obtained from the National Virology Reference Laboratory at the Ethiopian Public Health Institute in Addis Ababa, Ethiopia, which serves as the first national COVID-19 testing laboratory. They were

initially collected for clinical diagnosis of COVID-19 in viral transport media. The samples were retrieved using the unique numbers assigned to each specimen in the laboratory register. These specimens were retested using the EFDH technique, and the results were compared with those from the extraction-based detection method. The clinical samples were handled and processed in a Biosafety Level 2 laboratory at the virology laboratory.

2.2. Method validation

We intentionally chose six specimens with an average Ct value of 19 and an internal control (with Victoria [VIC] dye) having an average Ct value of 19. These specimens were diluted 1:2 and 1:4 with RNase-free water. The diluted specimens were then heated at 62°C and 72°C for 10, 15, 20, and 30 min, and at 96°C for 5, 10, 15, and 20 min using an Eppendorf thermomixer. Considering these heated and diluted specimens as an RNA eluate, we added 10 µL of the eluate to 20 µL of master mix and performed RT-qPCR on a thermal cycler (ABI 7500, Thermo-Fisher Inc., United States of America) using DAAN detection kits (Cat. no. DA0932, Daan Gene Co., Ltd, China). For the SARS-CoV-2 DAAN detection kits, the primary targets are the *N* and *ORF1ab* genes. The kit targets the *N* and *ORF1ab* genes, with the *N* gene detected on the FAM channel, the *ORF1ab* gene on the VIC channel, and the internal control on the Cy5 channel. These genes are crucial for detecting and identifying SARS-CoV-2 using PCR-based assays. The results show that after 15 min and 30 min incubation at 72°C, the average *N* and *ORF1ab* Ct values were 24 and 26, respectively; and the VIC average values were 26 and 27, respectively. The validation experiment showed that 1:2 diluted specimens exposed to 72°C for 15 min yielded results comparable to the standard RNA extraction-based method for SARS-CoV-2 detection. Therefore, we evaluated this EFDH method on the 300 samples using a 1:2 dilution and exposure at 72°C for 15 min.

2.3. Extraction-based protocol (reference method)

Extraction was performed manually using the QIAamp Viral RNA Kit (Cat. no. 52904, QIAGEN, Germany) following the standard protocol.[32] The process began with sample lysis under highly denaturing conditions, effectively inactivating RNases and protecting the integrity of viral RNA. These conditions ensured that the RNA remained intact throughout the extraction process. Next, the buffering conditions were carefully adjusted to facilitate the binding of RNA to the specialized membrane of the QIAamp plates. This step was crucial for capturing the RNA while preventing the co-binding of contaminants. To ensure the removal of impurities, the bound RNA underwent a series of washes using two wash buffers followed by a final ethanol wash. These steps effectively eliminated contaminants, including proteins, nucleases, and other potential inhibitors, yielding high-purity RNA. The purified RNA was then eluted using a specially formulated RNase-free buffer, making it immediately available for downstream applications or safe for long-term storage without degradation. The unique QIAamp membrane played a pivotal role in the process, ensuring exceptionally high recovery rates of pure and intact RNA. The methodology avoided the use of phenol/chloroform extraction or alcohol precipitation, making it a safe and efficient alternative for obtaining high-quality RNA suitable for sensitive molecular diagnostic procedures.

2.4. PCR reagent preparation (master mix preparation)

The PCR solutions A and B were thawed at room temperature and thoroughly mixed. The solution was then centrifuged at 8,000 rpm for a few seconds. The number of specimens to be tested, along with the number of *ORF1ab* and *N* negative controls and positive controls, was labeled on the PCR tubes. Next, 20 µL of the solution was aliquoted into each PCR tube, then 5 µL of the negative control material, RNA from the specimens to be tested, or positive control material was added to the respective PCR tubes. After securely covering the tubes, they were transferred to the amplification detection area following a brief centrifugation at 8,000 rpm. Finally, the reaction tubes were placed in the instrument's sample sink.

