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Short Communication

Microbiology Section

Incidence of Non Amplification of *N* Gene of SARS-CoV-2 using Commercially Available Real Time Polymerase Chain Reaction Kit and its Comparison with Two Other Different Kits

RAJEEV KUMAR JAIN[1], NAGARAJ PERUMAL[2], DEEPTI CHAURASIA[3], KAMLESH KUMAR AHIRWAR[4], ARCHA SHARMA[5], RAKESH SHRIVASTAVA[6], JAYA LALWANI[7]

ABSTRACT

Introduction: Novel Coronavirus Disease-2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) has resulted in an unprecedented global pandemic. Real Time Polymerase Chain Reaction (RT-PCR) tests are being used in the diagnosis of COVID-19 worldwide however mutations in the SARS-CoV-2 genome have generated many SARS-CoV-2 genome variants and which may affect the correct Reverse transcriptase-Real time Polymerase Chain Reaction (RT-PCR) diagnosis.

Aim: To confirm and study the incidence of the non amplification of the SARS-CoV-2 Nucleocapsid gene (*N* gene) target among known SARS-CoV-2 positive samples.

Materials and Methods: This retrospective observational study was carried out at the State Virology Laboratory, Gandhi Medical College, Bhopal, Madhya Pradesh, India, during January to May 2021. During the study period, a total of 159 SARS-CoV-2 positive samples were failed to amplify the *N* gene target. To investigate the non amplification of *N* gene target of SARS-CoV-2, a total of 20 samples were selected and retested using the initially used RT-PCR kit (VIRALDTECT RT-PCR kit) and also with the two different RT-PCR kits (TaqPath RT-PCR kit and Hi-PCR RT-PCR kit) which also contain primers/probes for the SARS-CoV-2 *N* gene target.

Results: Amplification and detection of the SARS-CoV-2 *N* target gene was not observed in VIRALDTECT RT-PCR test results. In contrast, amplification was detected in the *N* gene target of SARS-CoV-2 while using the TaqPath and Hi-PCR kits. Obtained results confirm the failure of the annealing of VIRALDTECT kit *N* gene primer/probe and suggest the possible mutation event in the SARS-CoV-2 *N* gene among the *N* gene non amplified samples.

Conclusion: Present study reports, the incidence of non amplification of SARS-CoV-2 *N* gene, where the RT-PCR kit failed to detect *N* gene target and seriously affect RT-PCR diagnosis. Hence, the study emphasises the revalidation of commercially available SARS-CoV-2, RT-PCR kits to identify these kinds of failure incidence.

Keywords: False negativity, Gene mutation, Severe acute respiratory syndrome coronavirus-2, Sequencing

INTRODUCTION

The SARS-CoV-2, source of fatal COVID-19 disease was reported from Wuhan, China and has caused global pandemic since December 2019 [1,2]. Timely and accurate diagnosis of COVID-19 disease has become indispensable to stop the spread of SARS-CoV-2 virus [3]. RT-PCR technique has been playing a vital role in the diagnosis of COVID-19 [4]. Several SARS-CoV-2 specific gene targets such as Envelope *(E)-gene*, Open Reading Frame *(ORF1ab)*, *N*-gene, Spike protein gene (*S*-gene) and RNA-dependent RNA polymerase *(RdRp)* have been commonly used for RT-PCR diagnostics [5]. SARS-CoV-2, like all other Ribonucleic Acid (RNA) viruses, has high mutagenic capability and go through adaptive evolution and variations in the genome. Mutation events in the SARS-CoV-2 genome have produced numerous Variants Of Concern (VOCs) and Variant of Interest (VOIs) and among them B.1.1.7 variant has been associated with higher mortality and transmission risk [6,10]. These mutational events lead to the major challenges in the RT-PCR diagnosis of COVID-19 and directly affect the sensitivity of the test kit and therefore increases the false negative cases [11,12]. Various studies have also documented the non amplification of SARS-CoV-2 gene targets in the RT-PCR test due to the Single Nucleotide Polymorphism (SNP) and novel insertion/deletion mutation in SARS-CoV-2 genome [13-16]. Some studies indicated the *N* gene has the highest mutation density which was also evidenced by the mutation rate and mutation *h*-index value of the SARS-CoV-2 genomes [17-18]. Various non synonymous *N* gene mutations were also identified and reported among Indian variants of SARS-CoV-2 [19]. Herein, this study also reported the incidence of non amplification of SARS-CoV-2 *N* gene in routinely used commercially available SARS-CoV-2 RT-PCR kit (VIRALDTECT II) and secondary objectives was to compare this kit with two other commercially available RT-PCR kits.

