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Research Article

DUAL TARGET REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION FOR SARS-COV-2 FOR ENHANCING ACCURACY WITH E GENE AND ORF1B GENE: A CROSS-SECTIONAL STUDY

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ABSTRACT

Objectives: The pandemic of the recently emerged 2019- novel coronavirus infection was a challenge to public health. The current gold standard for the molecular diagnosis of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection is the reverse transcription polymerase chain reaction (RT-PCR) for the qualitative and quantitative detection of viral nucleic acids. However, superior combination of gene targets for SARS-CoV-2 detection is underexplored. To find out the possible combination of target genes for maximizing RT-PCR accuracy in SARS-CoV-2 detection.

Methods: This study is an observational, cross-sectional study conducted at a tertiary care hospital in Kolkata. Study population included all SAR-CoV-2-infected patients attended either inpatient or outpatient department in a tertiary care hospital in eastern India. 870 patient's respiratory tract samples with the clinical diagnosis of COVID-19 were collected. E gene, N gene, RNA dependent RNA polymerase (RdRp) (open reading frame [ORF1ab]) gene, ORF1b gene, and human RNase P (Internal control) gene targets were detected.

Results: Among all the genes, E gene was the most frequently detected (n=665; 86.70%) closely followed by N gene (n=429; 83.30%). ORF1b could be detected in 69.73% (n=159). RdRp was least frequently detected in only 44.64% (n=175). The E gene and ORF1b could simultaneously be detected in 85.12% samples when tested together. E gene and N gene could be detected together in 71.16% and E gene and RdRp could be detected in only 53.41%.

Conclusion: Our study found that the combination of E gene and ORF1b gene as optimal targets for assay design as these two genes are expressed simultaneously, that will minimize inconclusive results and maximize diagnostic yield.

Keywords: Severe acute respiratory syndrome coronavirus-2, E gene, N gene, open reading frame 1b gene, RdRp gene, pandemic.

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INTRODUCTION

COVID-19, a disease caused by the novel virus severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), was originally reported in Wuhan, Hubei province, China, in December 2019. It has caused the first major pandemic of the new millennium. It is a single-stranded positive sense RNA virus with a genome size of almost 30kb (largest RNA virus), having genome structure like other coronaviruses. The genome order of the virus is 5-replicase (open reading frame [ORF1b)-structural proteins (spike [S]-envelope [E]-membrane [M]-nucleocapsid [N])-poly(T)-3. The genome is predicted to have 14 functional ORFs. Two large 5-terminal ORFs, ORFs 1a and 1b, encode 16 non-structural proteins of which nsp12 is RNA dependent RNA polymerase (RdRp) [1]. Initially, corona viruses were first reported in 1931, however, the first human infection was detected in the 1960s. Mainly two groups of viruses were responsible for some cases of cold and respiratory tract infection [2-5]. Recently, a new human corona virus came out in December 2019, named as novel coronavirus (2019-nCoV) by the World Health Organization (WHO). The current pandemic is flourishing fast, and between 70,000 and 85,000 new cases/day was reported over past month in India. Due to the huge spread of COVID-19, a scientifically sound detection method is needed for tracking the patients worldwide to control the disease burden. The SARS-CoV-2 nucleic acid can be detected in nasal and pharyngeal swab specimens, bronchoalveolar lavage fluid, sputum, bronchial aspirates, blood, anal swab, and other samples by a Real-time reverse transcriptase-polymerase chain reaction (RT-PCR). After the whole genome sequencing of SARS CoV-2 was successful, the WHO announced various primer and probe sets for SARS-CoV-2 and various manufacturers have opted different set of genes out of many genes of SARS-CoV-2. Hence, every assay has varied degree of sensitivity [6]. Besides, to improve the sensitivity and specificity of detection, most manufacturers choose two or more regions of viral nucleic acid sequence for detection, including the ORF1b sequence, E gene, N gene, and S gene of the SARS-CoV-2 genome. Our aim of the study was to find out the most sensitive sets of gene to detect SARS-CoV2 virus from nasopharyngeal and oro-pharyngeal swab specimens of patients admitted in a tertiary care hospital.

METHODS

Study settings and study design

The study was conducted in a tertiary care hospital in Eastern India. It was cross-sectional, descriptive and observational in nature.

Study population

Our study population included all SARS-CoV-2 infected patients who attended either inpatient or outpatient department in a tertiary care hospital in eastern India during the study period of 3 months from June 01st, 2020, to August 30th, 2020. Data were collected from 870 patients.

Study tool

Sample collection

Respiratory tract samples (oropharyngeal swabs and nasopharyngeal swabs) with the clinical diagnosis of COVID-19 were collected from

870 patients according to the manufacturer's guidance in adequately labelled viral transportation tubes in triple layer packing and sent to RT-PCR laboratory at the Department of Microbiology, Medical college and hospital, Kolkata, in cold box immediately.

