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## Evaluation of recombinase-based isothermal amplification assays for point-of-need detection of SARS-CoV-2 in resource-limited settings

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### ABSTRACT

**Objectives:** The democratization of diagnostics is one of the key challenges towards containing the transmission of coronavirus disease 2019 (COVID-19) around the globe. The operational complexities of existing PCR-based methods, including sample transfer to advanced central laboratories with expensive equipment, limit their use in resource-limited settings. However, with the advent of isothermal technologies, the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is possible at decentralized facilities.

**Methods:** In this study, two recombinase-based isothermal techniques, reverse transcription recombinase polymerase amplification (RT-RPA) and reverse transcription recombinase-aided amplification (RT-RAA), were evaluated for the detection of SARS-CoV-2 in clinical samples. A total of 76 real-time reverse transcription PCR (real-time RT-PCR) confirmed COVID-19 cases and 100 negative controls were evaluated to determine the diagnostic performance of the isothermal methods.

**Results:** This investigation revealed equally promising diagnostic accuracy of the two methods, with a sensitivity of 76.32% (95% confidence interval 65.18–85.32%) when the target genes were RdRP and ORF1ab for RT-RPA and RT-RAA, respectively; the combination of N and RdRP in RT-RPA augmented the accuracy of the assay at a sensitivity of 85.53% (95% confidence interval 75.58–92.55%). Furthermore, high specificity was observed for each of the methods, ranging from 94.00% to 98.00% (95% confidence interval 87.40–9.76%).

**Conclusions:** Considering the diagnostic accuracies, both RT-RPA and RT-RAA appear to be suitable assays for point-of-need deployment for the detection of the pathogen, understanding its epidemiology, case management, and curbing transmission.

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## 1. Introduction

The unprecedented devastation caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has brought a tremendous threat to millions of lives and disrupted the global economy (Chih-Cheng Lai et al., 2020). To date, more than four million deaths have been attributed to coronavirus disease 2019 (COVID-19) worldwide, along with an immeasurable degree of morbidity (JHU, 2021). SARS-CoV-2 is the seventh member

of the coronavirus family capable of causing human disease, and since its discovery in the city of Wuhan in China, this airborne virus has devastated and continues to devastate millions of lives and livelihoods through active and passive ongoing transmission (Tao Wua et al., 2020).

COVID-19 typically manifests as general flu-like symptoms including fever, cough, fatigue, dyspnea, and gastrointestinal issues (Chen et al., 2020; Huang et al., 2020). Owing to the overlap of symptoms with those of other viral infections, the early-stage diagnosis of SARS-CoV-2 infection is problematic (Xue et al., 2020). Furthermore, no 100% effective therapeutic or preventive intervention has yet been developed, and the isolation of infected individuals is the mainstay to curb the transmission of this pathogen (Lotfi et al., 2020). Lockdown fatigue and the urgent need for an income to provide the most basic needs is putting pressure on this control measure, which is cruelly having the greatest impact on the poorest populations that are most at risk. Therefore, an accurate and rapid diagnostic method that can be applied in resource-poor settings is highly desired for controlling the spread of the virus/disease and directing treatment to infected individuals.

A handful of direct techniques including virus isolation, plaque assays, electron microscopy, and immuno-electron microscopy are available for the diagnosis of SARS-CoV-2 infection/COVID-19. However, considering the superior sensitivity and specificity, as well as the high-throughput capacity of real-time reverse transcriptase PCR (real-time RT-PCR), this method has become the gold standard for detection (Hemida, 2021). Moreover, the quantitative ability of the method has also been exploited for prognosis (Ulinici et al., 2021). Despite its promising diagnostic efficacy, the requirements for a highly qualified laboratory, trained personnel, and expensive reagents limit the application of this technique at decentralized facilities. This is a critical gap in most developing countries.

To overcome such challenges, many antigen/antibody tests for SARS-CoV-2 have been developed and validated. These tests are regularly used globally, and especially in low and middle income (LMIC) settings. Among them, a few antigen-based tests including Lumipulse G, LuminraDx, and Standard Q Nasal have shown promising diagnostic efficacy, with sensitivity >80% and specificity >95% (Brümmer, 2021). However, most of the antigen and antibody-based tests do not qualify for the desired accuracy (Deeks et al., 2020). Consequently, there is a pressing need for a diagnostic tool that can be used at decentralized facilities without decreased accuracy and reliability relative to real-time RT-PCR tests.