MATERIALS AND METHODS

This retrospective observational study was carried out in a period of one month (June 2021) at the State Virology Laboratory, Gandhi Medical College, Bhopal, Madhya Pradesh, India. Ethical approval was obtained from Institutional Ethical Committee (IEC) (13083/MC/IEC/2020). During the period of January to May 2021, in the routine SARS-CoV-2 molecular diagnosis, a total of 159 SARS-CoV-2 positive samples were found to be failed to amplify the *N* gene target of SARS-CoV-2 using Indian Council of Medical Research (ICMR) validated commercially available RT-PCR kit. Those *N* gene failed samples were earlier tested using VIRALDTECT II Multiplex real time RT-PCR for COVID-19 (Genes2me Pvt., Ltd., India) and declared SARS-CoV-2 positive based on other two targets, with Cycle threshold (Ct) values ranging between 12 and 35 for the *E* gene, and ranging between 13 and 35 for the *RdRp* gene.

Specimen Selection Criteria

Samples were selected for this study on the basis of: (a) *N* gene non amplification in the routine testing; and (b) *E* gene Ct; values ranging between 12 and 35 and *RdRp* gene Ct value between 13 and 35. Samples which did not fulfilled above criteria were excluded. To analysis this incidence of non amplification of *N* gene, Out of those 159 samples, a total of 20 samples were randomly retrieved and divided in two groups on the basis of two different Ct value ranges (Group I- Ct <30

and Group II- Ct >30). Integrity of the archived samples was checked by performing RNA extraction and RT-PCR test and qualified samples were further used for this study (data not shown). These selected 20 samples were retested for *N* gene amplification failure by using two different RT-PCR kits and also using the initial tested kit.

RNA Extraction and Real Time-PCR Assay

The RNA was extracted using an automated RNA extractor Genolution Nextractor® NX-48S instrument and NX-48S Viral Nucleic Acid (NA) kit according to the manufacturer's instructions. The extracted viral RNA was evaluated by initial VIRALDTECT RT-PCR kit and two other commercially available multiplex RT-PCR assays (TaqPath COVID-19 Combo Kit (Applied Bio-Systems, USA) and Hi-PCR Coronavirus (COVID-19) Multiplex Probe PCR Kit/HIMEDIA/India) as both the kit contains SARS-CoV-2 *N* gene primer/probe in addition to other SARS-CoV-2 specific gene primers/probes [Table/Fig-1]. Thermo cycling settings and result interpretation were performed as per the kit manufacturer's instructions [Table/Fig-2]. None of the manufacturers were involved in the assessment and interpretation of the study results.

Kit name/Manufacturer/Country	SARS-CoV-2 specific genes	In-house control gene
VIRALDTECT II Multiplex real time RTPCR for COVID-19/Genes2me Pvt. Ltd., Gurugram, Haryana, India	*N* gene	*RNase P* gene
	RdRp gene	
	E gene	
TaqPath COVID-19 Combo Kit/Applied Bio-Systems (USA)	*N* gene	*Bacteriophage-emesvirus zinderi* Phage
	Orf1ab gene	
	S gene	
Hi-PCR Coronavirus (COVID-19) Multiplex Probe PCR Kit/HIMEDIA/India	*N* gene	*Ribonuclease P RNA componenet H1* gene
	RdRp gene	
	E gene	

[Table/Fig-1]: Summary of various RT-PCR kits used in the study.