2.5. EFDH protocols (index method)

Each sample (100 µL) was transferred to a heat-resistant 1.5 mL microcentrifuge tube and heated at 72°C for 15 min using a thermomixer (Eppendorf, Germany). The eluates were then stored at −20°C for up to 4 h while waiting for other clinical samples to be processed in batches. Afterward, 10 µL of the eluate was mixed with 20 µL of the master mix, following a procedure similar to that for the extraction-based eluted samples. RT-qPCR was then performed on a thermocycler (ABI7500, Thermo-Fisher Inc., United States of America) following the protocol as shown in Figure 1.

2.6. Detection of SARS-CoV-2 (RT-qPCR)

The detection of SARS-CoV-2 using RT-qPCR was performed using a thermocycler (ABI7500, Thermo-Fisher Inc., United States of America), along with the DAAN kits (Cat. no. DA0932, Daan Gene Co., Ltd, China). The protocol consisted of a single cycle of reverse transcription at 50°C for 15 min, followed by a single cycle of polymerase activation at 95°C for 2 min. This was followed by 40 amplification cycles, each consisting of

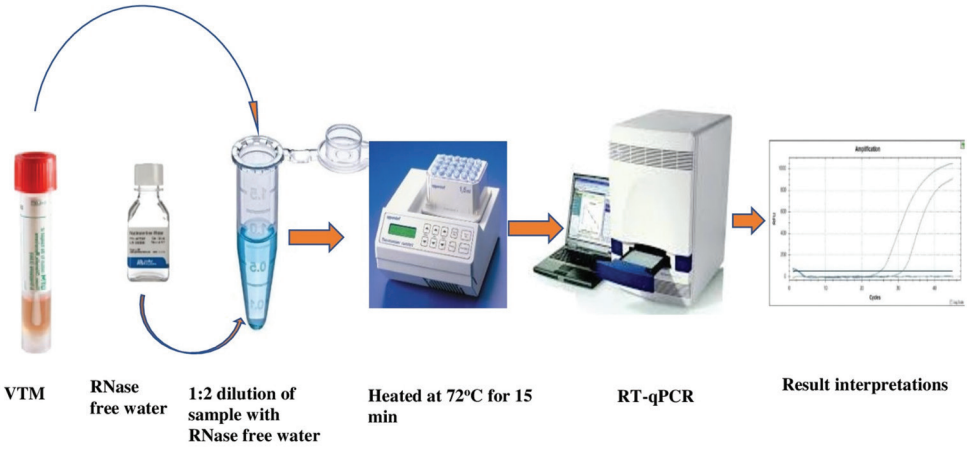

Figure 1. Process of sample dilution and heating for the detection and interpretation of severe acute respiratory syndrome coronavirus 2
Abbreviations: RT-qPCR: Real-time quantitative polymerase chain reaction; VTM: Viral transport media.

15 s at 95°C and 60 s at 60°C. The purpose of this assay was to detect the presence of SARS-CoV-2 using primers and probes targeting the *N* and *ORF1ab* gene. For comparison, the standard EB technique served as the reference method, alongside the EFDH method. The RT-qPCR results were then interpreted. A Ct value greater than 40, or the absence of an amplification curve in both FAM and VIC channels, indicated the absence of SARS-CoV-2 RNA in the sample. Conversely, a clear amplification curve in the VIC channel with a Ct value of 40 or less indicated a positive result. Samples with borderline amplification or ambiguous VIC signals were retested, and the result of the repeat run was considered final. The repeated result was considered the final result. At each step of both methods, strict quality control measures were taken. In addition to the positive and negative controls provided in the kit for the extraction method, we also used an extraction control. All measuring devices in the laboratory, including the thermal cycler, were within their calibration dates.

2.7. Ethics statement

This study was reviewed and approved by the Institutional Review Board of Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University in Ethiopia, under protocol number ALIPB IRB/52/2013/21. A permission letter was obtained from the Ethiopian Public Health Institute with reference number WG12/19 on April 21, 2021. The studies were conducted in accordance with the local legislation and institutional requirements.

