Assessment of RT PCR diagnostic assays for SARS CoV-2 detection in a Molecular diagnostic & Research Lab.

Running Title: Analysis of RT PCR assays for COVID-19 S testing in Molecular diagnostic lab .

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Abstract

Background: SARS CoV-2 infection took the whole world by storm in the final month of 2019.Different measures have been taken to reduce its spread by timely and accurate detection of COVID 19(coronavirus disease 2019) infection in suspected patients and theircontacts.

Aim: This study was conducted to assess commercially available five Rt pcr (reverse transcriptase polymerase chain reaction) kits from different manufacturers available in our center for diagnostic testing ofSARS CoV-2 infection .94 oropharyngeal clinical samples, previously confirmed as 64 positive and 30 negative for SARS CoV-2 were extracted and amplified separately by each of the five Rt pcr kitsand the results compared.

Results: The performance of different kits was in was satisfactory and above 90 percent in agreement with the standard kit for samples (n=47) with low Ct values (Ct values<30) . There was a significant variation in performance among the five kits while testing high Ct values (Ct value >30) samples (n=17).Significant variation in Ct values of E gene ,RdRp gene and N gene was observed in the Rt pcr kits results .

Conclusion: We conclude that it is necessary to assess the diagnostic performance of different Rt pcr kits for COVID 19 clinical samples from time to time to study the variation in Ct values,

sensitivity of different gene targets of SARS CoV2 virus, with proper co -ordination with other laboratories , for development of reliable COVID 19 diagnostic centers at every level.

Keywords: Rt pcrkits ,COVID- 19 ,SARS CoV-2,diagnostic

Introduction

A novel Coronavirus(SARS-CoV-2) that originated from Wuhan ,China linked to the outbreak of severe respiratory infections in humans was first reported on December 31,2019.[1]Since then it has spread across the globe to as many as 224 countries with 98,925,221 confirmed cases and 21,27,294 confirmed deaths, as on 26 January 2021.[2]

As stated by WHO ,the most effective way to prevent the infections and save lives is to break the chain of transmission and increase the Covid 19(disease caused by the new coronavirus 2019)) testing facilities exponentially to match the pace of spread of infection .[3]The laboratory diagnosis of Covid 19 just like all infectious diseases is divided into Molecular (PCR)assays and Immunoassays .[4]Real time reverse transcription polymerase chain reaction (RT PCR) test is the most sensitive and specific assay out of the various molecular assays and thus has become the current standard diagnostic method for diagnosis of Covid 19 . [5]The most important step in the RT PCR diagnostic labs is to identify RTPCR kits with high sensitivity and specificity. [6]There are around 328 RTPCR kits also known as mastermix kits validated and approved by ICMR to be used in RT PCRMolecular labs all over India.[7]

Coronaviruses are positive-stranded RNA viruses that express their replication and transcription complex, including their RNA-dependent RNA polymerase (*RdRp*), from a single, large open reading frame referred to as *ORF1ab* . The coronavirus structural proteins, including the envelope (E), nucleocapsid (N), and spike (S) proteins, are expressed via the production of sub-genomic messenger RNAs, which during certain stages of the replication cycle far outnumber (anti)genomic RNAs. The *ORF1ab/RdRp, E, N*, and *S genes* are the targets most frequently used for SARS-CoV-2 detection by RT-PCR kits . [8]

In our lab too many RT PCR kits with different gene targets combination have been used . The sensitivity of Real time PCR assays appears to be also affected by the target gene set used to test the samples .As carrying out large number of tests in economically poor countries is already putting a lot of burden , it is essential that commercially available kits used are of high sensitivities and specificities to avoid repeat testing to detect the viral nucleic acid in suspectedCOVID-19 patient. Comparison of performance of different RT-PCR kits for COVID-19 isstill limited from this part of India .Therefore this study was conducted to compare and evaluate the performance of five commercial RT PCRkits available and in use in our lab for testing SARS –CoV-2 virus infection.

Material & Methods

Study Design

The study was conducted in December –January2021. Oropharyngeal samples received from symptomatic, suspected Covid19 patients in routine in the laboratory were included in the study . Out of them, 94 samples were taken ,64 positive and 30 negative for Covid 19 virus RNA as confirmed by testing them by ICMR approved , standard NIV RT PCR kit(*RdRp a*nd *orf*gene targets) .

