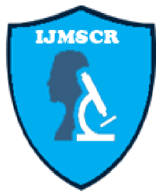

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An Assessment Of Pooling Methodology Of Testing in SARS-CoV-2

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Abstract

The SARS-CoV-2 or novel COVID-19 virus belonging to Coronaviridae family has already taken million lives across the world and this menace is not completely disappearing anytime soon and predicted to return in waves looking into the current trends. India, fought her battle against this pandemic till this moment but any wave of this virus might come or come in which form. Testing laboratories adopted techniques to process larger sample size in shorter duration to ramp up the contact tracing needed for containment of this virus. In this experiment viral RNA was determined from pools of one confirmed positive sample with other negative samples. This continued to 10th number and RNAs were extracted by two most popular procedures, viz, Spin column based and Magnetic bead based to observe the changes in results. Receiver Operator Characteristic (ROC) curve was constructed to evaluate the data obtained from Real-Time PCR platform to identify a suitable pool to discriminate a true sample from a false one. This resulted that combination of one true positive sample with 5 negative (pool no 6) and one positive with 7 negative sample (pool no 8) can be a limit to get a pool for least faulty result in both type of extraction methods to detect N and ORF1ab genes respectively. RNA from consecutive pools has shown significant decrease in purity and quantity if we increase the negative samples in a pool beyond abovementioned specific number. Fold change study showed dominant expression of ORF1ab gene compared to N gene in human swab samples.

Keywords: SARS-CoV-2, Spin column, Magnetic bead, ROC, N gene, ORF1ab, Fold change

Introduction

"*The secret of change is to focus all of your energy, not on fighting the old, but on building the new*" Socrates.

Definitely the nature of taking challenge in overcoming obstacles like unknown disease or other calamities, human developed scientific temperament, taking role of Physician or a Scientist, are always known as pivotal character of saving mankind decades to decades. The same focus gained during the fight against recent SARS-CoV-2 or novel COVID-19 pandemic, the scientific community has managed to develop many more as well as advanced diagnostic kits for fast and accurate determination of presence of this virus in human body and finally a solution, the most awaited vaccine as preventive medicine. The SARS-CoV-2 virus belongs to *Coronaviridae* family and orders *Nidovirales* which are responsible for respiratory, enteric, hepatic and

neurological diseases infamously known for its pandemic wrath upon mankind for more than one year and bringing mortality to human more than any pathogen within last century. The genome structure of the single-stranded non-segmented positive-sense RNA of the CoVs includes two-thirds of RNA which are responsible for encoding viral polymerase RNA-dependent RNA polymerase (RdRp), RNA synthesis materials, and two large nonstructural polyproteins that are not involved in host response modulation, open reading frames (ORF1a-ORF1b). The other one-third of the genome encodes four structural proteins; spike (S), envelope (E), membrane (M), nucleocapsid (N), and other helper proteins (Li , 2016 ; Sahin et al., 2020).

Basically, for fast and valid detection of this viral particles or respective antibody or nucleic acid in human body two different types of assays are commonly being practiced during this pandemic viz, Immunological assays and Amplification based techniques. In spite of so many diagnostic techniques developed under those types, RT-PCR is still the gold standard (Lee et al., 2020; Younes et al., 2020). RT-PCR is a type of PCR methods that uses reverse transcriptase enzyme to convert RNA molecules to cDNA molecules. Then cDNA works as a template sequence for the PCR reaction (Shahi et al., 2018). Quantitative PCR determines a DNA molecule with the help of fluorescent dye or fluorophore-attached DNA probe such as TaqMan and monitored with a Real-Time Machine platform. However developing an efficient RNA extraction procedure for large volume of sample altogether is still a need of the hour. Meanwhile in order to screen positive samples from negatives "pooling" technique was developed by scientists to Fast-Track RT-PCR based diagnosis. Recently, Loop-mediated isothermal amplification (LAMP) or Clustered regularly interspaced short palindromic repeats (CRISPR) perhaps outrun the costly RT-PCR technique in near future but scientists has yet to work on their over sensitive detection process. RT-PCR based study hold its specialty as it gives its user a significant token of data evaluation - the Threshold cycle (Ct) value. Ct value of a target gene (whether N and ORF1ab uniplexed or multiplexed) in qRT-PCR based diagnosis not only gives information about the presence or absence of Corona virus, apart it also helps in understanding the dynamics in due course of infection as well as viral load; however, clinical importance is limited due to various laboratory complications (Wishaupt et al.,2017; Chang et al., 2020). Ct values are inversely related to viral load and can provide an indirect method of quantifying the copy number of viral RNA in the sample; eventually the viral load (Rao et al., 2020).

However, which may be the platform or technique used for determination of respective gene in the selected amplicon, none but all depends on the quality of the nuclear material, viz, viral RNA to be collected from saliva, urine, blood or any body fluid. In India, when the first case on January 30, 2020 expanded to a level of community transmission in mid-September, 2020 with a number of >96,000 SARS-COV-2 infected people (John Hopkins University COVID-19 database), the diagnostic teams all over the country offered a smart solution to fast extraction of RNA from a large population so to separate unhealthy people quickly from other non-suspected population. Such strategy helped state like Assam, which attained almost stability by November 2020 during the 1st wave through this procedure called "pooling". This also helped during highest level of infection and death was monitored in Indian hospitals, which became a combat technique to isolate uninfected from other spreaders. While pooling of respiratory samples for the detection of RNA viruses such as influenza viruses has been evaluated (Praharaj et al., 2020) ,but it cannot vaguely be used for diagnosis for overcoming a scenario of pandemic which is related to sensitivity of RT-PCR results vis a vis the inequality of SARS-CoV-2 spread to diverse diaspora of India. When pooling of raw samples are the way to obtain cocktail of RNA of large population in a tube, mathematical models and epidemiological projections have suggested different pooling sizes, which might be feasible and effective for handling testing needs in regions with different positivity rates (Deckert et al., 2020). Till date we are using 4-5 samples in a pool mostly, this experiment was done in order to see if we can actually include more samples in pool or not? This question is crucial especially in case of a largely populated country India, where reduction of time of sample processing through a smart strategy could only help the diagnostic facilities spread out in this diverse country. This attempt also made the authors of this manuscript to understand the behaviour of