With the consistent demand for an accurate, rapid, less expensive, and easy-to-operate diagnostic tool, isothermal amplification methods such as loop-mediated isothermal amplification (LAMP), CRISPR-based fluorescent application, and nicking enzyme-assisted reaction (NEAR) have been exploited to detect SARS-CoV-2 (James and Alwneh, 2020). These methods require neither thermostable enzymes nor expensive equipment. Notably, a handful of studies have diagnosed COVID-19 through LAMP, and 10 reverse transcription (RT)-LAMP tests have already been approved by the US Food and Drug Administration (FDA) for emergency use (FDA, 2021). Recently, recombinase polymerase amplification (RPA) assays have emerged as another promising alternative to real-time PCR for the diagnosis of infectious diseases (A and J, 2015; Macdonald et al., 2018; P et al., 2021). The RPA method has multifarious advantages over other isothermal amplification technologies, including LAMP. RT-RPA is comparatively rapid (10–15 min), requires simple equipment, and is easy to operate at ambient temperature (37–42°C) (Qian et al., 2020).

The versatile advantages of RT-RPA assays has led to several creative applications of these assays for the detection of coronaviruses such as Middle East respiratory syndrome coronavirus (MERS-

CoV) and bovine coronavirus (BCoV) (Behrmann et al., 2020). Further, the method has been incorporated into a 'suitcase laboratory' for the point-of-care (POC) diagnosis of H7N9 avian influenza virus, Ebola virus, and visceral leishmaniasis, each of which has demonstrated the great potential of this isothermal technology as a diagnostic tool for use in rapidly evolving and resource-constrained outbreak situations (A et al., 2015; Mondal et al., 2016; O et al., 2015). In parallel to other isothermal technologies, RT-RPA for the diagnosis of SARS-CoV-2 infection has been explored in several studies (Behrmann et al., 2020; Nassir et al., 2020; Subsoontorn et al., 2020; el Wahed et al., 2021).

Another recombinase-based assay – reverse transcription recombinase-aided amplification (RT-RAA) – has recently been developed to detect infectious pathogens (Tao Wua et al., 2020). The diagnostic efficacy of the assay to detect SARS-CoV-2 has been well validated in a multicenter study (Wang et al., 2020).

RT-RPA and RT-RAA are both based on isothermal amplification, in which the double-stranded DNA denaturation and strand invasion is accomplished by a cocktail of recombinase enzymes, single-stranded binding proteins, and DNA polymerases. The only difference between the methods is the source of recombinase, which is phage-derived for RT-RPA and bacteria/fungi-derived for RT-RAA (Tao Wua et al., 2020).

Considering the various advantages of the isothermal methods, this study was performed to evaluate the ability of RT-RPA targeting the E, RdRP, and N genes and RT-RAA amplifying ORF1ab to diagnose SARS-CoV-2 infection. The positive results of this study will facilitate the use of these rapid and accurate diagnostic tools at decentralized facilities such as community clinics, especially in LMIC including Bangladesh.

## 2. Materials and methods

### 2.1. Study site and population

A diagnostic evaluation study was performed, in which a case-control design was followed to evaluate the diagnostic efficiency of both RT-RPA and RT-RAA. The study was conducted in the Virology Laboratory and the Emerging Infections and Parasitology Laboratory of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b); institutional review board approval was obtained (PR-20043). Archived RNA samples were used to evaluate assay performance. Following RNA isolation from the clinical samples (nasopharyngeal swab), real-time RT-PCR was performed in the Virology Laboratory. The RNA samples were then archived at –80°C before RT-RPA and RT-RAA assays were performed in the Emerging Infections and Parasitology Laboratory.

The clinical samples included in this study were collected from suspected COVID-19 patients at icddr,b during the pandemic. The staff of icddr,b and their family members were instructed to contact the icddr,b staff clinic if they had any clinical symptoms of COVID-19. Following clinical evaluation, suspected patients and their contacts were referred to the COVID-19 facility, Dhaka Hospital of icddr,b for nasopharyngeal swab sample collection. The collected swab samples were transferred in viral transport medium to the Virology Laboratory, icddr,b for laboratory investigation to detect SARS-CoV-2 through RT-PCR. The samples were collected from all patients irrespective of age.