Five Covid 19RT PCR diagnostic Kits I ,II, III, IV ,V(-*blackbio co ltd , Bhopal;Huweii life sciences ltd, Hyderabad;Genes2me Pvt Ltd,gurugram;Genetix Biotech Ltd ,Delhi; General Biologicals corporation Ltd ,Taiwan respectively* **.)** approved and validated by ICMR, India and available in the lab during the period of study were taken for comparison in the study .The basic information about the kits is summarized in Table .1. All the five kits were compatible with Biorad CFX-96 touch ^{TM}RT PCR machine(California,U.S.A) and the detection targets included *E* gene, *N* gene,*RdRp* gene ,*RdRp+N* gene and *orf*1ab gene in different combinations as mentioned in table .1. None of the manufacturers were involved in the assessment and interpretation of the results .

Nucleic acid extraction and amplification

The samples were opened in Biosafety cabinet II B2 with utmost precautions .300µl of the oropharyngeal sample from VTM vial was pipetted in the sample rack and extracted by selected protocol-Chemagenic VIRAL 300 360 H96 prefilling short VD200626.che by Chemagenic Viral DNA/RNA kit of Chemagenic^{TM} 360 (Perkin Elmer,Massachusetts, U.S.A magnetic bead Assay) machine as per manufacturers instructions .After 32 minutes protocol run 100 µl of viral RNA elute was obtained in elution buffer rack .The extracted RNA from each of 94 samples was amplified separately by all five RT PCR kits ,according to the kits instructions,on the Biorad CFX-96 touch ^{TM} RT PCR machine.

The positive samples taken included those withCt value>=30(n=17)andCt value<= 30 (n =47).The kits targeted screening and confirmatory genes like *E*, *RdRp,RdRp+N, N, ORF 1ab gene.*

Statistics :

For Statistical analysis, Data was collected and analyzed using GraphPad Prism (San Diageo California, USA), version 8.4. Descriptive analysis was done on the reported cycling threshold (Ct) values and results were compared by the commercial RT-PCR kits and targets. ANOVA test and unpaired "t" test and post hoc tukey test was used to compare the Ct values of different gene targets reported by the commercial RT-PCR kits . Boxplots was used to show the distribution of Ct values and detection results by the different commercial kits. All P values were considered to be statistically significant at alpha < 0.001.

Results:

The RT PCR test results were determined based on the cycle threshold (Ct) of the amplification curve of various gene targets .Ct is the number of replication cycles required to produce a detectable fluorescent signal ;with lower Ct values indicating higher viral RNA load and vice versa.[9]The cut off value for concluding the test positive for SARS-CoV-2 infection for various genes in different kits have been mentioned in Table 1.

Table .1. Basic information of Rt pcr kits used in the study

Rt pcr KIT	Target genes	Nucleic acid volume	No of cycles	Ct* Value	LOD	Internal control	
I	E, Rdrp+N	10µl	38	S shaped amplification curve and Ct<=35	1000copies/ml	Rnase P	
II	E, N	10µl	40	S shaped amplification curve and Ct<=37	25 copies/rxn	IC	
III	E , N , RdRp	11.5µl	40	S shaped amplification curve and Ct<=37	Not mentioned	Rnase P	
IV	E ,RdRp	05 µl	45	S shaped amplification curve and Ct<=36	1000copies/ml	Rnase P	
V	E ,Orf	10µl	45	S shaped amplification curve and Ct<=37	500 copies/ml	IC	

The sensitivity, accuracy of kit I was maximum among the five kits .In this study sensitivity and accuracy percentages among all kits were satisfactory and around 90percent (table .3.,figure 1) As shown in table 4, the five RT PCR kits showed varied sensitivity for positive samples with low viral load i.e. with Ct value>30(n=17).The sensitivity for detecting these positive samples was significantly lower in kit III($p<0.001$, table.6.,figure 2).

Table 2. PCR CYCLING CONDITIONS USED FOR EACH OF THE RT PCR KITS IN the STUDY.

