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Detection of SARS-CoV-19 Delta Mutant by RT-PCR

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Abstract:- Since March-2020, after WHO announced that novel coronavirus (COVID-19) outbreak, a global pandemic, the virus has caused significant causalities more than 200 countries across the world. Recently there has been surge in the infections due to mutation of the SARS Covid-19 virus of which delta mutants with its lineages have been prominent. At present, mutations are monitored by a consortium using NGS (Next-generation sequencing) technology which is accurate but expensive and time consuming. As a rapid, inexpensive but accurate method we have explored RT-PCR (Real Time-Polymerase Chain Reaction) to detect the mutants. This would help in the early diagnosis and surveillance of this pandemic. One hundred Covid positive samples archived with us between Jan-2021 to July-2021 were studied by RT-PCR using commercial delta mutant kit. Sanger sequencing was done to confirm the RT-PCR findings. RT-PCR method was able to detect delta mutants in 08 of the 100 samples studied (8%). The simple RT-PCR technique is found to be sensitive and specific for the detection of Cov-19 delta mutant with less turnaround time than NGS. This technique may be deployed in all the approved laboratories for Screening and surveillance to contain the spread of delta mutant in the community. This simple method can be exploited to detect other published mutants of Covid-19, when the situation arises.

Keywords:- *Covid-19, Delta Mutant, Gene Sequencing, RT-PCR, Surveillance.*

I. INTRODUCTION

The coronavirus SARS-CoV-2 is an enveloped, positive sensed, single stranded RNA (Ribonucleic Acid) genome with a size of 29.Kb [1] The SARS-CoV-2 has a typical gene characteristics known for SARS CoVs, with two overlapping ORFs (Open reading Frames); ORF1a and ORF1b located at 5' end occupying about two- thirds of genome, encoding proteins which are auto proteolytically processed into 16 non-structural proteins Nsp1 to NSP 16 [2]. The 3' end of genome encodes structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins and 9 accessory proteins, encoded by ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10 [3]. Although the structural proteins are important to form the complete viral particle, the ~200 kDa glycoprotein S plays the vital role in attachment of the virus to the host cell surface receptor ACE2 (angiotensin-converting enzyme receptor), membrane fusion and cell entry [4]. Covid -19 RNA viruses have high mutation rates due to the RNA-dependent RNA polymerase (RdRP) lacking the proofreading activity, which is usually correlated with enhanced transmission considered beneficial for viruses [5]. The Indian SARS-CoV-2 Genomics Consortium (INSACOG) initiated by the Government of India is a consortium of 28 National Laboratories to monitor the genomic variations in the SARS-CoV-2. [6]. The network carries out whole genome sequencing of SARS-CoV-2 virus across the nation, aiding the understanding of evolution and spread of the virus which is useful in aiding public health response. One of the disadvantages in this sequencing program is that the turnaround time is between 7-9 days [7].It is inevitable that we will have more mutants in the future increasing transmissibility of the virus which might bring in more morbidity and mortality among general population. So there is an urgent need to increase the surveillance of Covid-19 by detecting mutants by rapid method like RT- PCR. We have explored the possibility of detecting Covid-19 delta mutant by simple RT-PCR.

II. MATERIALS AND METHODS

This study was approved by Rangadore Hospital Ethics Committee and has been registered at CTRI (CTRI/2021/10/037034)

A. Sample Collection

This retrospective observational study was designed and conducted at Cancyte Technologies Private Limited, Bangalore, and Karnataka, India. Samples from Individuals who had Covid like symptoms, travelers and primary contacts of confirmed Covid cases were collected by healthcare workers as per the guidelines of Government of India. Details of all these individuals were collected, verified and entered in the RT-PCR app developed by NIC (National Informatics Centre), once SRF ID (sample referral form identifier) is generated by the mobile app, the individuals were sent to designated collection area for sample collection. Two samples were collected from each individuals, one from the nasopharyngeal and the other from oropharyngeal area using nylon swabs. After collection, swabs were immersed into single Viral Transport Medium- VTM. After collecting the sample, individuals were instructed to self-quarantine till the RT-PCR results were provided [8].

B. Viral Rna Extraction

Samples were collected from all the individuals and subjected for RNA extraction using Nucleic acid extraction kits. We have used both magnetic bead based automated extractor supplied by Genetix^{R} (Delhi, India) and as well as column based nucleic acid extraction supplied by NeoDX^{R} (Bangalore, India). Briefly 200ul of collected sample was taken for both automated method and manual method, in both

the methods as a first step, the specimen was lysed in lysis buffer and then continued for RNA extraction according to the manufacturer's instructions. Viral nucleic acid is purified by binding to magnetic beads and eluted in buffer as a final step.