A total of 76 suspected COVID-19 cases were confirmed through real-time RT-PCR. One hundred negative control samples were investigated to directly compare the isothermal assays. Samples with a cycle threshold (Ct) value  $\leq 37$  were considered as positive in the RT-PCR. A random code was generated by the study investigator for each sample to ensure that the laboratory personnel were blinded to the classification or type of sample while performing the assays.

The operational definitions for the study participants from whom the clinical samples were collected were as follows: a 'COVID-19 suspect' was a suspected COVID-19 case defined in a manner consistent with the World Health Organization (WHO) guidelines, including (1) a patient with an acute respiratory illness (fever and at least one sign/symptom of respiratory disease, e.g., cough, shortness of breath) AND a history of travel to or residence in a location reporting community transmission of COVID-19 disease during the 14 days prior to symptom onset, OR (2) a patient with any acute respiratory illness AND having been in contact with a confirmed or probable COVID-19 case in the last 14 days prior to symptom onset, OR (3) a patient with a severe acute respiratory illness (fever and at least one sign/symptom of respiratory disease, e.g., cough, shortness of breath, AND requiring hospitalization) AND in the absence of an alternative diagnosis that fully explains the clinical presentation. A 'confirmed COVID-19 case' was a suspected COVID-19 case with a positive real-time RT-PCR test. Controls were individuals with flu-like symptoms who had negative COVID-19 real-time RT-PCR results.

## 2.2. Nucleic acid extraction and real-time RT-PCR

Viral RNA was extracted and purified from the clinical samples (nasopharyngeal swabs) using the InviMag Pathogen Kit on an automated extractor (KingFisher Flex 96 system). SARS-CoV-2 detection was performed by real-time RT-PCR assay targeting the SARS-CoV-2 RNA-dependent RNA polymerase (RdRP) and nucleocapsid protein (NP) genes according to the real-time RT-qPCR protocol as outlined by the Chinese Center for Disease Control and Prevention for the detection of RdRP and N genes of SARS-CoV-2 recommended by the WHO (WHO, 2021). The primers and probes used to amplify the targets are provided in **Supplementary Material File 1**.

The real-time RT-PCR was performed in a 25- $\mu$ l reaction volume consisting of 5  $\mu$ l viral RNA, 12.5  $\mu$ l of 2  $\times$  reaction buffer provided with the Superscript III One Step RT-PCR system with Platinum Taq polymerase (Invitrogen, Darmstadt, Germany; containing 0.4 mM of each deoxyribonucleotide triphosphate (dNTP) and 3.2 mM magnesium sulfate), 1  $\mu$ l of reverse transcriptase/Taq mixture from the kit, 0.4  $\mu$ l of a 50 mM magnesium sulfate solution (Invitrogen), and 1  $\mu$ g of non-acetylated bovine serum albumin (Roche). The steps of the amplification cycles were as follows: reverse transcription for 20 min at 55°C, followed by denaturation at 95°C for 3 min, 50 cycles of amplification at 95°C for 15 s and annealing–extension at 58°C for 30 s, and cooling the thermal cycler (CFX-96; Bio-Rad, USA) at 40°C for 30 s. To control for batch-to-batch variation, a positive control of known Ct value was included in each run. If both targets (open reading frame 1ab, nucleocapsid protein) were positive by specific real-time RT-PCR, the case was considered a laboratory-confirmed case.

