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DIAGNOSIS OF SARS-COV-2 VIRUS WITH REAL TIME PCR IN AMBEDKAR NAGAR DISTRICT OF UTTAR PRADESH: A NEAR CARE APPROACH

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ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) is an emerging human-to-human infectious disease that broke out in early December 2019 and threatens global public health, causing widespread concern. This respiratory disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The development of reliable techniques for COVID-19 diagnosis is a significant step to prevent further infection. Thus in this study we see the prevalence of Covid 19 cases in eastern part of Uttar Pradesh by a molecular diagnostic method.

Aim and Objective: To study the diagnosis of sars-cov-2 virus with real time PCR in patients attending the tertiary care centre.

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Material and Methods: This was a Retrospective study carried out in the Department of Microbiology between September 2020 to June 2022. The Samples were collected from COVID-19 clinically suspected patients at GMC, Ambedkarnagar and evaluated with real time reverse transcriptase polymerase chain reaction (RT-PCR) tests. Also few samples were sent for genome sequencing and analysed.

Results: In our study a total of 2999 patients were diagnosed with COVID-19. Five samples showed emerging delta strain in mid part of study.

Conclusion: RT-PCR amplification of viral nucleic acid has been widely recognized as the gold standard for diagnosis of COVID-19 and can effectively confirm timely and accurate SARS-CoV-2 infection.

Keywords: SARS-CoV-2, RT PCR, Nucleic acid amplification, COVID-19 Laboratory diagnosis, Genome Sequencing

INTRODUCTION

COVID-19, an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, was first reported in Wuhan, China in December 2019[1]. In December 2019, an outbreak of coronavirus disease 2019 (COVID-19) began in Wuhan, China and causing rapid person-to-person transmission led to a global pandemic[2]. In March 2020, the WHO declared the COVID19 outbreak a pandemic. As of November 2021, COVID-19 has accounted for over 250 million cases and over 5 million deaths globally[3] .

Whole genome sequencing of viruses in lower respiratory tract samples revealed that the outbreak was caused by anovel beta-coronavirus[4] with phylogenetic similarity to SARS-CoV. The novelcoronavirus was named severe acute respiratorysyndrome coronavirus 2 (SARSCoV-2) by the International Committee on Taxonomy of Viruses.

The clinical features of COVID-19 arecomplex and similar to those of other diseases including influenza virus infection.Symptoms include fever, cough, sore throat and fatigue. Therefore, rapid diagnosis of COVID-19 in suspected cases is essential to curb viral transmission and to initiate appropriate therapies. Thus, serological tests are increasingly applied for diagnosis of SARS-CoV-2 infection[5].

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Reducing the transmission of SARS-CoV-2 from asymptomatic and pre-symptomatic patients by laboratory testing is critical in controlling the circulation of the virus[6] can be done extensive testing for SARS-CoV-2.

In fact, SARS-CoV-2 molecular assays have become an integral component in a multipronged strategy (case identification (i.e., through testing), quarantine or isolation of exposed/infected individuals, and contact tracing) aimed at reducing transmission of the virus[7]. Timely and accurate reporting can lead to proper contact tracing and effective containment measures.

The first step in managing COVID-19 is the rapid and accurate detection of SARS-CoV-2 enabled by the real-time reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR detects SARS-CoV-2 nucleic acids present in nasopharyngeal fluids. Testing is used to prevent infectious spread between persons and communities that include asymptomatic infected persons whose viral shedding can inadvertently spread the infection to the elderly and those with disease comorbities[6].

Molecular tests, such as real-time PCR, have become a cornerstone in the diagnosis of infectious diseases and have been the most common laboratory method utilized during the COVID-19 pandemic. Quantitative fluorescence-real time reverse transcriptase polymerase chain reaction (RTPCR)-based assays are regarded as the gold standard test for COVID-19 pandemic[8] because of the inherent sensitivity, specificity[9], cost effective, reliability[10]and simple quantitative analysisof real-time PCR that allows for limit of detection at detection at 100 copies/ml or less of target nucleic acid in clinical samples[11].This corresponds to the viral detection at 2–3 days before the onset of symptoms[12]. However, PCR-based detection has many limitations such as the requirement of high purity sample, expensive laboratory equipment, expensive kits, trained technicians[13]. Furthermore, RT-PCR requires respiratory tract specimens, such as throat swabs, nose swabs or sputum samples, which could delay diagnosis and treatment[14]. As per the published literature its sensitivity is estimated to be 70–98% and specificity is approximately 95%[15].

At present, definite diagnosis of COVID-19 is mainly based on positive results reverse transcriptase polymerase chain reaction (RT-PCR)amplification of viral nucleic acids from respiratory tract specimens[16].

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Thus in this study, we comprehensively analyzed the COVID-19 patients at eastern part of Uttar Pradesh. We assessed prevalence and analyzed the prevalent mutation strain of COVID-19.