Mastermix		Kit I	Kit II	Kit III	Kit IV	Kit V
1 cycle of	Reverse transcription &Taq activation	50°C for 15 min	53°C for 10 min	55°C for 10 min	50°C for 15 min	48°C for 15 min

		95° C for 5 min	95°C for 15 min	95°C for 3 min	95°C for 3 min	95°C for 10 min
Amplification cycles &temp	Denaturation & Annealing, Extension	38 cycles 95 ° C for 5 sec 60 °C for 40 sec 72°C for 15 sec	40 cycles 95°C 15 sec 60°C for 30 sec	40 cycles 95°C 15 seconds 60° C 60 seconds	45 cycles 95°C for 10 sec 60°C for 30 sec	45 cycles 95°C for 15 sec 60°C for 30 sec
Total cycling Time		1hr 38 minutes	1hr 37 minutes	1hr 41 minutes	1hr 29 minutes	1hr 40 minutes

Table 3 . Overall Diagnostic efficacy of kits used in the study

	Kit I		Kit II		Kit III		Kit IV		Kit V	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Sensitivity	64/64	100.00	62/64	96.88	51/64	79.69	54/64	84.38	58/64	90.6
Specificity	30/30	100.00	28/30	93.3	30/30	100.00	30/30	100.00	30/30	100.0
PPV	64/64	100.00	62/62	100.00	51/51	100.00	54/54	100.00	58/58	100.0
NPV	30/30	100.00	28/29	96.55	30/39	76.92	30/36	83.33	30/33	90.9
Accuracy	94/94	100.00	92/94	97.87	81/94	86.17	84/94	89.36	88/94	89.9

Table 4 .%efficacy in detecting samples with ct value >30 and ct value <30

NIV RT PCR KIT	KIT I	II	III	IV	V
POSITIVE SAMPLES WITH CT >30 (N=17)	**POSITIVE :17**	**POSITIVE :15** INCONCLUSIVE :1 UNSATISFACTORY CURVE:01	**POSITIVE :06** INCONCLUSIVE :4 UNSATISFACTORY CURVE:07	**POSITIVE :12** INCONCLUSIVE :04 UNSATISFACTORY CURVE:01	**POSITIVE :13** INCONCLUSIVE :01 UNSATISFACTORY CURVE:03
% DETECTED amongct>30	100	88.2	35.29	70.58	76.4
POSITIVE SAMPLES WITH ct<30 (N=47)	**POSITIVE :47** INCONCLUSIVE :0 UNSATISFACTORY CURVE:0	**POSITIVE :47** INCONCLUSIVE :0 UNSATISFACTORY CURVE:0	**POSITIVE :46** INCONCLUSIVE :0 UNSATISFACTORY CURVE:01	**POSITIVE :42** INCONCLUSIVE :0 UNSATISFACTORY CURVE:05	**POSITIVE :45** INCONCLUSIVE :2 UNSATISFACTORY CURVE:0
%	100	100	97.87	89.36	95.7

DETECTED amongct<= 30					
TOTAL (N=64	**POSITIVE =64** INCONCLUSIVE :0 UNSATISFACTOR Y CURVE:0	**POSITIVE 62** INCONCLUSIVE :1 UNSATISFACTOR Y CURVE:01	**POSITIVE 51** INCONCLUSIVE :4 UNSATISFACTOR Y CURVE:09	**POSITIVE :53** INCONCLUSIVE :4 UNSATISFACTOR Y CURVE:06	**POSITIVE 58** INCONCLUSIVE :3 UNSATISFACTOR Y CURVE:03
CONFIRMED NEGATIVE (N=30)	**NEGATIVE =30**	**NEGATIVE 28 IR= 2**	**NEGATIVE =30**	**NEGATIVE =30**	**NEGATIVE =30**

table 5 . Overall summary of Ct values of different genes in the five RT PCR kits used in the study

Gene	Kit	N	Meanct value	SD	Medianct value	Min. Ct value	Max. Ct value	'p' value*	Significant difference between**
E	I	64	27.14	4.52	26	17	35	<0.001	III, IV
	II	64	27.16	5.11	28	15	39		III, IV
	III	64	23.28	10.95	27	0	35		I, II, V
	IV	64	23.1	9.45	25	0	34.5		I ,II, V
	V	64	28.55	7.66	28.9	0	39		III, IV
Rdrp	I	64	27.73	4.27	28	19	35	<0.001	II, III
	II	64	21.5	11.78	25	0	36		I
	III	64	21.85	10.28	26.05	0	33		I
N	II	64	28.78	4.77	29.7	18	40	<0.001#	
	III	64	20.86	12.31	25	0	38		
ORF1ab	V	64	26.4	9.58	28	0	38	NA	

*** ANOVA - Analysis of Variance** ****Post hoc Tukey HSD** **#Unpaired 't' test 5**
N = no of positive samples taken in study
SD=standard deviation

Table 6 .comparison of variation of results of 17 samples with ct>30 with 5 different kits in comparison with standard Niv Rt pcr kit results