## 2.3. RT-RPA assay

The RT-RPA assays for the E, RdRP, and N genes were performed according to the method described by el Wahed et al., with minor modification (el Wahed et al., 2021). For RT-RPA, the TwistAmp exo kit (TwistDx, Cambridge, UK) was used in combination with lyophilized RevertAid reverse transcriptase (Life Technologies, Darmstadt, Germany). For each reaction, 29.5  $\mu$ l of rehydration buffer, 2.5  $\mu$ l of RevertAid reverse transcriptase (200 U/ $\mu$ l), 5.7  $\mu$ l of H<sub>2</sub>O, 2.1  $\mu$ l of forward primer (10 pmol/ $\mu$ l), 2.1  $\mu$ l of reverse primer (20 pmol/ $\mu$ l), 0.6  $\mu$ l of exo-probe (10 pmol/ $\mu$ l), 2.5  $\mu$ l of 280 mM magnesium acetate, and 5  $\mu$ l of the template were added into the lid of the reaction tube containing the freeze-dried pellet. After capping the tube, the contents were mixed well and placed immediately into a T8 isothermal fluorescence reader

(Axxin, Fairfield, Australia). The reaction was incubated at 42°C for 15 min. A mixing step was conducted after 230 s for the RdRP assay and after 320 s for the N RT-RPA assay. The threshold time (TT) was calculated as the starting point of the amplification curve above the threshold of the negative control (water as template) in the first derivative analysis in the T8 desktop software (Axxin, Fairfield, Australia).

## 2.4. RT-RAA assay

The RT-RAA assay was performed according to the manufacturer's instruction (Jiangsu Qitian Bio-Tech Co. Ltd, China). The assay has been described elsewhere by Wang et al. (Wang et al., 2020). The commercial kit for the detection of SARS-CoV-2 comprises a design-locked single tube containing lyophilized primer/probe and enzymes to perform a single reaction. First, 40  $\mu$ l buffer VII and 4  $\mu$ l of nuclease-free water were added to each reaction unit tube with a pipette, and 5  $\mu$ l magnesium acetate I solution was added to the inside of the reaction tube cover. Subsequently, 1  $\mu$ l of extracted nucleic acid or 1  $\mu$ l negative/positive control was added, and the tube was closed and mixed properly. After mixing, the tube was briefly centrifuged and then transferred immediately into the T8 isothermal fluorescence reader (Axxin, Fairfield, Australia). The reaction temperature was set at 39°C for 15 min, and a mixing step was performed after 230 s. The time taken for each sample to reach the threshold (TT) was measured in real time by the fluorescence signal detector.

## 2.5. Data analysis

Standard statistical methods including contingency tests and binomial confidence intervals were performed to determine the sensitivity and specificity of the assays with 95% confidence interval (CI). Parametric and non-parametric tests were performed based on the distribution of data. Cohen's kappa coefficient ( $\kappa$ ) and the McNemar test were performed to determine the concordance and discordance among the diagnostic methods. The values of the Cohen  $\kappa$  coefficients were interpreted according to Landis and Koch: 1.00–0.81, excellent agreement; 0.80–0.61, good agreement; 0.60–0.41, moderate agreement; 0.40–0.21, weak agreement; 0.20–0.00, negligible agreement. Continuous variables were summarized using the mean and standard deviation. In addition, a receiver operating characteristic (ROC) curve analysis was performed to determine the accuracy of the assays. A Venn diagram was prepared using an interactive online tool called Venny (Venny 2.1.0.n.d.) (Venny 2021). All statistical analyses were performed using IBM SPSS Statistics version 25.0 and GraphPad Prism version 8.1.2. A *P*-value <0.05 was considered as statistically significant.

## 3. Results

### 3.1. Performance of the isothermal amplification methods

Among the 76 confirmed COVID-19 patients, 63 were found to be positive by RT-RPA when the target gene was N (Table 1). Although a small decline in the percentage sensitivity of the method was observed when the target gene was RdRP, the combination of the target genes augmented the overall sensitivity of the RT-RPA assay to 85.53% (95% CI 75.58–92.55%) (Table 1). RT-RPA and RT-RAA showed equal sensitivity of 76.32% (95% CI 65.18–85.32%) when the target for the former was RdRP and for the latter was ORF1ab.