MATERIALS AND METHODS

Study population and sample collection

PCR test was performed at Mahamaya Rajkiya Allopathic Medical College, Ambedkarnagar. RT-PCR was carried from September 2020 to June 2022. Improper collection of specimens may lead to false negative test results. The specimens most commonly preferred from upper respiratory tract are nasopharyngeal and/or oropharyngeal swab, as recommended by the World Health Organization[17].

The patients included cases with detectable SARS-CoV-2 RNA in respiratory samples since disease onset (defined as RT-PCR positive cases) and cases with clinical manifestations characteristic of COVID-19 but no detectable viral RNA in upper respiratory tract samples (defined as RT-PCR negative cases).

Procedures: We have tested and reported on approximately 663940 samples during the period of September 2020- June 2022, using multiple diagnostic kits as well as different platforms. But here we are discussing the process of one kit of them. A volume of 200 µl of VTM from each of the individual samples was used for pooling. Therefore, the total volume of a 5-sample pool was 1 ml. RNA was extracted from both 5-sample, as well as few individual samples using the RNA extraction kit. In other studies concordance between individual sample testing and pooled sample testing was calculated and expressed in percentages[18,19,].

A volume of 200 µl of the pooled sample/individual sample was used for RNA extraction.

RNA isolation

In the current study the Viral RNA was extracted from nasal/throat swabs collected in virus transport medium (VTM) which were sent to the microbiology laboratory at Government Medical college, Ambedkar Nagar (MRAMC) for SARS-CoV-2 detection. RNA extraction was performed from 200 µL of the VTM using the GB's Aura Pure Corona Virus (Covid-19) RNA Isolation kit (Spin Column based) according to the manufacturer's instructions.

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The procedure employed the glass-fiber membrane technology for the fastest and the most convenient of high purity RNA isolation. Sample lysis was achieved by incubation with a lysis buffer. During RNA extraction, 20µl of GB RNA IC was added to the lysate after the lysis step (after adding ethanol). Then continue the column binding step to ensure the quality of subsequent experiments. Viral RNA isolation kit buffer system provides the efficient binding condition of RNA to glass-fiber membrane and impurities on the membrane are washed to remove contaminants using two different wash buffers. Residual ethanol from previous wash steps is removed by air drying. Finally, highly pure RNA is eluted with low-salt elution buffer or nuclease free water.

Real-time PCR Protocol

Real-time RT-PCR assay was done using a SARS-CoV-2 nucleic acid detection kit (GB SARS-CoV-2 Real-Time RT-PCR), which mainly targets the open reading frame 1ab (ORF1ab)and E gene. The corresponding reaction mixture that includes RT-Master mix(master reagents specific for RNA) and GB SR (enzyme mixture required for RT-PCR). then, multiplex Mix (primers and TaqMan probes which is specific to the E gene and ORF1ab region of SARS-CoV-2) was mixed, according to the manufacturer's protocol. It was centrifuged briefly before adding into reaction tubes. 15µL of the reagent mixture was dispensed into each reaction well. Then was added 10 µL of RNA extracted from the specimen, positive control (PC, SARS-CoV-2) and Negative Control (NC,H_2O) to the well

rRT-PCR reaction

The mixture was dispensed in 96-well plates (MicroAmp™ Fast Optical 96-well reaction Plate 0.1 mL, Applied Biosystems) and sealed with optical film (MicroAmp™ Optical Adhesive Film, Applied Biosystems) and centrifuged briefly. Meanwhile, RNase/DNase-free ddH_2O was added to the negative control tubes to check any contamination or primer dimer.

Quantitation experiments were performed in a real-time PCR instrument (CFX 96 RTPCR). Then, the qPCR reaction conditions were adjusted as follow: 1) Reverse transcription at 48°C for 15 min, 2) Taq activation at 95°C for 10 min, 3) 45 cycles of denaturation at 95°C for 15 sec and annealing/Extending at 60°C for 30 sec. The reporter dye channel sets as FAM for viral *E* gene;

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and Texas Red for *ORF1* abgene; and VIC for *Internal control* gene. Results were considered positive if the cycle threshold value was less than 37 and the sample was positive for both ORF1ab and E genes, negative if the cycle threshold value was more than 37, and retesting was recommended when result of the VIC is more than or equal to 37.

***Genome sequencing of* SARS-CoV-2**

Oropharyngeal swab samples in the viral transport medium (VTM) previously tested positive for SARS-CoV-2, and appropriately stored at −80°C, were used. While sequencing is important to monitor on-going evolution of the SARS-CoV-2 genome and as genome sequencing of all samples were not possible so 13 out of 25 samples were selected of a lot were sent to IGIB, New Delhi for WGS.

RESULTS

In the present study a total of 663940 COVID-19 patients were enrolled in the study. During a study period , out of subjects tested, a total of 2999 patients were diagnosed with COVID-19 (Table 1). Thirteen RT-PCR positive nasopharyngeal secretion samples were sent for whole genome sequencing. The analysis of the strains revealed that 69 % of the sequences belonged to the B.1.617.2 (delta strain) and rest 30.7% showed none variant (Table 2).

Table 1.Detection of COVID-19 patients by RT-PCR.