2.8. Statistical analysis

The data were summarized as mean ± standard error of the mean (SEM) and coefficient of determination (R^2). The D'Agostino and Pearson test, using a 95% confidence interval (CI), was used to assess normality. CIs for sensitivity, specificity, positive and negative predictive values, and accuracy were calculated using R software (v.3.6.0, R Foundation for Statistical Computing, Austria). The Wilcoxon signed-rank test was used to compare the two groups. The correlation between the Ct values of the two techniques was determined with the two-tailed Pearson's correlation coefficient using STATA (v17, StataCorp LP, United States of America). A $p<0.05$ indicated statistical significance. The Ct values were grouped accordingly (Ct < 20, 20–35, and >35), and performance characteristics in each group were compared.

3. Results

The EFDH method detected 85.8% (163/190) of the positive samples and 100% (110/110) of the negative samples. However, 15 false-negative results were recorded, most of which had average Ct values >36 by the extraction-based detection method. In addition, the EFDH method yielded 12 invalid results where the internal control did not amplify. To compare the two methods, we excluded the invalid results, using 178 positive and 110 negative results detected by the extraction-based detection method, as shown in Table 2.

The extraction-based method yielded a mean Ct value of 24.1 (95% CI: 23.3–24.8) with an SEM of 0.4. This method detected a range of Ct values from 16.0 to 34.1. On the other hand, the EFDH method yielded an average Ct value of 27.7 (95% CI: 26.8–28.7) with an SEM of 0.5. This method also detected a range of Ct values from 18.0 to 38.9. The true prevalence among study participants was 62% (178/288) (95% CI: 56–67%). The apparent prevalence, defined as the proportion of true positives out of the total tested, was 57% (163/288) (95% CI: 51–62%).

3.1. Diagnostic performance of the EFDH method

The EFDH method showed an overall sensitivity and specificity of 92% (95% CI: 86–95%) and 100% (95% CI: 97–100%), respectively. The positive predictive value was 100% (95% CI: 97–100%), while the negative predictive value was 88% (95% CI: 81–93%). The negative likelihood ratio, which measures the ability of a test to rule out a disease, was found to be 0.08 (95% CI: 0.05–0.14). The study also revealed an overall agreement (accuracy) of 95% (95% CI: 92–97%) for the EFDH method, with a kappa value of 0.89 (p<0.001) (Table 3).

The correlation between the two methods is represented by Equation (1):

$$Y = -0.12+1.16\ X \quad (1)$$

With R^2 = 0.99, p=0.001, showing a strong and significant linear relationship between the extraction-based and EFDH detection methods. For every unit increase in the Ct value by the extraction-based detection methods, the Ct value by the EFDH detection method increases by approximately 1.16 units (Figure 2). The Wilcoxon signed-rank test (z = 11.08, p=0.0001) indicated a statistically significant difference in Ct values between the extraction-based and EFDH detection methods. The extraction-based method generally produces lower Ct values compared to the EFDH method across all observations.

Table 2. Comparison of the extraction-based method and the extraction-free dilution and heating (EFDH) methods

EFDH	Extraction-based method		
	Positive	Negative	Total
Positive	163	0	163
Negative	15	110	125
Total	178	110	288

Note: Pearson's χ^2<0.001.

Table 3. Performance characteristics of the extraction-based and extraction-free dilution and heating methods for severe acute respiratory syndrome coronavirus in Ethiopia

Statistic	Value (%)	95% CI
Apparent prevalence	57	51–62%
True prevalence	62	56–67%
Sensitivity	92	86–95%
Specificity	100	85–100%
Negative likelihood ratio	0.08	0.05–0.14
Positive predictive value	100	98–100%
Negative predictive value	88	81–93%
Accuracy/agreement	95	92–97%

Abbreviation: CI: Confidence interval.

3.2. Comparison of performance characteristics in terms of cycle threshold value

Regarding the samples with a Ct value <20 (38 samples), both techniques correctly identified all samples as true positives. Of the 124 results with a Ct value ranging from 20 to 35, 121 (98%) were true positives. For the remaining 119 test results with Ct values higher than 35, the EFDH method yielded nine false negatives. These findings demonstrated that both techniques were highly sensitive to the Ct < 20 group, accurately identifying all samples as positive. In the 20–35 Ct value group, both techniques detected most of the positive samples, but the EFDH method showed 2% false-negative results (3 samples). Finally, the EFDH technique showed low sensitivity in the group with Ct > 35. These results suggested that the EFDH method is more reliable for detecting SARS-CoV-2 in samples with higher viral loads (Table 4).