Although, all 76 samples were positive for both the RdRP and the N gene by real-time RT-PCR, the sensitivity of the two isothermal methods varied across the Ct values for each target gene (Table 2). It is worth noting, however, that the real-time RT-PCR Ct

**Table 1**

Diagnostic performance of the reverse transcription recombinase polymerase amplification (RT-RPA) assay and reverse transcription recombinase-aided amplification (RT-RAA) assay when the methods were evaluated with confirmed COVID-19 cases and controls

Assay type	Target	Sensitivity (95% CI) (n/N)	Specificity (95% CI) (n/N)
RT-RPA	RdRP	76.32% (65.18–85.32%) (58/76)	98.00% (92.96–99.76%) (98/100)
	N	82.89% (72.53–90.57%) (63/76)	95.00% (88.72–98.36%) (95/100)
	N+RdRP	85.53% (75.58–92.55%) (65/76)	94.00% (87.40–97.77%) (94/100)
RT-RAA	ORF1ab	76.32% (65.18–85.32%) (58/76)	97.00% (91.48–99.38%) (97/100)

CI, confidence interval.

**Table 2**

Sensitivity of the isothermal methods spanning different ranges of Ct values found in RT-PCR

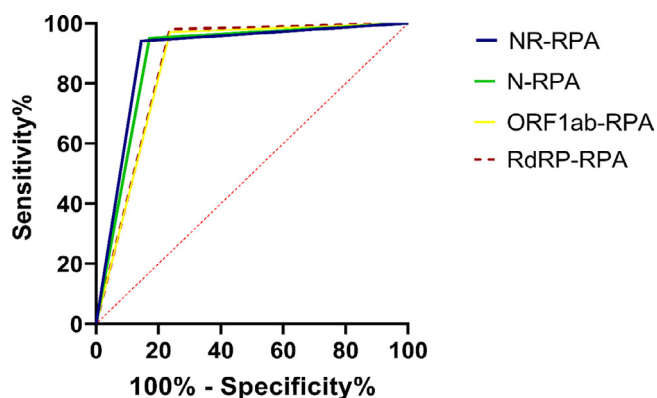
RT-PCR Ct range	Target for RT-PCR	Assay	Assay target	Sensitivity (95% CI)
0–30	RdRP gene	RT-RPA	RdRP	92.50% (79.61–98.43%)
		RPA	N	95.00% (83.08–99.39%)
		N+R*		87.50% (73.20–95.81%)
RT-RAA		ORF1ab	97.50% (86.84–99.94%)	
31–35		RT-RPA	RdRP	46.15% (26.59–66.63%)
		RPA	N	57.69% (36.92–76.65%)
	N+R*		73.08% (52.21–88.43%)	
36–40	RT-RAA	ORF1ab	34.62% (17.21–55.67%)	
	RT-RPA	RdRP	10.00% (0.25–44.50%)	
	RPA	N	20.00% (2.52–55.61%)	
0–30	N gene	N+R*		40.00% (12.16–73.76%)
		RT-RAA	ORF1ab	10.00% (0.25–44.50%)
		RT-RPA	RdRP	97.37% (86.19–99.93%)
RPA		N	97.37% (86.19–99.93%)	
N+R*			92.11% (78.62–98.34%)	
RT-RAA		ORF1ab	100.00% (90.75–100.00%)	
31–35	RT-RPA	RdRP	47.62% (25.71–70.22%)	
	RPA	N	71.43% (47.82–88.72%)	
	N+R*		80.95% (58.09–94.55%)	
36–40	RT-RAA	ORF1ab	47.62% (25.71–70.22%)	
	RT-RPA	RdRP	25.00% (7.27–52.38%)	
	RPA	N	12.50% (1.55–38.35%)	
>40	N+R*		37.50% (15.20–64.57%)	
	RT-RAA	ORF1ab	18.75% (4.05–45.65%)	
	RT-RPA	RdRP	0.00% (0.00–97.50%)	
RPA	N	100.00% (2.50–100.00%)		
N+R*		100.00% (2.50–100.00%)		
RT-RAA	ORF1ab	0.00% (0.00–97.50%)		

Ct, cycle threshold; RT-RPA, reverse transcription recombinase polymerase amplification assay; RT-RAA, reverse transcription recombinase-aided amplification assay. \*RdRP→R.

values also differed between the targets. Similarly, the sensitivity of each of the methods was found to vary for each of the target genes. While all of the samples positive for the RdRP gene emerged between Ct 0 and 40 in the real-time RT-PCR, most of the positives in the RT-RPA and RT-RAA assays became positive between 0 and 30 cycles, at sensitivities of 92.50% (95% CI 79.61–98.43%) and 97.50% (95% CI 86.84–99.94%), respectively (Table 2). Similarly, for the N gene the maximum of the RT-RPA and RT-RAA positive samples were under 30 cycles and exhibited sensitivities of 97.37% (95% CI 86.19–99.93%) and 100.00% (95% CI 90.75–100.00%), respectively. Very few cases were positive only for N-RAA, where the Ct value was around 40.

