Original Contribution

SARS-CoV-2 Detection using Real Time RT PCR by a Commercial Diagnostic Kit

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There is a new public health problem around the world with the emergence and spread of 2019 novel corona virus (2019-nCoV). The disease "coronavirus disease 2019" (COVID-19) caused by SARS-CoV-2. As virus isolates are unavailable so the public laboratories are now facing a challenge for detecting the virus because there is growing evidence of the outbreak which is more widespread than initially thought. We aimed here to discuss about the current diagnostic methodology for detecting the SARS-CoV-2 in health laboratories. Here we use the Novel Corona virus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) which is a real time reverse transcription polymerase chain reaction (rRT -PCR) test. A total of 230 samples in the department of microbiology, Mymensingh Medical College from 1st, April 2020 were selected for this study. Among them 20(8.69%) were positive for SARS CoV-2 and remaining were negative. Among the positive samples 55% could amplify both the ORF 1ab and N genes. The single gene ORF 1ab or N was positive in 15% and 30% cases respectively. The Ct values (<38) of ORF 1ab gene indicated by FAM dye was 92.8% and N gene curve indicated by ROX dye was 100%. The presence of IC gene curve with Ct values (<38) indicated by CY5 dye among the positives were 70% and 100% in negatives. The Ct values (38-40) of IC (CY5) among the positives were 15%. The present study demonstrates the enormous response capacity of the study kit for detecting SARS-CoV-2 within the laboratories in Bangladesh.

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Key words: SARS-CoV-2, COVID-19, Real-time RT-PCR (rRT-PCR), Nasopharyngeal

Introduction

The modern era is now challenging with a threat of Covid-19 disease that is now more difficult to control among the countries of the world. The current outbreak of coronavirus disease (COVID-19) that was first reported from Wuhan, China, in December 2019. This epidemic had spread to 216 WHO countries and territories around the world and 2 international conveyances with 7,145,539 confirmed cases, including 408,025 deaths, as of June 10, 2020[1]. The World Health Organization declared it as a Public Health Emergency of worldwide[2]. On Feb. 11th, 2020, the new corona virus was officially renamed "SARS-CoV-2" from "2019-nCoV"[3]. Middle East respiratory syndrome coronavirus (MERS-CoV) had become a worldwide health concern. MERS-CoV originally reported in 2012. It affected more than 2000 people in 27 countries and 4 sub-continents in the Middle East[4]. While in 2003, the epidemic of SARS affected 26 countries and resulted in more than 8000 cases[4]. One of the biggest challenges in effective control of the COVID-19 outbreak is the fact that in compared to similar viruses, the SARS-CoV-2 is more contagious because of its environmental stability; having longer incubation, higher rate of non-symptomatic infections as demonstrated from multiple independent sources[5], making early detection of virus infection is one of the most critical tactics in the battle against the virus.

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Therefore, the virus is spreading easily in overcrowded areas. Most patients are only mild to moderate symptoms, such as high body temperature in conjunction with some respiratory symptoms such as cough, sore throat, and headache. Some people may have severe symptoms like pneumonia and acute respiratory distress syndrome[6]. Also, individuals with underlying co-morbid conditions such as heart disease, chronic lung disease, or diabetes potentially display more severe symptoms[7]. Preventive measures such as masks, frequent hand washing, staying home when sick, avoid public contact, and quarantines are being recommended for reducing the transmission. To date, no specific antiviral treatment is proven effective; hence, infected people initially rely on symptomatic treatments that showed encouraging profile for blocking the new coronavirus in early clinical trials. Among the foremost priorities to facilitate public health interventions it is necessary for reliable laboratory diagnosis. In acute respiratory infection, RT-PCR is routinely used to detect causative viruses from respiratory secretions[8].

SARS-CoV-2 was found to be a positive-sense, single-stranded RNA virus belonging to the *Betacorona* virus B lineage and is closely related to the SARS-CoV virus[9]. Full-length genome sequences were obtained and indicated that the SARS-CoV-2 genome shares 79.6% sequence identity with that of SARS-CoV[10]. The genome size of the SARS-CoV-2 varies from 29.8 kb to 29.9kb and its genome structure followed the gene characteristics of the 5′ more than two-thirds of the genome comprises ORF 1ab encoding ORF 1abpolyproteins, while the 3′ one third consists of genes encoding structural proteins including surface (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Figure 1)[11,12].