4. Discussion

We compared the EFDH method to the standard extraction-based SARS-CoV-2 detection method. The EFDH method showed high sensitivity, specificity, and positive and negative predictive values, especially at high SARS-CoV-2 viral loads. In addition, the EFDH method showed a remarkable agreement and correlation with the extraction-based method. The mean Ct values for the EFDH method were slightly higher compared to the extraction-based method; however, they were generally comparable to the gold standard method. The findings

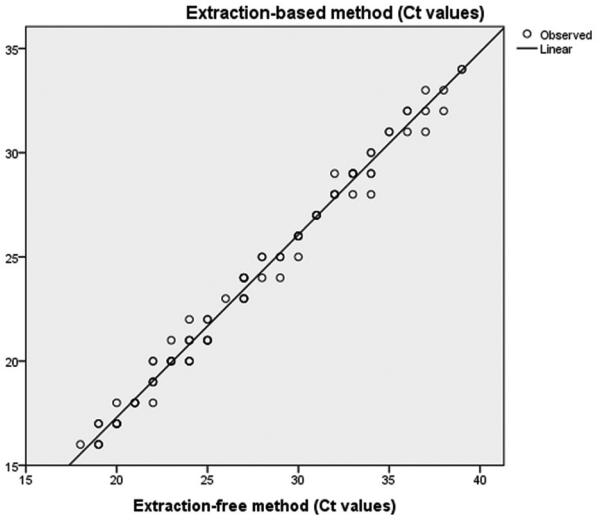

Figure 2. Correlation between extraction-free heating and dilution method and extraction-based severe acute respiratory syndrome coronavirus 2 detection methods in Ethiopia

Table 4. Performance of extraction-free and extraction-based methods across different ranges of cycle threshold value

Extraction-free method	Extraction-based method		
	Positive	Negative	Total
Ct<20			
Positive	38	0	38
Negative	0	0	0
Total	38	0	38
Ct=20–35			
Positive	121	0	121
Negative	3	0	3
Total	124	0	124
Ct>35			
Positive	0	0	0
Negative	9	110	119
Total	9	110	119

Abbreviation: Ct: Cycle threshold.

suggest that the EFDH method is particularly reliable for detecting SARS-CoV-2 in samples with higher viral loads. Despite limitations in samples with low viral loads or high Ct values, the EFDH method demonstrated overall high accuracy and closely aligned with the extraction-based method, indicating a strong performance and reliability.

In this study, the performance of the EFDH method was comparable to that of the extraction-based method. The EFDH method demonstrated good sensitivity and specificity. This finding aligns with a study conducted in India, which reported an overall sensitivity of 79% (95% CI: 71–86%) and specificity of 99% (95% CI: 98–100%).[33] Another study on extraction-free, multiplexed amplification of SARS-CoV-2 demonstrated a sensitivity of 86% and specificity of 100%.[34] Similarly, a study conducted at the Karolinska University Hospital in Stockholm, Sweden, in 2020, using a heating technique at 95°C for 5 min, reported a sensitivity of 96.0% and specificity of 99.8%.[17] In contrast, a study conducted in India and Italy reported lower overall sensitivity, specifically 78.9% (95% CI: 71–86%) and 57.3% (95% CI: 47.3–66.8%), respectively. However, both studies reported comparable specificity, at 99.9% (95% CI: 98–99.6%) and 100% (95% CI: 94.4–100%), respectively.[35]

This study reports higher positive and negative predictive values compared to previous studies. These findings indicate excellent specificity in identifying true positives, thus enhancing the reliability of the method for confirming the presence of the diseases. However, they also highlight the need to reduce false-negative rates, which would further enhance the method's utility for ruling out infection. False viral PCR results are caused by a combination of procedural and biological factors, often due to poor sample quality, low viral load, or inefficient RNA extraction, and may also arise from issues during specimen storage. In addition, contaminants in the sample can inhibit PCR amplification and compromise detection.[29,30] Similar findings were reported in a 2021 study conducted in the United Arab Emirates (UAE), showing positive and negative predictive values of 92% and 91%, respectively.[36] The finding aligns with another 2021 study in India, with positive and negative predictive values of 92% and 97%, respectively.[33]