RNA extraction

Collected samples were processed as soon as possible on receipt in the laboratory. In the event of any delay, they were stored at 2–8°C upto 24 h or −70°C for longer storage. Multiple freeze-thaw cycles of the specimens were avoided. RNA was extracted from the specimens using RNA extraction kit (QIAGEN or HIMEDIA) in Biosafety lab III b as per manufacturer's instructions. Briefly, 560 µL carriers RNA lysis solutions were added to the 140 µL sample and mix thoroughly, incubate for 10 min at room temperature. After that, centrifuge the sample, for 10 s; add 560 µL of ethanol and then transfer the lysate to spin column. Finally, after double wash elute the SARS CoV-2 RNA from sample and immediately subjected to RT-PCR.

RT-PCR for detection of gene

Freshly extracted RNA was used for RT-PCR testing; otherwise, it was stored in −20°C for 24 h and at −80°C if required to be stored for more than 48 h. RT-PCR reaction mix was prepared in a separate reagent preparation room using RT-PCR kits supplied by the Indian Council of Medical Research (National Institute for Research in Bacterial Infections) for COVID-19 as per the manufacturer's instructions. The specified amount of master mix, template RNA, positive control, and negative control were dispensed in the polymerase chain reaction (PCR) plate on ice box in Biosafety cabinet Class II B2 in a separate PCR room. The PCR plated was then sealed and loaded in the PCR instrument (CFX96™ real-time PCR detection system by bio-rad) after setting the PCR condition in the CFX software as per RT-PCR kit protocol.

E gene, N gene, RdRp (ORF1ab) gene, ORF1b gene and human R Nase P (internal control) gene targets were detected by means of fluorescent channel assigned and computed tomography (CT) analysis was done. Test validity and interpretation of each specimen result were done.

During the pandemic of COVID-19, samples were screened by RT-PCR at our institute for genes of SARS-CoV-2, using commercial kits. Most of the kits use a combination of two genes for detection of SARS-CoV-2 RNA. Those samples where at least one gene could be detected were run with a different RT-PCR kit targeting different set of genes. A total 870 samples were included in the study and a total of four genes were screened for, which includes nucleoprotein (N), envelope (E), RdRp, ORF1b.

Data analysis

Data analyses were performed using MS Excel software. Categorical variables were expressed in frequency and percentages and contingency tables were made for bivariate analysis.

Ethical issues

The study was approved by the institutional ethics committee. Informed consent was obtained from the study subjects. Anonymity and confidentiality of data were ensured. Accordingly, oropharyngeal swabs and nasopharyngeal swabs were obtained from the patients.

RESULTS

In our study, among all the genes, E gene was the most frequently detected (n=665; 86.70%) closely followed by N gene (n=429; 83.30%). ORF1b could be detected in 69.73% (n=159). RdRp was least frequently detected in only 44.64% (n=175). This makes the E gene as one of the targets while developing commercial kits for detection.

In the group where four genes (E gene, N gene, RdRp gene, ORF1b gene) were screened for, all four could be detected in only 2.46%(n=5), and three genes could be simultaneously detected only in 35.96% (n=73). The overall detection rate of positive cases (where at least two genes could be detected) was 77.34% and result was inconclusive in 14.28%. However, the significant finding is that, 52.70% (n=107) samples would have been tested inconclusive if additional two genes were not targeted in this group, and by this way, 38.42% (n=78) more samples could be confirmed positive. Hence, increasing the number of gene targets in any commercial assay could potentially increase the diagnostic yield of the assay.

In the group where three genes (E gene, N gene, ORF1b gene) were screened for, all three were detected in 10% (n=2), two were detected in 25%. Overall detection rate of positive cases were 35% only and inconclusive results were found in 65% cases.

Two genes (N gene/E gene and N gene/ORF1b gene) were screened for in 647 samples and both genes were detected in 91.03% (n=589) of them. The rate of detection of positive cases was 91.03% and inconclusive results were found 8.80%.

To find out which combination of target genes will be optimal to increase the diagnostic yield of the assay we tried to find out the concordance of positive results between target genes.

When three genes were tested together the highest concordance was found between N gene, E gene and ORF1b. These three genes were detected together is only 33.18%. However, if we take into consideration screening for two genes the results are as follows.

The E gene and ORF1b could simultaneously be detected in 85.12% samples when tested together. E gene and N gene could be detected together in 71.16% and E gene and RdRp could be detected in only 53.41%. This makes the combination of E gene and ORF1b as optimal targets for assay design as these two genes are expressed simultaneously, that will minimize inconclusive results and maximize diagnostic yield (Table 4).

N gene could be detected in isolation in 8.50% (n=74;) of the samples, however, it is the most common gene associated with inconclusive results (75.51%) and inclusion of N gene in assay that may potentially increase the number of inconclusive results in the assay (Table 5).