	I		II		III		IV		V	
	No.	%	No.	%	No.	%	No.	%	No.	%
Sensitivity	17/17	100.00	15/17	88.24	6/17	35.29	12/17	70.59	13/17	76.47
'p value*	NA		0.464		<0.001		0.053		0.111	

***'Z' test for difference of two proportions**

The lowest Ct value was seen with *E* gene (kit ,IV) and highest Ct value with *N* gene (kit II).(table 5)
The sensitivity for detecting high viral load samples withCt value<30 was satisfactory and around 90 percent in all five kits.(table .4.)There was concordance in results by all five kits for these samples .(figure 1)

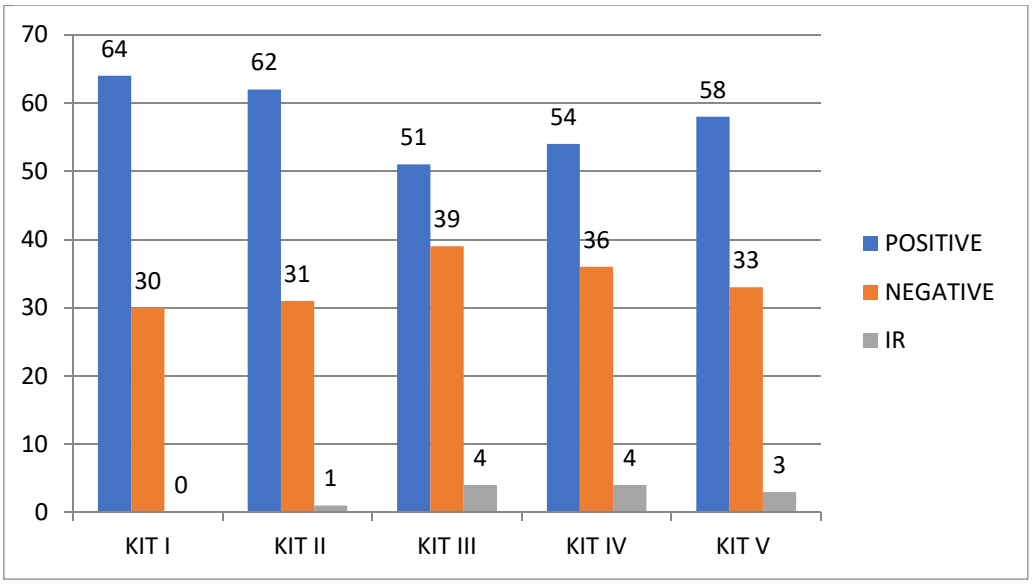


FIGURE 1 DISITRIBUTION OF RESULTS OF 94 SAMPLES BY ALL 5 KITS

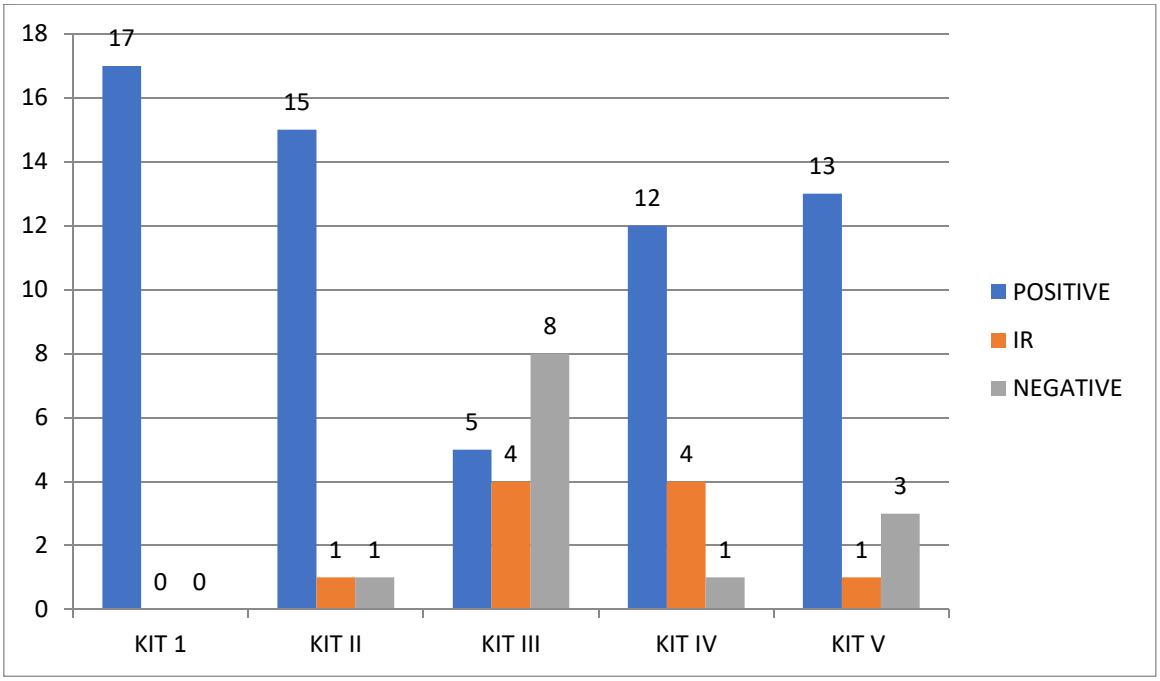


FIGURE .2. Distribution Of Results In 17 Positive samples With C_t Value > 30 with all five kits