In this study, the overall agreement between the extraction-based and EFDH methods was found to be 95%, with a kappa value of 0.89 (p=0.000). This indicates a high level of reliability in detecting SARS-CoV-2 infections. Similar findings were reported in a study conducted in India, where the agreement was 96.8% (k = 0.83, SEM = 0.03).[36]In addition, a study conducted in Sweden showed an accuracy of 98.8% (95% CI: 97.5–99.5%),[17] while a study in the UAE reported an overall agreement (kappa coefficient) of 0.797 (p<0.001).[36] However, different findings were reported from a study conducted at the clinical laboratory of the Institut Pasteur of M'sila, Algeria, where the overall agreement between extracted and heat-inactivated (65°C for 30 min) samples was only 45%, with a 95% CI of 37–52%.[37] In the current study, a near-perfect correlation (R^2 = 0.99, p=0.001) was found between the extraction-based and EFDH methods, supporting the consistency and reliability of the EFDH method in detecting SARS-CoV-2. These findings are further supported by a 2020 study conducted in Singapore, which showed an R^2 of 0.9986 for the detection of SARS-CoV-2 without extraction. Similar results were reported from the Karolinska University Hospital in Stockholm, Sweden, with an R^2 of 0.987.[17]

The consistent findings across studies utilizing the extraction-free method for SARS-CoV-2 detection can be attributed to the method's simplicity, the inherent properties of SARS-CoV-2, and its validation against established standards. These factors contribute to the method's robustness and reliability in detecting SARS-CoV-2, making it a valuable tool in overcoming the COVID-19 pandemic.[38] Despite the challenges, the technique's overall high performance supports its continued use in surveillance and outbreak control efforts. However, the potential for false negatives emphasizes the importance of comprehensive testing strategies, including targeted testing of high-risk populations and asymptomatic individuals.[39] This method's advantages in terms of speed and simplicity, combined with its high sensitivity and specificity, make it a compelling alternative for SARS-CoV-2 detection, especially in resource-limited settings or during rapid response scenarios.[40]

These findings highlight the strengths and limitations of the extraction-free method in detecting SARS-CoV-2 across different viral loads. Notably, the method demonstrated high sensitivity and specificity in samples with lower Ct values (<35). However, its performance decreases in detecting samples with low viral loads (higher Ct values), as evidenced by an increased rate of false negatives. A study conducted in Austria to evaluate extraction-free RT-qPCR methods for SARS-CoV-2 diagnostics showed an 8.2% false-negative rate for the EFDH method in samples with a Ct value >30.[38] Another study from Algeria on the detection of SARS-CoV-2 using heat inactivation at 65°C for 30 min correctly identified 100% of clinical samples with a high viral load (Ct value <30).[37] Accurate detection of SARS-CoV-2, even at high viral loads (lower Ct value), is crucial for early case identification, contact tracing, and isolation measures.

5. Limitations

The study utilized previously stored samples instead of freshly collected ones and compared the retest results with the initial findings. It did not assess the performance of the EFDH method across different storage conditions and time intervals. Furthermore, it did not determine the method's limit of detection.

6. Conclusion

The findings suggest that the EFDH method may serve as a reliable option for detecting SARS-CoV-2 in samples with low Ct values, corresponding to higher viral loads. The EFDH method offers a potential advantage in settings where resources are limited or rapid turnaround times are required. Despite the promising results, the study highlights areas for improvement. The number of invalid and false-negative results obtained with the EFDH method necessitates further investigation into the causes of these failures and the development of new strategies to mitigate them.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. Ethical review and approval were obtained from the Institutional Review Board of the Aklilu Lemma Institute of Pathobiology at Addis Ababa University, under protocol number ALIPB IRB/52/2013/21. A permission letter was obtained from the Ethiopian Public Health Institute to use leftover samples from repositories.

Consent for publication

Not applicable.

Availability of data

Data used in this work is available from the corresponding author upon reasonable request.

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