Variable			VIRALDTECT II kit		Taqpath kit		Hi-PCR kit	
Master mix volume			11 µL		20 µL		20 µL	
Template (RNA) volume			9 µL		5 µL		5 µL	
Total reaction volume			20 µL		25 µL		25 µL	
UNG incubation		Temp (°C)	NA		25	1 cycle	NA	
		Time (Min.)			02			
Reverse transcription		Temp (°C)	55	1 cycle	53	1 cycle	50	1 cycle
		Time (Min.)	10		10		15	
Initial denaturation		Temp (°C)	95	1 cycle	95	1 cycle	95	1 cycle
		Time (Min.)	03		02		03	
PCR	Amplification	Temp (°C)	95	40 cycle	95	40 cycle	95	40 cycle
		Time (Sec.)	15		03		15	
	Data collection (Fluorescence detection)	Temp (°C)	60		60		58	
		Time (Sec.)	60		30		30	
Approximate run time			~93 Min.		~67 Min.		~80 Min.	
Threshold Cut-off cycle (Ct)			≤37		≤37		≤38	

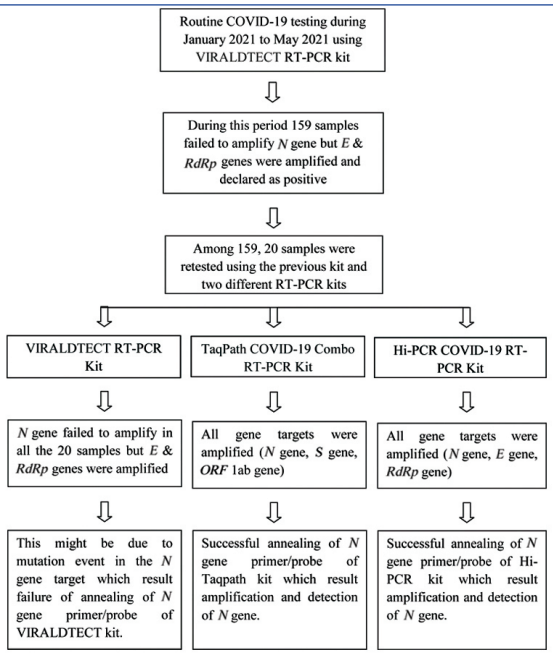
[Table/Fig-2]: RT-PCR reaction and RT-PCR amplification program conditions. UNG: Uracil N-glycosylase; PCR: Polymerase chain reaction; Min: Minute; Sec: Second; NA: Not applicable

STATISTICAL ANALYSIS

The Ct values of all SARS-CoV-2 genes of all the RT-PCR kits used in this study were recorded and Mean and Standard Deviation (SD) of the tested genes were analysed using Microsoft Excel 2010.

RESULTS

A total of 20 SARS-CoV-2 positive samples with two different Ct value ranges (Group I- Ct <30 and Group II- Ct >30) were used to confirm the incidence of the *N* gene non amplification. Again amplification and detection of SARS-CoV-2 *N* target gene was not observed in VIRALDTECT RT-PCR test results in both groups [Table/Fig-3]. However, other two genes (*E* and *RdRp*) of the kit were amplified satisfactorily. Group I samples mean Ct values of VIRALDTECT kit for *E* and *RdRp* genes were 28.83±4.4 and 25.59±3.6, respectively. Group II samples mean Ct values of VIRALDTECT kit for *E* and *RdRp* genes were 32.92±1.6 and 31.76±1.8, respectively. In contrast, *N* gene amplification was detected in the results of other two RT-PCR kits used (TaqPath and Hi-PCR kits). Remaining other SARS-CoV-2 target genes were also properly amplified in TaqPath and Hi-PCR kits tests in both group [Table/Fig-4,5]. This confirms the failure of annealing of the *N* gene primer/probe of VIRALDTECT kit and suggests the mutation event in the *N* gene sequence.



