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Molecular Diagnosis of COVID-19 by Targeting Envelope (E), RdRp and RNase P genes

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Abstract

Background: The SARS-CoV-2 infection responsible for COVID-19 pandemic. The RT-PCR frequently used assay for the diagnosis of SARS-CoV-2 infection due to its sensitivity.

Methods: The study involves the molecular analysis of swab sample of COVID-19 patients admitted at PIMS Hospital, Udaipur, Rajasthan, between November 2020 to June 2022. The present study based on amplification of two SARS-CoV-2 genes (E gene and RdRp) and a human RNase P gene simultaneously through multiplex RT-PCR detection method.

Results: The sensitivity of RT-PCR assay was tested on 201 symptomatic and 203 asymptomatic samples, which show 100% positive percent with a limit of detection (LOD) value of 10^3 copies/ml. The COVISure assay is found accurate, reliable, sensitive, and specific for the diagnosis of SARS-CoV-2 infection.

Conclusion: The COVISure assay shows a considerable impact on the screening for infection and COVID-19 patient management. The analytical sensitivity of the kit is 10^3 copies/ml.

Keywords: COVID-19, SARS-CoV-2, RT-PCR, COVISure

Introduction

The SARS-CoV-2 is the member of virus family *Coronaviridae* [1]. The various methods developed by the scientists for the diagnosis and treatment of SARS-CoV-2 infection during COVID-19 pandemic[2]. The real-time polymerase-chain-reaction (RT-PCR) method is based on the amplification of SARS-CoV-2 RNA[3] and its consider as the gold standard method for the COVID-19 screening [4].

The RT-PCR assay may also produce false-negative and false-positive results [5]. The patients with initial negative result may became positive within next 3-5 repeated swabs sampling [6, 7]. The rate of false-negative result range between 1 and 30% [8]. Therefore, to improve sensitivity multiplex PCR protocol should be preferred to target multiple genes in the same amplification reaction. Through COVISure assay, two viral genes (E gene and RdRp) and a human RNase P gene in the same rRT-PCR reaction were targeted, that help to increase the probability of diagnose the virus infection especially in the patients having low viral load.

The SARS-CoV-2 genome contains 14 ORFs that encodes 27 types of proteins [9]. The genome of SARS-CoV-2 consist of four major structural genes that are translated to spike protein (S), matrix protein (M), small envelope protein (E), and nucleocapsid

protein (N) [9], along with some accessory genes. The surface glycoproteins S1 bind to the host ACE2 receptor and S2 fuses with the host cell membrane, that allowing the virus to invade [10,11]. The other genes encoding non-structural proteins (Nsp) e.g., RdRP (Nsp12), RdRp responsible for the replication of the viral genome in the host cells [12, 13].

The various RT-PCR tests developed to target RdRP, E, N, or S genes [14, 15]. Among these, methods targeting RdRP gene were found to be highest analytical sensitivity [16]. Moreover, the human ribonuclease P (RNase P) gene is used as an internal control in multiplex RT-PCR protocols recommended by CDC and WHO [17, 18]. The real-time RT-PCR results may be affected by variations in viral RNA sequences [19].

In the present study, rRT-PCR assay designed and evaluated for the diagnosis of SARS-CoV-2. The study exhibits primer sets specific to SARS-CoV-2. The developed assay simultaneously detects viral E, RdRP and human RNase P genes in the same rRT-PCR reaction. The clinical performance of the test was screened with RNA samples from SARS-CoV-2 positive symptomatic and asymptomatic patients.

Methods

Sample collection and RNA isolation:

The RNA samples were extracted from nasopharyngeal swab or combined nasopharyngeal/ oral swab collected from the patients at Pacific Institute of Medical Sciences (PIMS) Hospital, Udaipur, Rajasthan, between March 2021 to December 2021. The swabs were collected in Virus Liquid Transport Medium-VTM as per manufacture instruction for collection and handling (COVISure, India). Specimens were transported to the lab in cool box and kept refrigerated at 6-8 ^{o}C for not more than 8 h till time of nucleic acid extraction.