C. Rt-Pcr For Covid-19 Detection

All the RT-PCR kits used for Covid-19 detection were approved by Government of India. The following kits were used for this study: Altona (Germany), Argene (France), Meril (India) and NeoDX (India). All these kits contain primers and probes for the following genes of Covid-19: E-gene, N-Gene and RdRp. In addition Altona kits contained primers and probes for the detection of S-gene. 10-15ul of isolated RNA was taken and subjected to RT-PCR according to the manufacturer's instructions. To perform RT-PCR, Thermo Quant fast 5 studio DX PCR machine (Thermo Scientific, USA) was used and operated according to the instructions given by the manufacturer. At the end of 45th cycle the signal obtained from each sample was analysed and interpreted according to the kit manual. As an Internal control for every sample, mainly to check PCR inhibitor and also to qualify the RT-PCR result hRNase P gene was used as a target in all the RT-PCR Kits.

D. Delta Variant Detection Kit

The kit used in this study was manufactured by Thermo Scientific (USA), Kit reference number: A51820, which could detect the mutation E484Q commonly present in B.1.617.1, B.1.617.3 (Indian Delta Variant) [9].This kit contained Sequence-specific forward and reverse primers to amplify the target sequence region. The reverse primers used in this kit helps in conversion of RNA to cDNA (complementary DNA). The kit also included two TaqMan minor groove binder (MGB) probes with non-fluorescent quenchers (NFQ), VIC dye labelled probe to detect the reference sequence and FAM dye labelled probe to detect the mutation sequence. The sample which gave positive signal for FAM dyed probe was considered as delta mutant. Totally 103 samples were subjected for delta mutant RT-PCR. After RT-PCR the data obtained was analysed using Design & Analysis software 2.6.0 provided by Thermo Scientific.

E. Sanger Sequencing

To confirm the mutation detected by RT-PCR method, we have designed the primers to amplify desired fragment of Spike region of SARS-Cov-2. After amplification, the obtained amplicons were purified and sent for Sanger sequencing using primers used for PCR.

III. RESULT

Samples were collected according to the guidelines given by ICMR and RNAs were extracted usiwell and 32 well RNA extractor. According to ICMR guidelines, samples showing positive signal must cross the set threshold of 0.2-0.4 within 35 cycles and those not crossing the threshold were considered as Negative for Cov-19 by RT-PCR [10]. In our study all the positive samples exhibited positive signals for the genes tested and Negative samples didn't show any signals for these genes except for hRNaseP which is an internal control. Three Covid-19 RT-PCR negative samples have been subjected for delta mutant study as a negative control.

A. Delta Mutant E484q

Totally 103 samples were subjected for detection of delta mutant by RT-PCR. Out of these, hundred samples were positive samples for Covid-19 and three were negative for Covid-19 by RT-PCR. The CT values of all positive samples chosen for this study were in the range of 18-25.

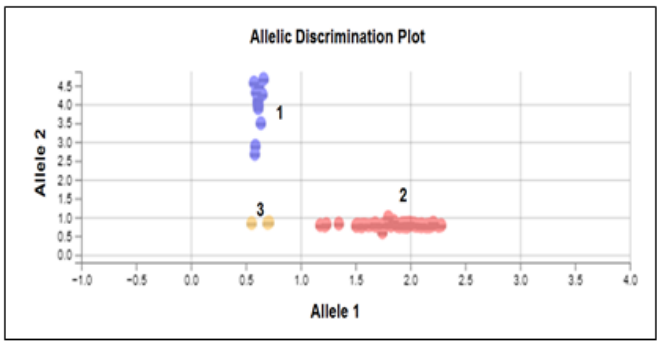

Fig.1 Allelic Discrimination Plot of RT-PCR

1. E484Q Delta Mutants (Allele 2) 2. Non E484Q -Delta Mutants (Allele 1) 3. Negative Samples. The x axis represents non-delta mutant, y axis represents delta mutant. The value crossing >1.0 on x or y axis are considered positive.

Three samples negative for Covid-19 were also subjected for RT-PCR by delta mutant kit and this acted as negative Out of 100 positive samples, 08 samples showed positive signal for Cov-19 Delta variant mutation, 92 samples showed no signals for delta mutant. As expected three samples (Negative Control) didn't show any positive signal for Delta mutants (Fig.1).

B. Sanger Sequencing Result

Sanger sequencing result mentioned in Fig 2.0 confirmed mutation at the position 484 in the Covid spike protein in all the 08 samples which were positive for delta mutant in the RT-PCR method. It showed the presence of glutamine (CAA) instead of glutamic acid (GAA) in the amino acid sequence studied. Of the remaining 92 samples which were negative for delta mutant by RT-PCR method, representative 20 samples were studied by Sanger sequencing. In all these 20 samples Sanger sequencing didn't show any mutation at position 484 in the spike protein confirming the result of RT-PCR. This also confirmed that there were no false positive or negative result with RT-PCR method.