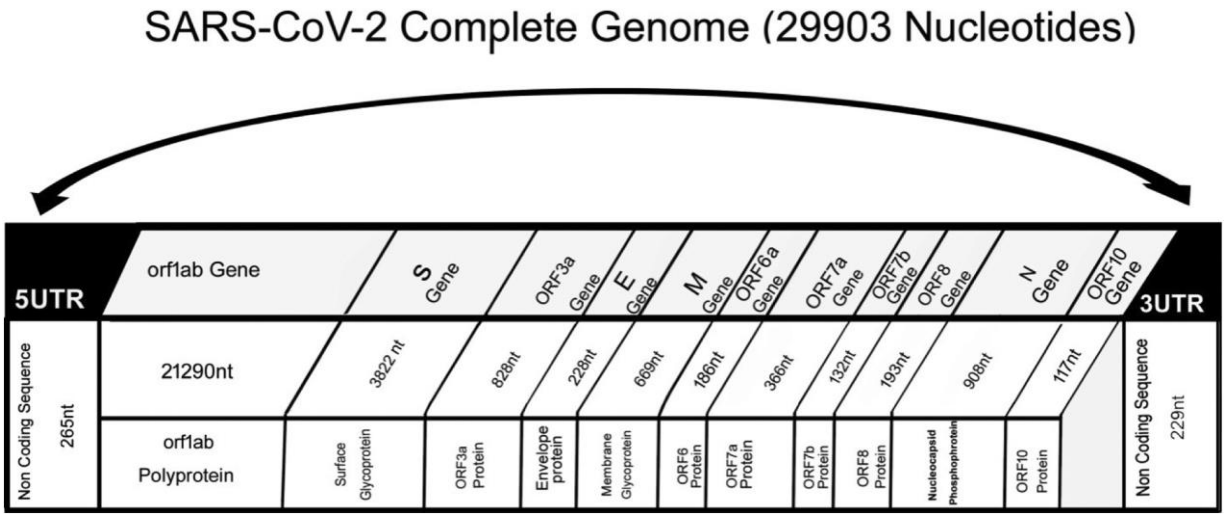

Figure 1: Structure of the SARS-CoV-2 genome[11,12]

The Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) is a real-time reverse transcription polymerase chain reaction (rRT -PCR) test. The 2019-nCoV primer and probe sets are designed to detect RNA from the 2019-nCoV in nasopharyngeal/ oropharyngeal swabs from patients who meet CDC 2019-nCoV clinical criterias

(e.g., signs and symptoms associated 2019-nCoV infection) in conjunction with CDC 2019-nCoV epidemiological criterias (e.g., history of residence in or travel to a geographic region with active 2019-nCoV transmission at the time of travel, or other epidemiological criteria for which 2019-nCoV testing may be indicated). This kit is used for qualitative detection of the ORF 1ab and N genes of 2019-nCoV. In this study we targeted to detect the positivity rate of SARS-CoV-2 virus detection and also to detect the presence rate of ORF 1ab gene and N gene by this kit in positive samples.

Methods

Samples like nasopharyngeal swabs were collected with the clinical criterias according to the National Guidelines on Clinical Management

of Coronavirus Disease 2019 (Covid-19) published by Directorate General of Health Services (DGHS) and Ministry of Health & Family Welfare (MOHFW), Government of the People's Republic of Bangladesh were collected. A total of 230 samples from Mymensingh Medical College which was started for SARS-CoV-2 laboratory in the department of microbiology from 1st, April 2020 were selected for this study.

RNA extraction

After collecting the sample the tube was centrifuged. Ten microliter samples were taken by a micropipette and mixed with ten microliter (10μL) lysis buffer containing PCR tube. All the procedures were done under BSL2 cabinet with maintaining standard protocol.

(rRT -PCR)

The primers and probes targeted the ORF 1ab and N of SARS-CoV-2 according to Chinese CDC protocol [13].

Target 1 (ORF 1ab),
forward: 5'-CCCTGTGGGTTTTACACTTAA-3',
reverse: 5'-ACGATTGTGCATCAGCTGA-3',
probe: 5'-FAM CCGTCTGCGGTATGTGGAAAGGTTATGG-3';

Target 2 (N),
Forward: 5'-GGGGAACTTCTCCTGCTAGAAT-3',
Reverse: 5'-CAGACATTTTGCTCTCAAGCTG-3',
Probe:
5'-ROX TTGCTGCTGCTTGACAGATT-3'.

Target 3 (RNAse P)[14]
Forward: 5'-AGATTTGGACCTGCGAGCG-3'
Reverse: 5'-GAGCGGCTGTCTCCACAAGT-3'

Probe
5'-CY5 TTCTGAACCTGAAGGCTCTGCGCG-3'.

According to the total number of specimens, 2019-nCoV-PCR-Positive Control and 2019-nCoV-PCR-Negative Control[15], calculated volume of 2019-nCoV-PCR Mix and 2019-nCoV-PCR Enzyme Mix (2019-nCoV-PCR Mix 26μL/test + 2019-nCoV-PCR-Enzyme Mix 4μL/test) were mix thoroughly to make a 2019-nCoV-PCR master mix. Add 30μL 2019-nCoV-PCR master mix into each PCR tube containing 20μL sample solution mentioned above. Carry out fluorescence quantitative PCR detection on the fluorescence PCR instrument. Place PCR reaction tubes into the specimen wells of the amplification equipment. Set up the 2019-nCoV-PCR-Positive Control, 2019-nCoV-PCR-Negative Control and specimens to be tested in the corresponding sequence and input specimen name. Then selection of PCR test channel, FAM (ORF 1ab region) and ROX (N gene) channels to test 2019-nCoV nucleic acid.