[Table/Fig-3]: Schematic representation of the work flow.

Sample ID code	VIRALDTECT II kit			Taqpath kit			Hi-PCR kit		
	SARS-CoV-2 genes								
	E	*N*	*RdRp*	*N*	*Orf 1ab*	*S*	*E*	*N*	*RdRp*
Group-1 (Cut-off threshold <30)									
SIC-001	19.72	ND	17.31	14.66	12.69	14.17	15.75	16.30	14.11
SIC-002	24.85	ND	22.57	21.79	20.10	21.43	23.83	24.23	22.35
SIC-003	32.71	ND	23.59	20.62	18.49	19.75	21.96	22.93	20.56
SIC-004	26.1	ND	24.99	24.86	22.62	24.42	27.15	27.64	25.15
SIC-005	28.98	ND	27.27	26.67	24.19	26.69	27.01	26.99	24.48
SIC-006	32.99	ND	27.53	25.14	25.49	25.79	26.8	25.78	25.79
SIC-007	29.4	ND	27.75	26.39	24.85	26.71	27.03	26.70	25.14
SIC-008	28.84	ND	27.87	25.53	25.25	27.52	26.71	25.18	27.32
SIC-009	34.78	ND	28.96	27.57	25.16	27.33	27.65	27.90	25.46
SIC-010	30.02	ND	28.15	27.37	27.33	28.64	27.78	27.61	29.4
Group-2 (Cut-off threshold >30)									
SIC-011	32.06	ND	30.02	29.82	26.04	28.99	29.68	32.69	31.22
SIC-012	31.41	ND	30.03	28.76	27.31	29.17	29.51	30.95	30.91
SIC-013	32.15	ND	30.15	28.81	26.78	28.49	31.68	32.03	29.78
SIC-014	32.69	ND	31.27	27.54	26.63	27.75	30.23	30.59	31.96
SIC-015	31.71	ND	31.42	28.35	27.84	29.51	29.03	28.29	30.09
SIC-016	32.99	ND	31.71	31.3	29.41	30.46	29.44	32.88	29.5
SIC-017	31.83	ND	30.81	30.74	29.72	30.78	30.82	33.18	32.52
SIC-018	36.29	ND	32.58	30.25	29.17	30.87	34.33	33.64	32.44
SIC-019	35.02	ND	34.53	33.33	33.21	34.64	33.02	34.52	34.91
SIC-020	33.05	ND	35.11	31.77	28.07	28.58	32.75	35.23	35.52

[Table/Fig-4]: Cut off threshold (Ct) value of the SARS-CoV-2 genes observed in the RT-PCR test. ND: Not detected

Threshold cycle (CT)	Gene	VIRALDTECT kit	Taqpath kit	Hi-PCR kit
Group I Ct <30	Cut off threshold (Ct) Mean±SD of SARS-CoV-2 genes			
	E gene	28.83±4.4	-	25.16±3.8
	RdRp gene	25.59±3.6	-	23.97±4.2
	Orf 1ab gene	-	22.61±4.4	-
	N gene	Not detected	24.06±4.0	25.12±3.5
	S gene	-	24.24±4.5	-
Group II Ct >30	*E* gene	32.92±1.6	-	31.04±1.8
	RdRp gene	31.76±1.8	-	31.88±2.0
	Orf 1ab gene	-	28.41±2.1	-
	N gene	Not detected	30.06±1.8	32.4±2.0
	S gene	-	29.92±1.9	-

[Table/Fig-5]: Mean and Standard Deviation (SD) of the threshold cycle (Ct) value of samples detected by different RT-PCR kits.