Both isothermal amplification methods showed high specificity in detecting true-negatives. The RdRP and N gene targeted in the RT-RPA assay showed specificities of 98.00% (95% CI 92.96–99.76%) and 95.00% (95% CI 88.72–98.36%), respectively. A small decline in specificity to 94.00% (95% CI 87.40–97.77%) was observed when the aforementioned targets were combined for the RT-RPA assay. Like RT-RPA, an increased specificity of 97.00% (95% CI 91.48–99.38%) was found for RT-RAA.

The ROC analysis substantiated the higher accuracy (area under the ROC curve (AUC) 0.897) of the RT-RPA when the N and RdRP genes were combined (Figure 1) (Table 4). Again, the RdRP- and ORF1ab- targeted RT-RPA and RT-RAA methods respectively showed parallel diagnostic accuracy. As the E target is specific



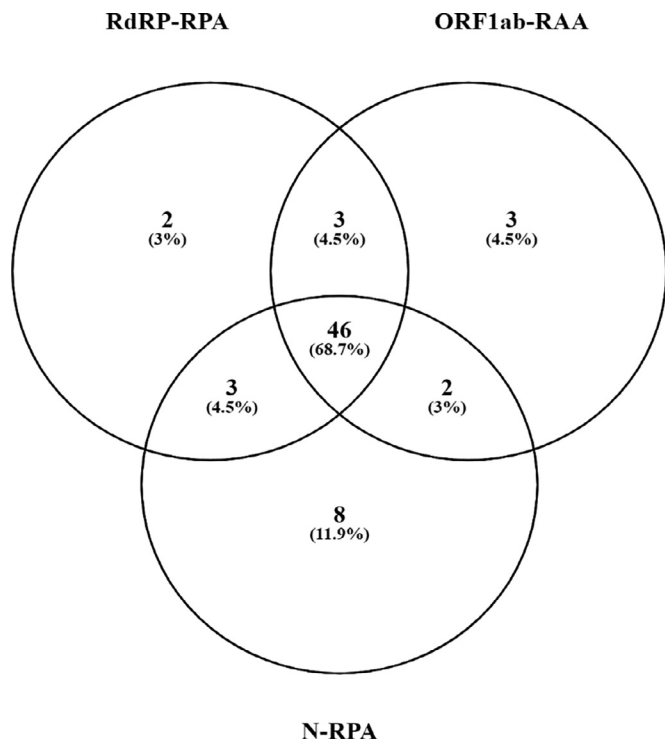
**Figure 1.** Multiple receiver operating characteristic (ROC) curve exhibiting the diagnostic accuracy of the isothermal assays.

for all betacoronaviruses (severe acute respiratory syndrome coronavirus (SARS-CoV), MERS-CoV, SARS-CoV-2, etc.) and the target gave a high number of false-positives (Supplementary Material File 1), the RT-RPA assay targeting the E gene was excluded from the analysis.

**Table 3**  
Kappa coefficient values for the degree of agreement between the methods

Assays	Measure of agreement (kappa)	P-value(McNemar test)
RdRP-RPA vs NR-RPA	0.770	0.019
RdRP-RPA vs ORF1ab-RAA	0.836	1.00
RdRP-RPA vs N-RPA	0.755	0.115
N-RPA vs ORF1ab-RAA	0.719	0.210
N-RPA vs NR-RPA	0.869	0.549
NR-RPA vs ORF1ab-RAA	0.662	0.087

RPA, recombinase polymerase amplification; RAA, recombinase-aided amplification.



**Figure 2.** Venn diagram presenting the distribution of positives by each method among the 76 confirmed COVID-19 cases. The distribution shows the highest number of positives through RT-RPA when the target gene was N (N-RPA). Equal numbers of positives were identified by RT-RPA and RT-RAA when the target genes were RdRP and ORF1ab, respectively (RdRP-RPA and ORF1ab-RAA).