Prior to RNA extraction, collection tubes were vortexed; 200 or 400 µl of VTM were transferred to 2 ml tubes for RNA extraction (Genetix Biotech Asia Pvt. Ltd., India). Finally, RNA extraction was done in automated RNA extractor and 5 µL of extracted RNA was used as template for the rRT-PCR.

rRT-PCR: The DTprime real-time PCR instrument (Genetix Biotech Asia Pvt. Ltd., India) was used for qualitative and quantitative analysis of RNA targets in rRT-PCR reactions. COVISure is Single Tube Multiplex Assay for qualitative detection of SARS CoV-2 infection with 100% specificity and sensitivity from nasopharyngeal, oropharyngeal, swabs and sputum samples from patients.

COVISure is recommended to use a standard RNA extraction kit for isolation of viral RNA. The performance of the kit validated using RNASure Virus Kit (CatLog numbers NP-67703, 67705, 67706 and 67707) and Automated RNA purification system, PurifierHT 96, using GeneMag viral RNA/ DNA purification kit (GxMY502T5-P-96).

Sample Preparation

Preference of COVISure real time RT-PCR diagnostic kit is depended upon the amount and quantity of template RNA purified from the clinical samples, including nasopharyngeal swab, oropharyngeal swab, and sputum. However, validation of the essay was performed on RNASure Mini Kit catalog number NP-67703 (300 Prep), bracket close NP-67005 (50 Prep), NP-67706 (100 prep) and NP-67707 (250 prep) bracket.

The following amplification conditions were applied for the rRT-PCR reaction:

Steps	Temperature	Time	Cycle
Reverse transcription	50 ^{o}C	15 min	Hold
Initial denaturation	95 ^{o}C	3 min	Hold
Denaturation	95 ^{o}C	10 sec	45 cycles * Acquisition
Annealing/	60 ^{o}C	30 se	

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extension			on FAM (465-510 nm) Hex (530-580 nm) and Quasar 670 (625-670 nm)

The fluorescence reading was performed at the amplification step. Amplification of RNaseP in samples suggested proper RNA extraction. Amplification in FAM and Quasar 670 channels indicates presence of SARS-CoV-2 in sample. The oligonucleotide primers and probes used for the detection of 2019-nCoV were selected from regions of the virus RNA dependent RNA polymerase (RdRp) gene and Envelope (E) gene. An additional primer/probe set to detect the human RNase P gene in clinical specimens is included in the kit. The analytical sensitivity of the kit is 10^3 copies/ml. This concentration serves as limit of detection (LOD).

Data analysis

Amplification curves of viral and human genes were considered to evaluate the results. After manual adjustment of the cycle threshold (Ct or Cq) line, the representative Ct value of each gene was determined by using ABI 7500 software. The positive cut-off value was set at cycle threshold number ≤ 36 with a sigmoidal curve. Any patient meets the criteria were accepted as positive.

Ethical approval

The study is approved by the ethics committee of Pacific Institute of Medical Sciences (PIMS), Sai Tirupati University Hospital, Udaipur, Rajasthan (Ref No. STU/IEC/2021/81). All methods were carried out in accordance with relevant guidelines and regulations.

Results

COVISure rRT-PCR assay was optimized for the diagnosis of SARS-CoV-2. The assay mix simultaneously targets three viral genes (E gene, RNase P and RdRp). The assay tested in in 201 positive symptomatic and 204 asymptomatic patient's RNA samples. The figure 1 and 2 exhibits the rRT-PCR outputs belonging to positive and negative COVID-19 individuals. In COVID-19 positive samples, E gene, RNase P and RdRp genes amplified simultaneously, forming S-shaped sigmoidal curves (Fig 2, Table 1).

Amplification in FAM and Quasar 670 channel indicates presence of SARS-CoV-2 in sample. Due to competition and limiting factors, sample with high viral copy may not show amplification in HEX (IC) channel or the curve is not proper sigmoid.