DISCUSSION

The pandemic of the recently emerged 2019-nCOV viral infection is a challenge to public health. During the pandemic situation, testing is crucial to track the spread of the disease, so that proper and timely public health interventions including isolation, quarantine, and appropriate clinical management of afflicted individuals can be taken. The current gold standard for the molecular diagnosis of SARS-CoV-2 infection is the real-time RT-PCR for the qualitative and quantitative detection of viral nucleic acids [7]. RNA is reverse transcribed to cDNA and subsequently amplified using a real-time quantitative RT-PCR (qRT-PCR) instrument. WHO announced various primer and probe sets for SARS-CoV-2, targeting different sections of the virus genetic sequence including the envelope E gene, ORF1a, ORF1b, the RdRp gene, and the N gene [8,9]. Various manufacturers or agencies have opted for different sets of genes and developed RT-PCR kits that detect SARS-CoV-2 virus either using two individual single-step RT-PCR assays for identification and amplification of any two genes of the viral genome or by multiplex assays using more than one gene in single reaction [6]. Based on the different sets of genes used, sensitivity and specificity of the RT-PCR is varied.

We had compared the different sets of genes to find out the best possible combination for identification 2019-nCoV from infected patients. Among the four genes detected (ORF1b, RdRp, E, N gene), E gene was detected most frequently followed by N gene in our study (Table 1). According to Corman, targeting the E gene reported highest sensitivity followed by the RdRp gene for confirmation [9]. However, according to Cheng *et al*., N protein is most abundantly expressed viral protein in infected cells, so may have a higher sensitivity but clinical

Table 1: Percentage of different gene detected by RT-PCR

Type of gene	Detected (%)	Not detected	Total
N gene	429 (83.30)	86	515
E gene	665 (86.70)	102	767
RdRp	175 (44.64)	217	392
ORF1b	159 (69.73)	69	228

N gene: Nucleocapsid gene, E gene : Envelope gene, RdRp: RNA-dependent RNA polymerase, ORF1b : Open reading frames, RT-PCR: Reverse transcription polymerase chain reaction

Table 2: Percentage of target genes detected in combination

Group	Sample number	Result (%)	
Group 1 four genes screened	203	All four detected	5 (2.46)
		Three detected	73 (35.96)
		Two detected	79 (38.91)
		One detected	29 (14.28)
		None detected	17 (8.37)
Group 2 three genes screened	20	All three detected	2 (10)
		Two detected	5 (25)
		One detected	13 (65)
		None detected	0
Group 3 two genes screened	647	Two detected	589 (91.03)
		One detected	57 (8.80)
		None detected	1 (0.15)

Table 3: Target genes detected simultaneously when at least three genes were targeted

Screening of genes	Sample tested	Concordance
N gene+E gene+ORF1b	223	74 (33.18)
N gene+E gene+RdRp	223	9 (4.03)
E gene+RdRp+ORF1b	223	7 (3.13)
N gene+RdRp+ORF1b	223	6 (2.69)

RdRp: RNA-dependent RNA polymerase, ORF1b: Open reading frame

Table 4: Target genes detected simultaneously when at least two genes were targeted

Screening of genes	Sample tested	Concordance
E gene+RdRp	322	172 (53.41)
N gene+E gene	482	343 (71.16)
E gene+ORF1b	363	309 (85.12)

RdRp: RNA-dependent RNA polymerase, ORF1b: Open reading frame

Table 5: Frequency of different target genes associated with inconclusive results

Gene	Inconclusive result
N gene	74 (75.51)
E gene	15 (15.30)
ORF1b	7 (7.14)
RdRp	2 (2.04)

RdRp: RNA-dependent RNA polymerase, ORF1b: Open reading frame

data are lacking. Ravi *et al*. compared various RT-PCR kits and observed that S gene target could be used to distinguish between SARS-CoV and SARS CoV-2 more conveniently followed by N gene (which shared 90% homology) [10]. Wang *et al*. found that RT-PCR assays targeting RdRP gene had the highest sensitivity and that the lower respiratory tract samples showed higher rate of positivity for this gene [11].

We found that the detection of positive cases could be made more confidently when two genes were targeted (91% cases) rather than targeting a single gene or more than two genes (Table 2). Inconclusive result was found in rest of the 9% cases where single gene was detected. Artesi *et al*. identified and reported a single nucleotide polymorphism in the E-gene of SARS-CoV-2 which was associated with the failure of qRT-PCR which targeted the E -gene in the cobas® SARS-CoV-2 dual target assay (Roche) [12]. The other target ORF1b was identified correctly. Another dual target (N and E gene) SARS-CoV-2 RT-PCR assay failed to identify the N gene target due to G29140U mutation [13]. Studies by Colton *et al*. suggested targeting E gene alone could account for non-specificity as it shared by various corona viruses and a further confirmation by RdRP gene amplification was essential [14]. They also observed that the CT values of both E and RdRP genes were the lowest around 48–72 h of symptom onset and the median CT value of RdRP was higher than that of E gene. Shirato *et al*. also emphasized need for dual targets as the mutant variants particularly in the primer/probe binding regions impact the efficiency of the qRT-PCR assays [15]. Further we studied the best combination of two genes and our finding concluded that set of E gene and ORF1b was the best possible combination showing 85% concordance compared to N and E gene (71% concordance) and E and RdRp (53% concordance) (Table 3).

CONCLUSION

According to our findings, targeting two genes, precisely E gene and ORF1b of SARS-CoV-2 genome in RT-PCR from nasopharyngeal swab has highest sensitivity and specificity in diagnosing COVID-19.

CONFLICT OF INTEREST

None.

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