### 3.2. Agreement between the RT-RPA and RT-RAA assays

Around two-thirds of the COVID-19 samples (68.7%) were positive by each of the methods when the assay outcomes of the target genes were independent of each other (Figure 2). When presented as a Venn diagram, the high degree of overlapping positives between RdRP-RPA and ORF1ab-RAA is readily observed. The higher kappa coefficient ( $\kappa = 0.836$ ) between the aforementioned methods further bolstered their excellent agreement (Table 3). As expected, N-RPA showed good agreement with both RdRP-RPA and ORF1ab-RAA, with kappa values of 0.755 and 0.719, respectively. Furthermore, a good agreement ( $\kappa = 0.662$ ) was observed between the RT-RAA and RT-RPA assays when the N and RdRP targets were combined (Table 3).

## 4. Discussion

In the absence of any magic bullet against COVID-19, the containment of SARS-CoV-2 infections has been the cornerstone for the disruption of transmission. Taking into account previous successes, the three Ts – test, trace, treat – have been considered indispensable towards moving to the ‘new normal’ era and allevi-

**Table 4**  
Area under the curve (AUC) values determined through receiver operating characteristic curve (ROC) analysis

Assay	AUC
RdRP-RPA	0.871
ORF1ab-RAA	0.866
NR-RPA	0.897
N-RPA	0.889

RPA, recombinase polymerase amplification; RAA, recombinase-aided amplification.

ating the crippling impact on the global economy by diminishing virus spread (Griffin, 2020; Ng, 2020; Zheng et al., 2021). In this regard, the democratization of COVID-19 testing facilities is still a distant and potentially unattainable goal in low-resource settings, because of the lack of universal operating procedures, as well as an absence of harmonization and implementation of truly widely available PCR-based diagnostic assays (Kevadiya et al., 2021). However, with the advent of isothermal amplification-based point-of-need assays, accessibility to diagnosis and faster screening, while maintaining the optimal diagnostic efficacy, is no longer a dream in the fight against the pandemic (James and Alwneh, 2020).

Critically, unlike RT-PCR strategies, isothermal amplification technologies with reduced turnaround times require neither professional expertise nor expensive infrastructure. Consequently, this technology has the potential to be deployed at the community level. Thus, the transfer of samples to a centralized laboratory is not required and the longer execution period associated with the standard COVID-19 real-time RT-PCR test disappears (Subsoontorn et al., 2020). Considering such a multitude of technical and practical advantages, the overarching goal of the present study was to evaluate both RT-RPA and RT-RAA assays as emerging isothermal techniques amenable to resource-limited settings for the detection of SARS-CoV-2. Both of the methods showed moderate sensitivity when compared to previous studies, while their specificities were comparable (el Wahed et al., 2021; Wang et al., 2020; Xue et al., 2020). The deviations in diagnostic efficiency of the tests can be attributed to the sample type, sample quality/quantity, different settings, and variable patient cohorts and operators.

The earlier study performed by Ahmed et al. included only 36 samples to evaluate the clinical efficiency of the RPA assay, whereas 176 samples (76 cases, 100 controls) were under investigation in the current study (el Wahed et al., 2021). Moreover, the various types of clinical samples used in the study performed by Wang et al. might be responsible for the elevated sensitivity of the RT-RAA assay (Wang et al., 2020). On the other hand, only nasopharyngeal swab samples were used to determine the clinical efficiency of the RT-RAA assay in the current study. It is noteworthy that in the previous multicenter study, 62.5% of the swab

samples were found to be positive for SARS-CoV-2 by RT-RAA test (Wang et al., 2020).

In the current study, both of the isothermal methods detected most of the samples at Ct values <30 in the real-time RT-PCR. A recent review by Francois et al. showed that infected individuals with  $10^6$  viral particles/ml are contagious – the detection limit of the nucleic acid amplification-based methods varies between  $10^5$  and  $10^3$  viral particles/ml for clinical samples (Berthiaume, 2021). Such infected individuals with high Ct values are therefore less likely to transmit the virus, and the isothermal techniques detected most of the likely super-spreaders. Even without this consideration, both of the tests evaluated achieved the qualification of a useful test by the estimation of sensitivity + specificity (1.5 value), as per the diagnostic test quality proposed by Power et al. (Power et al., 2013).