Duration	**Total cases**	**RT-PCR positive cases (n=2999)**	**RT-PCR negative cases**	**P value/Positivity rate**
Sept 2020 till June 2022	**663940**	**2999**	**660941**	**0.03**

Table 2. Whole genome sequencing of 13 samples

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S.NO	VoC or Mutation	Mutations Detected	Name of the variant
1.	Yes	210:G:T,241:C:T,ORF 1a	B.1.617.2
2.	Yes	210:G:T,241:C:T,ORF 1a	B.1.617.2
3.	Yes	210:G:T,241:C:T,ORF 1a	B.1.617.2
4.	Yes	210:G:T,241:C:T,ORF 1a	B.1.617.2
5.	Yes	210:G:T,241:C:T,ORF 1a	B.1.617.2
6	No	----	None
7.	No	ORF 1:E3909fs	None
8.	No	-----	None
9.	No	-----	None
10.	No	-----	None
11.	No	-----	None
12.	No	-----	None
13.	No	------	None

DISCUSSION

COVID-19 can cause serious, sometimes fatal, pneumonia[20] and asymptomatic COVID-19 patients may also be capable of transmission[21]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread to nearly every corner of the globe, causing societal instability. The resultant coronavirus disease 2019 (COVID-19) leads to fever, sore throat, cough, chest and muscle pain, dyspnoea, confusion, anosmia, ageusia and headache.

It is important to note that serological tests are effective methods for patients with clinical symptoms typical of COVID-19 or an epidemiological contact history but negative nucleic acid tests. However, it is found serological tests are not suitable for screening the general population in areas with low infection rates[22]. The Ct values of the reactions in our study are ranged in between 22 to 35. According to the CDC (Center of Disease Control and Prevention) and WHO recommendations, the samples with a Ct value of 37.01 or greater are considered as negative[23]. In other words, the Ct value should not exceed 37 to accept the sample as positive.

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Due to the possibility that SARS-CoV-2 molecular assays can remain persistently reactive in patients who have recovered from COVID-19 or remain asymptomatic, there has been interest in whether semiquantitative or quantitative data can assist in result interpretation. The *CT* value is inversely proportional to the amount of target nucleic acid and can be used as a relative indicator of the concentration of a pathogen in a clinical specimen. For example, a positive PCR result with an associated *CT* value of 15 would indicate a very high concentration of the target nucleic acid in a sample, whereas a *CT* of 35 may suggest that the target is present but near the assay's limit of detection. Since the C_T value is not normalized against a human gene internal control, it is possible that a high C_T value (e.g., >30) could be due to an inadequate sample collection rather than a low level of target nucleic acid or "noninfectious" virus.and, therefore, cannot be considered a quantitative result. This is because the *CT* value is dependent on a number of variables, including the assay's gene target, the extraction platform, PCR amplification chemistry, and even the quality of specimen collection. The Ct value of the *E* gene was in between 18 and 41 using the Cepheid`s system. COV2-kit revealed the Ct scores of the same gene as 27–35.5. CDC recommends the upper limit of the Ct as 37.

The utility of this test is limited by several false positive, false negative and inconclusive results at early stages of infection, scarcity of reagents and lack of well-equipped labs including trained staff. It has been observed in our study that patients with a strong clinical suspicion of COVID-19 with classical features showed negative results similar to other study. This could be because of primers used for the fragment of gene which may have mutations, amplification inhibitors, insufficient target (low copy number of virus in the patient) or sample degradation because of improper transport or handling of the samples[24]. Improper collection of specimens may lead to false negative test results. Moreover, appropriate sample collection and transport, standard laboratory protocols, stringent quality control norms, good quality RNA extraction kits, PCR kits with suitable primers can help in improving accuracy of the test results.

A careful assessment of clinical, radiological and molecular findings is required for identifying potential cases of COVID-19.

COVID-19 pandemic is still continuing, while new variants such as delta-variant[25] and omicron are spreading among the population. In addition, genetic diversity of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) plays an important role and may affect the results of the

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RT-PCR test[24]. Genomic studies have shown that SARS-CoV-2 shares approximately80% identity sequencing with SARS-CoV, The analysis of nine Genomic sequences of SARS-CoV-2 isolated in our study revealed the emergence of delta variant in May – June 2021 which have continued to merge as the variant circulates around the world at the time of writing. In addition to investigating the molecular test positivity, genomic surveillance could qualify the epidemiological investigation allowing the identification of clusters, transmission sites, and timely actions for the control and prevention of hospital infection[26].Screening and isolation of asymptomatic health care workers/ staff and general population using RT-PCR possibly had an impact on intra hospital viral circulation, significantly reducing tests positivity due to COVID-19 at a time of increased community transmission in the city and in hospitalized patients[6].

CONCLUSION

Timely and accurate diagnosis requires an RT-PCR to prevent the transmission of disease. Continued genome sequencing supports the monitoring of the disease's spread and evolution of the virus.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declarations:

Conflicts of interest: There is no any conflict of interest associated with this study

Consent to participate: We have consent to participate.

Consent for publication: We have consent for the publication of this paper.

Authors' contributions: All the authors equally contributed the work.

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