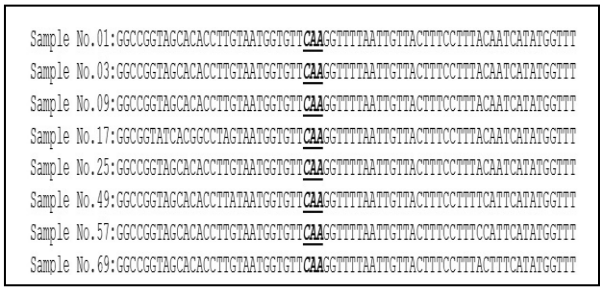

Fig.2 Sanger Sequecing result.

Sanger Sequencing showing change of amino acid, glutamic acid (GAA) to glutamine (CAA) at 484th position in delta mutant positive samples by RT-PCR.

IV. DISCUSSION

Since March-2020 after WHO announced that novel coronavirus (COVID-19) outbreak is a global pandemic, this pandemic has affected lives throughout the world. To prevent the spread of this contagious disease, the lockdown has been enforced in various parts of the world which has resulted in slowing down of the economy, agriculture, health, education and various aspects of life [11].

As the virus is spreading to many countries it also has evolved itself by changing its genome to survive among the population. In the globe so far eight notable mutants have been identified. The mutants have been named, by convention using Greek alphabets as Alpha, Beta, Gamma, Delta, Eta, Lambda, Iota, Kappa and Lambda [12]. These mutations are monitored by sequencing the positive samples by health authorities so that the disease can be contained and eventually can be eradicated. Researchers across the world have been involved in developing drugs and vaccines analyzing and utilizing the data from the sequencing consortiums.

Researchers have suggested that RNA virus mutation rates have evolved to be just under the threshold for lethal mutagenesis sometimes referred to as error threshold and that selection for genetic diversity and other consequences of a high mutation rate may push RNA viruses to near their catastrophic limits [13].

To identify the mutants of SARS-Cov-2 initially two units in the world started dealing with sequencing of SARS-CoV-19, GISAID and COVID-19 Genomics UK Consortium. So far GISAID is the largest open-access portal, hosting the genome sequences and related epidemiological and clinical data of more than 5.1 million SARS-CoV-2 strains as of Nov-12-2021 [14]. In the ongoing genomic surveillance using GISAID data, several new SARS-CoV-2 variants, such as B.1.1.7 (Alpha; first identified in the United Kingdom), B.1.351 (Beta; first identified in South Africa), B.1.1.28 (Gamma; P.1, first identified in Brazil), B.1.617.2 (Delta; first identified in India), B.1.617.1 (Kappa; first identified in India), P.3 (Theta; first identified in the Philippines), and B.1.427 and B.1.429 (Epsilon; first identified in the United States), have been identified. This information has been used to influence public health policies for the control of COVID-19 infection. Considering the benefits of genomic surveillance, scientists have pressurized countries to increase their sequencing ability, and this has led to several initiatives, such as COG-UK (United Kingdom; https://www.Cogconsort -um.uk/), INSA-COG (India; https://pib.gov.in/ PressReleseDetailm.aspx?PRID=1684782), NGS-SA (South Africa; http://www. krisp.org.za/ngs-sa/ngsa_network_for_genomic_surveillance_south_africa/)andSPHERES(UnitedStates;https://www.cdc.gov/coronavirus/2019-ncov/covid-data/spheres.Ht ml)[15].Although an increasing number of COVID-19 positive samples are being sequenced , an equally challenging issue is to upload the data quickly by the consortium . Rapid submission is important as it enables the international community to analyze the variants emerging around the world quickly and provide actionable information to governments.

Phylogenetic analysis of the data from GISAID revealed that newly identified lineages B.1.617.1 and B.1.617.2 were predominantly circulating in India (named as Delta lineage). The signature mutations possessed by these strains were L452R, T478K, E484Q, D614G and P681R in the spike protein, included within the receptor-binding domain (RBD) [16]. Of these, the mutations at residue positions 452, 484 and 681 have also been reported in other globally circulating lineages like alpha and beta. The structural analysis of RBD mutations L452R, T478K and E484Q revealed that these may possibly result in increased ACE2 binding while P681R in the furin cleavage site could increase the rate of S1-S2 cleavage, resulting in increased transmissibility. The two RBD mutations in delta variant L452R and E484Q interferes with binding of monoclonal antibodies (mAbs) to the virus and this may affect neutralization potential of the antibodies [17].

The detection of selected mutants by RT-PCR technique is a well-known method in cancer and infectious diseases [18] and agricultural biotechnology [19]. The current gold standard diagnostic tools available for the detection of SARS-CoV-19 recommended by WHO and CDC (World Health Organization/Centre for Disease Control) is RT-PCR [20].

The advantages of using RT-PCR for the detection of the mutants are rapidity, simplicity, scalability and widespread utilization. In this study we have shown that the delta mutant can be identified by RT-PCR. This can be employed by all ICMR approved laboratories across the country to provide rapid identification and better management of the mutants as a public health initiative.

Limitation of this study is the number of samples studied are limited and the detection can be done only for the known sequence as it targets only one gene. Not with standing this we have demonstrated that known delta mutants can be detected by simple RT-PCR reliably and quickly.

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