In the COVID-19 negative specimen, the internal control human RNase P was the only gene amplified with a sigmoidal amplification curve (Fig 1, Table 1). In the positive control reactions, pseudoviral RNA including E and RdRP regions and a human RNase P mRNA was used as template. The amplification curves were obtained for all targeted genes. In the negative control reactions, ddH_2O was used as the template, which led no amplification line without primer dimer formation (homo-dimer or hetero-dimer).

Table 1. Multiplex detection. Manual (threshold) method (B, F); threshold FAM = 7.9, and threshold HEX = 5.3

Well no	Cp, Fam	Cp, Hex	Cp, Rox	Cp, Cy5	Well no	Cp, Fam	Cp, Hex	Cp, Rox	Cp, Cy5
A1		23.1			B1	25.4	29.2		28.0
G4					G4				

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H4	20.4	25.4		20.4	H4	20.4	25.4		
Negative					Positive				

Figure 1. Determination of the limit of detection (LOD) for RdRP and E primers. The 5 × 104 copy/µl pseudoviral RNA was serially diluted. The graph indicates amplification plots (a, b) and the amplification efficiencies for negative patients.

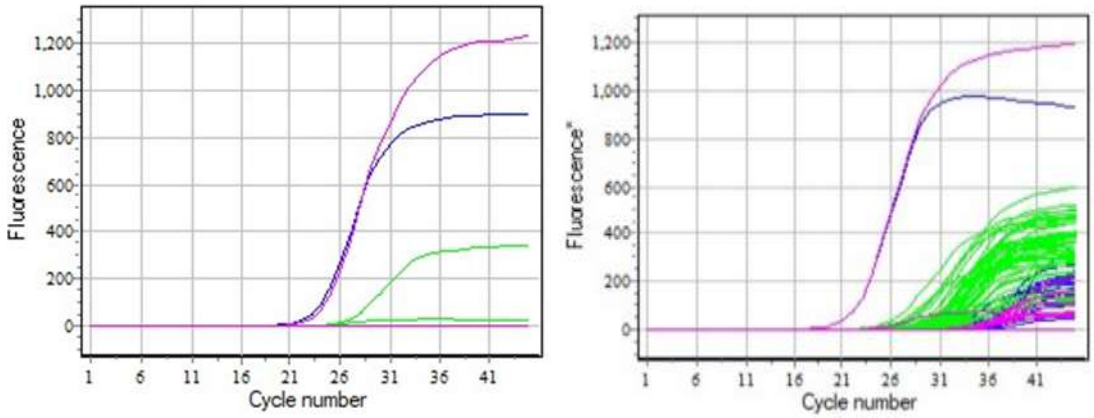
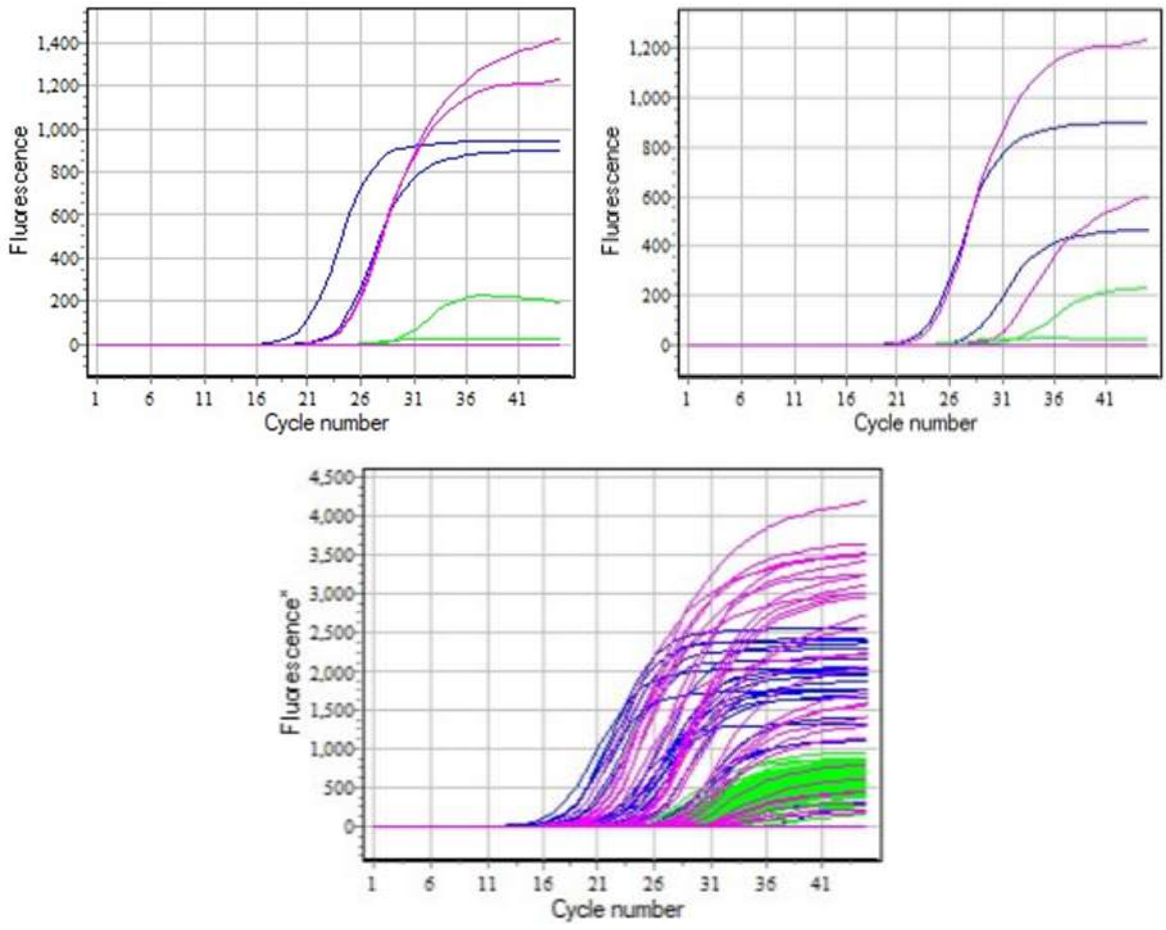