The overall Ct value summary reported by all five kits targeting the *E* gene is given in table 5,figure 3.KitsI,II,V showed significant difference in *E* gene values in comparison to kit III and IV ($p<0.001$, test of ANOVA and Post hoc Tukey HSD test).
Similarly there was a significant difference inCt value of*RdRp* genein kit I in comparison to *RdRp* geneCt values in kit II , III.(table 5,figure 4).
Similarly there was a significant difference in Ct value of *N* genein kit II in comparison to *N* geneCt values in kit III.(P value <0.001 ,Unpaired "t" test ,table 5,figure 5).

Figure 3 Descriptive statistics of E gene by various kits(y axis -Ct value and x axis- type of kits)

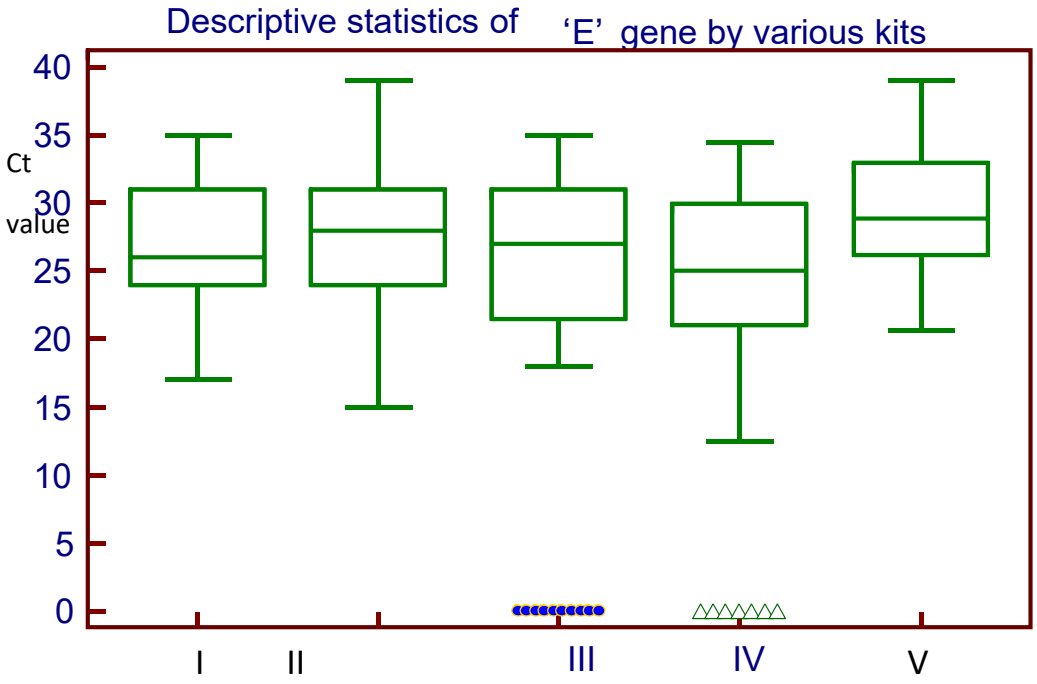


Figure. 4. Descriptive statistics of rdrp gene by various kits(y axis-ct value and x axis -type of kits)