DISCUSSION

The SARS-CoV-2, like all other RNA viruses, has high mutagenic capability and drives viral evolution. Mutational events cause genetic variation in the population of circulating viral strains over the course of the COVID-19 pandemic. Mutation events in the SARS-CoV-2 genome have produced many VOC and created key challenges in the RT-PCR diagnosis of COVID-19. Due to this, false negative cases have increased which adversely influences SARS-CoV-2 pandemic control program. In recent time, many studies have described failure of the RT-PCR assay and posed a situation of negative reporting of particular SARS-CoV-2 target gene [17,20-25]. Early detection of such events is crucial and can be beneficial to control SARS-CoV-2 pandemic.

E gene, *N* gene, *RdRp* gene, *Orf 1ab* gene and *S* gene are the most familiar SARS-CoV-2 genes and have different structural and non structural functions and commonly used as target genes in commercially available SARS-CoV-2 RT-PCR assays. The *N* gene expresses protein implicated structural components of SARS-CoV-2, plays important role in the viral life cycle and these features make key targets for the diagnosis and vaccine development [26,27]. *N* gene has the highest mutation density which was evidenced by the mutation rate and mutation *h*-index value of the SARS-CoV-2 genomes [18,28]. In different Indian SARS-CoV-2 variants, *N* gene mutations were also identified and reported [Table/Fig-6] [19].

Variant	Protein position	Protein mutation	Prevalence	Percentage (%)
G28881A	N:203	R203K	3519	39.93
G28882A	N:203	R203K	3506	39.79
G28883C	N:204	G204R	3497	39.68
C28854T	N:194	S194L	2214	25.12
C28311T	N:13	P13L	730	8.28
T28277C	N:2	S2P	661	7.5

[Table/Fig-6]: Most prevalent SARS-CoV-2 *N* gene mutations in India. (Source: https://data.ccmb.res.in/gear19/ last accessed: 29.08.2021, [19])

In this study, it was frequently observed in the routine COVID-19 diagnosis using VIRALDTECT II RT-PCR kit that the cut-off signal in the *N* gene in some of the samples were negative. However, other SARS-CoV-2 gene targets *RdRp* and *E* genes were still correctly annealed, amplified, showed positivity with cut-off signal and declared as SARS-CoV-2 positive. To confirm this non amplification phenomenon in the *N* gene in VIRALDTECT RT-PCR kit, other commercially available kits (Taqpath and Hi-PCR) were used, which also contain SARS-CoV-2 *N* gene primers and probes. Purposely two different make RT-PCR kits were used to make sure whether all other SARS-CoV-2 gene targets were amplified or not. In addition to SARS-CoV-2 *N* gene, Taqpath kit contains *Orf1ab* and *S* gene and Hi-PCR kit having *E* and *RdRp* gene. On the whole all SARS-CoV-2 target genes were covered for the verification by two other RT-PCR kits.

As expected, again *N* gene was not amplified in the 20 samples used with VIRALDTECT RT-PCR kit. Surprisingly, all SARS-CoV-2 target genes showed good amplification in both (Taqpath and Hi-PCR) kits and primers/probes of all the gene targets of both kits were correctly annealed and simultaneously amplified [Table/Fig-3]. These retesting results of other RT-PCR kits confirm a mutation event in the sequence of the *N* gene of those SARS-CoV-2 positive samples which was associated with the failure of the *N* gene amplification by VIRALDTECT II kit. Earlier this sort of SARS-CoV-2 *N* gene mutation phenomenon was detected and reported. Novel SARS-CoV-2 *N* gene mutation (C29200A) in three patients was reported by Hasan MR et al., whereas Ziegler K et al., found SNP in the *N* gene of SARS-CoV-2 infected patient and novel insertion/deletion mutation in the *N* gene which ultimately cause negative results for SARS-CoV-2 was revealed by Lee S et al., [5,14,29]. In all these studies, RT-PCR kits were failed to amplify *N* gene target and detected as negative for SARS-CoV-2 *N* gene.