Next selection of CY5 channel to test internal control. Finally set cycle parameters[15]:

	Steps	Temperature	Time	Cycle
1	Reverse transcription	50	30	01
2	cDNA predenaturation	95	01	01
3	Denaturation	95	15	45
	Annealing, extension and fluorescence collection	60	30	
4	Device cooling	25	10	01

When the settings are completed, save the settings and carry out the reaction procedure.

Results

A total of 230 nasopharyngeal swabs from patients with or without signs and symptoms of infection that were suspected of COVID-19 or had a contact history with COVID-19 were analyzed by rRT-PCR. We have detected 20(8.69%) positive and 210(91.3%) negative for SARS - CoV-2. Among the positive samples 55% could amplify both the ORF 1ab and N genes. The single gene ORF 1ab or N was positive in 15% and 30% cases respectively. The Ct values (<38) of ORF 1ab gene indicated by FAM dye was 92.8% and N gene curve indicated by ROX dye was 100%. The presence of IC gene curve with Ct values (<38) indicated by CY5 dye among the positives were 70% and 100% in negatives. The Ct values (38-40) of IC (CY5) among the positives were 15%.

For positive interpretation

If the N gene, the ORF 1ab gene and Internal control (IC) gene are positive (Ct ≤40) or both the

N gene and the ORF 1ab gene are positive (Ct ≤40) without IC gene, then the sample test result is positive.
If only the N gene or the ORF 1ab gene is positive (Ct ≤40) and IC gene is with or without, then the sample test result is positive.

Table I: Presence of target genes in positive samples (n=20)

Different genes	Number	Percent (%)
Both (ORF 1ab+N)	11	55
ORF 1ab	03	15
N	06	30
Total	20	100

Table II: Different Ct values among positive samples (n=20)

Genes	Ct values (%)	
	(<38)	(38-40)
ORF 1ab (FAM) (14)	13 (92.8)	1 (07.14)
N (ROX) (6)	06 (100)	0 (00.00)

Table III: Presence of Internal control gene (CY5) among the total samples (n=230)

Results	Ct values (%)	
	(<38)	(38-40)
Positive (20)	14 (70)	3 (15)
Negative (210)	210 (100)	0 (0)

For negative interpretation
Presence of internal control (CY5) gene (Ct<40), with no signals detected for ORF 1ab gene (FAM), N gene (ROX) or with ORF 1ab gene (FAM), N gene (ROX) both Ct>40.

Discussion
The present report describes the establishment of a diagnostic workflow for detection of an emerging virus by rRT-PCR. In this study, a total of 230 nasopharyngeal swabs from patients with or without signs and symptoms of infection that were suspected of COVID-19 or had a contact history with COVID-19 were analyzed by rRT-PCR. We have detected 20 (8.69%) positive and 210(91.3%) negative for SARS-CoV-2. Among the positive samples 55% could amplify both the ORF 1ab and N genes. The single gene ORF 1ab or N was positive in 15% and 30% cases respectively. The ORF 1ab has the highest specificity for confirmation of the target gene, but is considered to be less sensitive than other targets in clinical application[16].
In our study, we got the Ct values (<38) of ORF 1ab gene indicated by FAM dye was 92.8% and N gene curve indicated by ROX dye was 100%. Some scholars believe that the detection of N gene is an accurate, rapid, early and simple diagnostic method for COVID-19[17]. An increasing number of articles showed that the SARS-CoV-2 is undergoing rapid mutation[18,19]. Ninety-three mutations were found over the entire genomes of SARS-CoV-2 with twenty-nine missense mutations in the ORF 1ab region and four in the N region[20]. Fortunately, through gene comparison on BLAST, the primer or probe sequences published by CDC, China and WHO were not in these mutation regions[21].
Here internal control (IC) gene is targeting human RNAse P gene. It is used here to monitor the quality of sample collection, sample handling and rRT-PCR process to avoid false negative results. The low concentration of the IC gene in master mix ensures that the specificity and sensitivity of the test are not affected by competitive amplification of other cDNAs[22]. The IC gene amplification signal may disappear in positive samples where the pathogen cDNA is present in high amounts[22]. In this study, the presence of IC gene with Ct values (<38) and (38-40) indicated by CY5 dye among the positives were respectively 70% and 15%. The IC gene signal should always be detected in negative samples (absence of the pathogen). Here, we found IC gene with Ct values (<38) indicated by CY5 dye among all the negative samples (100%).

Conclusion
With an increasing number of potential cases emerge; the SARS-CoV-2 poses a major threat to global public health. Early detection and differential diagnosis of respiratory infections increase the chances for successful control of COVID-19 disease. The rRT-PCR test is used as a widely deployed in diagnostic virology and is regarded as the current standard for molecular diagnosis with high sensitivity.

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