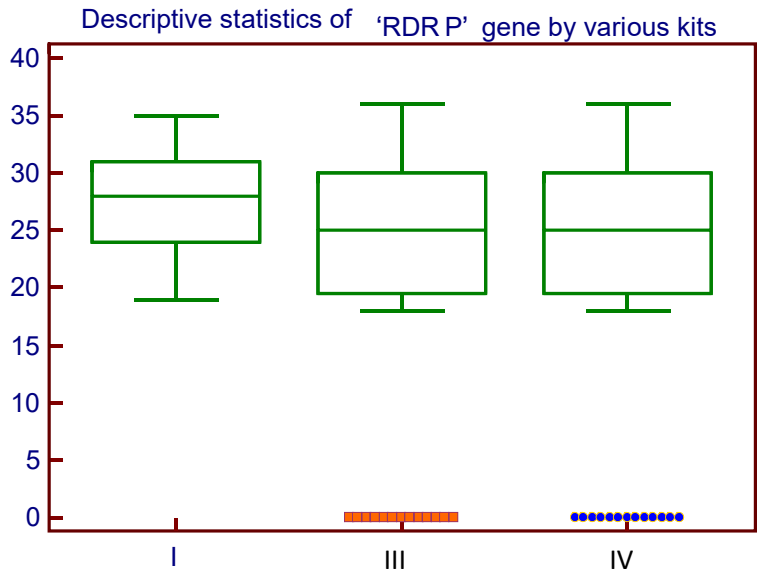
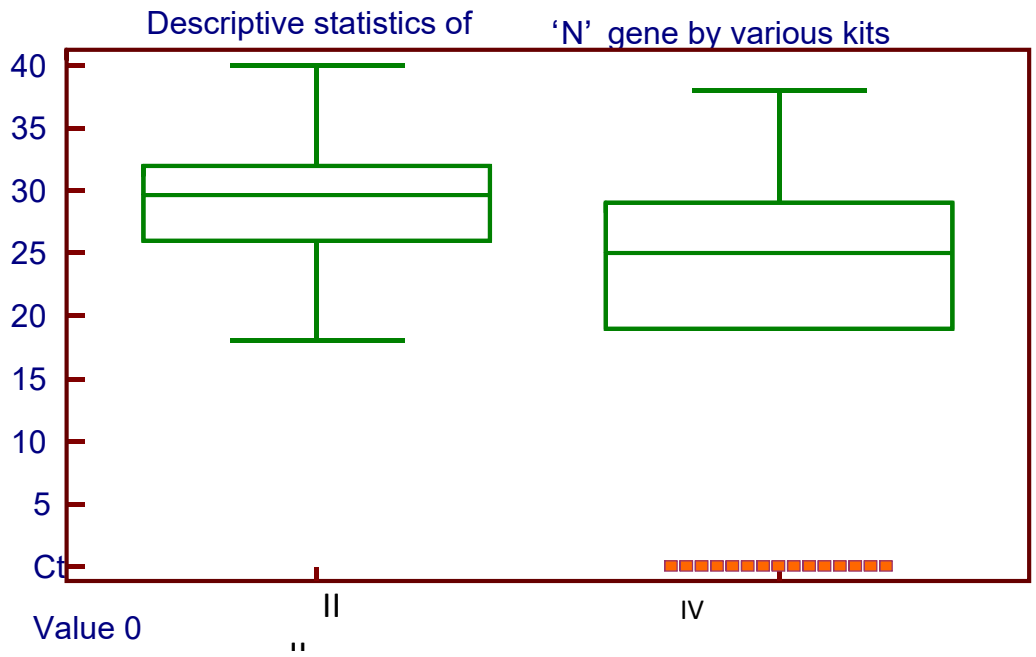


Figure 5 Descriptive statistics of N gene by various kits (y axis-ct values ,x axis type of kits)



*Ct =Cycle threshold

**All kits above were approved by ICMR ,India

Discussion :

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged among humans during the final months of 2019, causing severe acute respiratory diseases, multiple organ injuries, and fatal outcomes. The resulting disease, therefore, has been named coronavirus disease (COVID-19). SARS-CoV-2 is a human coronavirus (HCoV). HCoVs are enveloped viruses with a single-stranded, positive-sense RNA and belong to the order Nidovirale.These viruses are divided into seven species, including HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2.SARS-CoV-2 has a single-stranded positive-sense RNA genome that is 26 to 32 kilobases in length, encoding 27 proteins including an RNA-dependent RNA polymerase (*RdRp*) and four structural proteins include the spike surface glycoprotein (S), nucleocapsid protein (N), small envelope protein (E) and matrix protein (M).