Genome sequencing is the only method to correctly identify the mutation events and positive or negative cases of SARS-CoV-2 [30,31] and has been used for the validation of such events and however, limited numbers of specimens are asked by the government institutions with definite criteria [32]. Since, it is extremely expensive method, rapid diagnosis of thousands of the samples each day using this method is not possible. Due to the lack of sequencing facility, the present study does not have sequencing data of those SARS-CoV-2 positive RNA samples. However, this report still highlights the importance of the reference RT-PCR kits which contain primers/probes targeting emerging SARS-CoV-2 VOC in a particular region.

Mutations in the genome of SARS-CoV-2 have generated many VOIs and VOCs and were responsible for the deadly second wave of this pandemic. Due to mutations in the primers/probes specific region of the SARS-CoV-2 gene, RT-PCR kits are not able to perform correctly, therefore non amplification phenomenon happens. In India, SARS-CoV-2 RT-PCR kits were validated by ICMR, New Delhi with particular batch number and those kits are sold commercially [33]. Batch number changes as per the production of those kits and there are possibilities that new batch kit not able to detect upcoming mutant version of the SARS-CoV-2 genes. For that reason, some reference kits should always be there to address these problems. Present study also emphasises production and validation of the reference kits which contain primer/probes of all the gene targets of the circulating mutant version of the SARS-CoV-2. It is urgently required to find reference sequences of new SARS-CoV-2 variants and share these knowledge for the designing of the primers and probes for accurately diagnosing COVID-19 disease.

Most recently, SARS-CoV-2 Variant of Concern Omicron (B.1.1.529) was reported from South Africa and this variant has a large number of mutations in *S* gene. As per World Health Organisation (WHO), current SARS-CoV-2 RT PCR kits continue to detect this variant, however *S* gene dropout or *S* gene target failure may be encountered and this non amplification of *S* gene can therefore be used as marker for this variant [34].

In addition, genomic sequencing must be done of those SARS-CoV-2 positive samples where all target genes were not completely amplified in the RT-PCR test. This study also underline the possibility of escape of SARS-CoV-2 cases especially if tested by single gene based kits and further emphasise the importance of multiple gene target RT-PCR kits for the diagnosis of SARS-CoV-2.

Limitation(s)

The limitation of the study was small sample size, lack sequencing data and only two SARS-CoV-2 RT-PCR kits were used for the confirmation of the *N* gene non amplification phenomenon.

CONCLUSION(S)

Present study reports the incidence of non amplification of SARS-CoV-2 *N* gene, where the RT-PCR kit failed to correctly amplify *N* gene target and detected SARS-CoV-2 positive specimens as negative. This study emphasise the importance/production of the reference/new RT-PCR kits containing primers/probes which can targets various SARS-CoV-2 VOC in a particular region and also the regular

validation of the commercially available SARS-CoV-2 RT-PCRs kit at the scheduled interval using the circulation new variants. This will helpful in timely identification of these kinds of failure incidence.

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PARTICULARS OF CONTRIBUTORS:
1. Scientist-C, Department of Microbiology, Gandhi Medical College, Bhopal, Madhya Pradesh, India.
2. Scientist-B, Department of Microbiology, Gandhi Medical College, Bhopal, Madhya Pradesh, India.
3. Professor, Department of Microbiology, Gandhi Medical College, Bhopal, Madhya Pradesh, India.
4. Assistant Research Officer, Department of Microbiology, Gandhi Medical College, Bhopal, Madhya Pradesh, India.
5. Assistant Professor, Department of Microbiology, Gandhi Medical College, Bhopal, Madhya Pradesh, India.
6. Associate Professor, Department of Microbiology, Gandhi Medical College, Bhopal, Madhya Pradesh, India.
7. Associate Professor, Department of Microbiology, Gandhi Medical College, Bhopal, Madhya Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Rajeev Kumar Jain,
Scientist-C, State Virology Laboratory, Department of Microbiology,
Gandhi Medical College, Bhopal-462001, Madhya Pradesh, India.
E-mail: rageevjain@gmail.com

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