It appears that this is the first study to directly compare RT-RPA and RT-RAA assays for the detection of SARS-CoV-2 in clinical samples. The major limitations of this study include the use of archived samples and the use of similar types of samples, both of which might influence the true clinical efficiency of the tests. However, random samples spanning a range of RT-PCR Ct values with variable viral loads were used to more robustly assess the efficiency of each test. Furthermore, several aliquots of the archived RNA samples were used to avoid the introduction of freeze-thaw as an impacting variable. Furthermore, the laboratory personnel were blinded to the types of samples they were evaluating to nullify any operator bias. Finally, apart from the technical aspects, the assays have a limitation in terms of throughput, as the portable device can detect eight samples in a single run. However, the developed capacity would be adaptable in resource-limited settings to detect other pathogens as well when the pandemic is over.

Considering the current need, the diagnostic efficiencies, and the feasibility of performing the assays in resource-constrained settings, both of the isothermal methods demonstrate promise for application as point-of-need tests for the diagnosis of SARS-CoV-2 infection/COVID-19. The RdRP-targeted RT-RPA and ORF1ab-targeted RT-RAA appear sufficient to specifically detect SARS-CoV-2, and their use would reduce the operational costs. Moreover, the earlier studies confer the detection of the virus through ORF1ab, where both RdRP and ORF1ab flank the same region of the genome of SARS-CoV-2 (Corman et al., 2020; el Wahed et al., 2021; Wang et al., 2020). This also explains the similar diagnostic efficiency or excellent agreement of the methods while using the aforementioned targets for two different tests. The technical advantages of the RT-RAA are its simple pipetting scheme and the availability of lyophilized all-in-one primer in pellet form, which contains the RT, RAA enzyme, and protein, as well as the SARS-CoV-2 oligonucleotide. Recently, coupling of the methods with CRISPR Cas9/12a has been investigated to develop a lateral flow device for further facilitating POC detection (Sun et al., 2021; Xiong et al., 2021; Yu et al., 2021). However, further methodological improvisation of the methods is warranted prior to large-scale application.

In the last decade, the applications of isothermal amplification technologies have been advancing, with recombinase-based isothermal assays contributing around 10% of the global isothermal amplification technology market (Li et al., 2019). A number of isothermal amplification techniques have received emergency use authorization (EUA) from the FDA (FDA, 2021). Recently, several POC molecular tests including OptiGene RT-LAMP, QuantuMDx Q-POC, and ID Now have shown promising diagnostic accuracy with high sensitivity and specificity ranging from 73.0% to 96.8% and from 99.1% to 100%, respectively, depending on the type of clinical sample and trade-off Ct values obtained in RT-PCR (Caffry, 2021; Graham, 2021; GOV.UK 2021) <https://www.gov.uk/government/publications/>

[rapid-evaluation-of-optigene-rt-lamp-assay-direct-and-rna-formats/rapid-evaluation-of-optigene-rt-lamp-assay-direct-and-rna-formats/](#)). The large-scale application of the POC assays mentioned above warrants the development and validation of new POC tests feasible for use in resource-constrained settings to accelerate the diagnosis of COVID-19.

Since the recombinase-based RT-RAA and RT-RPA assays are feasible to perform in point-of-need settings, the findings of this study could facilitate the use of these isothermal methods on a large scale and in a broad array of settings. Finally, incorporation into the 'suitcase laboratory' format, along with a rapid nucleic acid extraction technique, would augment the utility of the tests by allowing deployment in decentralized facilities (rural community clinics, shopping and travel hubs, airports, etc.) to combat outbreak situations.

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## Ethics statement

This study was approved by the Institutional Review Board (IRB) and Ethics Review Committee (ERC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) (research protocol number PR-20043). The authors confirm that the ethical policies of the journal, as noted in the journal guidelines, have been adhered to.

## Data availability statement

The data that support the findings of the study are available in the supplementary material of this article.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijid.2021.11.007](https://doi.org/10.1016/j.ijid.2021.11.007).

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