Real-time reverse transcription–polymerase chain reaction (RTPCR) is the most sensitive and specific assay that can provide crucial etiological evidence for this new coronavirus species diagnosis. Recently, the efficacy of RT-PCR for COVID-19 diagnosis has been questioned. Although several COVID-19 RT-PCR diagnostic kits are commercially available, the detection rates of SARS-CoV-2 infection have been unsatisfactory, and several cases have been detected following negative detection results obtained from repeated RT-PCR laboratory diagnostic tests and COVID-19 features already observed on computed tomography images.[10]

In this study we tried to compare the performance of the different kits,so that to avoid false negative reporting ;kits with high sensitivity could be used for testing in centers like ours which deals with large number of samples on daily basis .

The sensitivity of kit I, II , III , IV ,V was 100, 96.8 ,79.6 , 84.3, 90.6 percent and accuracy was 100 , 97.8 , 86.17 , 89.36 and 89.9 percent respectively (table 3).This was overall satisfactory performance for use of these kits in routine in the molecular lab. As stated in studies that a diagnostic test method should have sufficient sensitivity and accuracy to make appropriate clinical decisions rapidly during a pandemic. [11]

As for Kit I(fromBlack bio diagnostics ltd) 100 percent sensitivity and accuracy values correlates with another study conducted by Garg A et al in Lucknow ,India.[12]The sensitivity ofRT PCR is around 45 to 60 percent, other studies have shown the sensitivity was around 63 to 72 percent depending on the sample.[5,6]Therefore the sensitivity of real time RT PCR test kits appears to be affected by multiple factors including the primer –probe set combinationsused,sample type, potential cross contamination, samplestorage, reagent storage requirements among other factors stated in the literature.[13,14]

There have been reports of variation in results with same RT PCR kits when there are used for amplification of extracted RNA on different available compatible RTPCR machines. [15]

However it was observed in this study that Kit III had significantly lower sensitivity for detecting samples with low viral load (Ct >30).The Lower limit of detection (L.O.D, mentioned in all the kits except in Kit III (table 1)) ,gives an idea of the lowest concentration of RNA detected by that particular kit .The presence of SARSCoV -2 RNA viral load below the kit LOD will also give false negative results .In general clinical samples that have low viral loads (Ct value >30) have higher sensitivity requirements for the RT PCR test detection kits .If the

minimum detection limit cannot reach the detection concentration ,weakly positive samples might show a false negative result.[5]
False negativeresults can occur due to numerous reasons including suboptimal specimen collection, testing too early in the disease process, low analytic sensitivity, inappropriate specimen type, low viral load, or variability in viral shedding.[16]Similarly technical problems including contamination during sampling (eg, a swab accidentally touches a contaminated glove or surface), contamination by PCR amplicons, contamination of reagents, sample crosscontamination, and cross-reactions with other viruses or genetic material could also be responsible for falsepositive results.[17]
Another reason would be ,the kit III detected 6/17 low viral load samples and 4/17 were IR and 7 /17 negative (table 6) i.e it was able to detect these(4/17 ,and 7 /17) samples with good sigmoid graph but either at high CT value which was interpreted as negative or only one gene target was amplified as sigmoid graph out of the required two gene targets which was interpreted IR (inconclusive, repeat sample) respectively according to kit manufacturer's instructions .Thus it necessitates that RT PCR curves should be analyzed beyond the manufacturers recommended cut off threshold and should be repeated if required.
As mentioned in the literature some primer probe combinations were prone to background amplification ,which impairs the ability to distinguish between true positives and negative results at low virus concentrations . [18]
As in this study only 17 out of 64 positive samples were of low viral load the results for the kits cannot be taken as a generalized performance .Furthermore the sensitivity for the same type of kit can vary with change of batch and lot numbers .This inference thus cannot be generalized for Kit III before checking its sensitivity for detecting low viral load samples in larger numbers and with more batches of the same kit (kit III).
The result of batch effects for the Rt pcr kit with same gene targets need to be anlayzed as variations have been documented in some recent studies .[6]
The highest Ct value was noted of N gene of Kit II (Ct =40),then of E gene of kit II(Ct=39) indicating high sensitivities of both these genes.The WHO recommended that the E gene assay for screening followed by a confirmatory assay using the RdRp gene can be utilized for firstline screening of COVID-19 cases as E gene is said to be more sensitive and RdRp gene more specific; and in the United States the CDC had asked to use more sensitive two nucleocapsid protein targets [N1 and N2] as a molecular assay. [19,20]Studies conducted by He et al and Fang et al with RT PCR kits using Rdrp gene concluded that the sensitivity of test to be 79% and 71% respectively .Ishige et al. in their study developed a multiplex PCR targeting 3 genes Sarbeco-E gene, N-gene, and human abl1 as an internal control. This kit results perfectly matched with simplex PCR results with different targets and gave sensitivity of 100 percent . [21]Muenchoff et al. in a multicenter comparative study (seven laboratories)found study using RT PCR kit based on Nucleocapsid gene (N) Envelope gene (E), the RNA-Dependent RNA Polymerase (RdRp) gene, found RdRp to have lower sensitivity with the need to improve its sensitivity but found CDC N1 primer/probe-based kits highly useful and sensitive. [22] The N and E gene were among the most sensitive according to the Ct values in our study which correlates well with findings available in above mentioned studies .
However a study published from Hong Kong, China found that RdRp/Hel assay had the lowest limit of detection in vitro and have higher sensitivity and specificity among the three developed novel real-time RT-PCR assays targeting the RdRp/Hel, S, and N genes of SARS-CoV-2 . [23]