Figure 2. Determination of the limit of detection (LOD) for RdRP and M primers. The 5 × 104 copy/µl pseudoviral RNA was serially diluted. The graph indicates amplification plots (a, b, c) and the amplification efficiencies for positive patients.



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Discussion

The results showed that the multiplex primer and probe design successfully amplify all targeted genes both in SARS-CoV-2 positive specimen and synthetic positive control samples without forming primer dimer or self-amplification.

Validation of the assay

Since COVID-19 is highly infectious, we expect that new technique accelerate the development of accurate COVID-19 diagnostic, and reduce the viral infection spread [21, 22]. The validation of the results has been performed by using COVISure kit (Genetix, India), that are targeting different genes such as E gene, RdRp and RNase P. Accordingly, the Ct cut off value for FAM and Quasar 67 is $\leq$ 36 is accepted as positive. Besides, in both assays, the Ct score of those negative samples was $\geq$ 36, [20]. In this case, the assay exhibited 100% positive percent agreement with those commercial assays [23].

We urge testing laboratories to carefully consider the E gene, RdRp and RNase P as a target to optimise SARS-CoV-2 diagnostics.

Conclusion

COVISure is a multiplex assay for qualitative detection of SARS-CoV-2 infection with 100% specificity and sensitivity. The COVISure kit primers and probes selected from regions of the virus genomic e.g., RdRp gene and Envelope (E) gene. An additional primer/probe set to detect the human RNase P gene in clinical specimens is included in the kit. The analytical sensitivity of the kit is 10^3 copies/ml. COVISure assay show considerable impact on screening of infection and COVID-19 patient management.

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Ethical approval: Research project approved by the ethics committee (Ref. No. STU/IEC/2021/81) of Pacific Institute of Medical Sciences, Umarda Udaipur- 313005, Rajasthan, India**.**

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