Thus it is advisable to use, at least two molecular targets to avoid the situation of a potential genetic drift of SARS-CoV-2 and the cross-reaction with other endemic coronaviruses as well, However, the ideal design would include at least one conserved region and one specific region to mitigate against the effects of genetic drift, especially as the virus evolves within new populations .[24]WHO guidelines of RT PCR targets for SARS CoV-2 detection i.e. at least 2 targets namely, one sarbecovirus specific E-gene and other SARS CoV-2 specific gene (N, RdRp or ORF1b, etc.) positivity is most essential and followed by most of the kits available.[25]

As seen in this study there was significant variation in same gene target Ct values like *E*gene ,*N* gene,*RdRp* gene between results by the five kits. As same set of positive samples were used it was interesting to note that starting from same initial quantities of RNA in samples different kits gave different yield of sometimes same set of gene targets and thus variable sensitivities .
A correspondence letter by David et al clears mentionsCt-values can vary significantly between and within methods. It stated that the median Ct-values reported by the instruments for different methods varied by as much as 14 cycles. Within a single test performed on the same instrument, the difference in the median Ct-values for different targets was as high as 3.0 cycles. Finally, within a single gene target for a single method, up to 12.0 cycle differences were seen across all laboratories. Many clinical laboratories are using multiple tests that assess different gene targets for SARS-CoV-2 and are performing testing on different platforms. This adds to the potential variability of Ct-values produced by a single laboratory.[26]Additionally Ct values and cutoffs are assay- and method-specific. A specimen with a Ct of 35 by one pcr assay will not necessarily have the same Ct value by other assays. These values can vary up to two to three logs from test to test due to how the tests are designed. There can be a difference in the relative sensitivities of standardized and approved tests which may also impact Ct values. According to comparison data recently published by FDA using a standard panel, there can be as much as a 1000-fold difference between the various Rt pcrassays.[27]
Thus a thorough research is extremely valuable in advancing our understanding of Ct-value variation in SARS-CoV-2 molecular testing.
In general ,as observed in other studies too it is difficult to understand the reason for difference in performance of different molecular tests due to natural variations in sample processing and reference material used for validations in different laboratories .
A proper correlation of diagnostic efficacy, Ct values of gene targets of differentRT PCR kits in different molecular testing labs in different cities ,states ,countries with a proper inter- linked system will truly help in producing consistent and reliable results of patients samples for COVID 19 laboratory/molecular diagnosis.
Thus it is recommend that regardless of the laboratory choice of diagnostic commercial kit for the clinical detection of COVID-19 patients the need for good plan for validation and collaboration with exterior laboratories as well as establishment of Quality control labs is essential in order to monitor the virus changes overtime, procedures variations, differences in results due to technicians, and the different kits performances. It is crucial that PCR kits are thoroughly evaluated prior to using them ,especially when sensitivity of the PCR kit is not declared by the manufacturer and an enormous number of RT PCR kits have already flooded the markets .

A good balance between ease of availability ,accessibility and diagnostic efficacy of these RT PCR kits will be the key for reliable and timely diagnosis of SARS CoV virus infection by RT PCR tests .

Limitations of the study

There has been many limitations of the study .It could have been planned with a larger no of samples , particularly low viral load samples to accurately compare the sensitivity of the kits.In this study we didn't evaluate the LOD of the kits (because of unavailability of RNA transcript and digital pcr facility ,).The true specificity of the different kits couldn't be assessed by using them for testing samples which were positive for respiratory viruses other than SARS-CoV 2 virus . We were not able to analyze difference in performance of different batches of the kits used in the study .

Conflicts of interest

None declared

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