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expression of 2 sets confirmatory COVID-19 genes in response to their extraction method through various pool creation, through two most favorite nucleic acid extraction procedure and observing the changes happened to a constant positive sample when analyzed through RT-PCR. This work may have informative value in the field of molecular diagnosis of viruses obtained from pools and so to discriminate a false positive as well as limit of formation of pools when a bigger wave of any virus may strike again in near future.

Materials And Methodology

Materials

Study Method

Samples were collected from patients suspected for SARS-CoV-2 and who have travelled from affected countries as well as from other parts of the states. Details of the patients which include were recorded in standard Specimen referral forms as per ICMR guidelines.

Laboratory Confirmation

Nasopharyngeal & oropharyngeal swabs were collected in Viral Transport media (VTM) and sent to Viral Research & Diagnostics Laboratory for RT-PCR test for the presence of SARS-CoV-2. Samples were tested for SARS-CoV-2 as per protocol (manufacturer guidelines) by using Viral RNA extraction kit and RT-PCR kits which was supplied by ICMR, New Delhi, India.

Experiment setup

For development of pools of samples 1 confirmed positive and 9 confirmed negative samples were arbitrarily chosen from sample cold storage. 10 pools were developed by mixing the positive sample to one negative to create first pool, then two negative and so on till 10th pool. Each pools were designated as S1(1 positive and 0 negative sample), S2(1 positive and 1 negative samples and so on till S10 (1 positive and 9 negative samples) (refer to Table 1).

Table 1. The various pool construction strategy where increasing number of negative samples (-) were kept adding to one confirmed SARS-CoV-2 positive sample (+) to get up to 10 pools

Pool names	Pooling by negative (-) and Positive (+) samples									
S1										+
S2									-	+
S3								-	-	+
S4							-	-	-	+
S5						-	-	-	-	+
S6					-	-	-	-	-	+
S7				-	-	-	-	-	-	+
S8			-	-	-	-	-	-	-	+
S9		-	-	-	-	-	-	-	-	+
S10	-	-	-	-	-	-	-	-	-	+

Viral RNA extraction

For comparison of pools made of positive and negative samples, the two widely used method of viral RNA extraction was used, viz., Spin-Colum Based RNA extraction method and Magnetic bead based nucleic acid extraction method. First for each pool of increasing numbers of samples, 100 µl from 9 each sample containing VTM collected from SARS-CoV-2 negative and 1 positive patient were poured in separate 1.5 ml centrifuge tubes to obtain progressive

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manner. From all these cocktails 140 µl of sample was taken for experiment. For Spin-Colum Based RNA extraction, QIAmp Viral RNA Mini kit was used and for Magnetic bead based method, MGI nucleic acid extraction kit was used. The protocols followed were obtained from the kit manuals [**Cat no**: 166031621 (Qiagen), **Cat no**: Item No. : 1000020471 (MGI)].

The extracted RNA was taken out stored at -20^0 C freezer for recent use and stored at -70^0 C for further use. The RNA samples after extraction were tested for their purity and then subjected to Real-Time PCR based detection of certain genes associating SARS-CoV-2 genome.

Evaluation of purity of viral RNA

RNA quantity and purity were assessed by measuring UV absorption at 260, 280 and 230 nm using a Multiscan sky (Thermo Scientific, Waltham, MA, USA) accessorized with a µ-drop plate. The 260:280 and 260:230 ratio were calculated to evaluate purity of RNA and contamination of DNA or other material.

Real-Time PCR assays for detection of SARS-CoV-2

Single-step real-time RT-PCR for SARS-CoV-2 targeting the N gene and ORF-1ab was performed on the extracted RNA from the individual positive sample as well as the same sample in 9 progressive pools. The platform used for PCR was Applied Biosystems 7500 model. For performing reverse transcription plus polymerase chain reaction reagents from MERIL COVID-19 One Step RT-PCR kit. In this multi-plex PCR, 9 µl of resuspended master mix, 1 µl of ORF1ab/N/ IC primer & probe mix diluted with 5 µl RNAse free water for 5 µl of sample RNA. In the machine platform FAM and HEX/VIC channel were selected for detection of ORF-1ab and N-gene in COVID-19 RNA. ROX was selected for the Internal Control (RnaseP/β actin). The temperature program follows: **1**. Reverse Transcription (50^{0}C/15 min/1 cycle), **2**. cDNA initial denaturation (95^{0}C/3 min/1 cycle), **3**. Denaturation (95^{0}C/15 sec) & Annealing-Extension-Data capture for˜45 cycles and **4**. Cooling (25^{0}C/10 sec/ 1cycle). The Ct value for positive controls appeared ≤ 35 for the respective genes and hence considered in house positive cut-off for discriminations.

Statistical Analysis And Gene Expression Study

The statistics and data analysis was done by using Microsoft Office Excel tools and Origin 8.5 software. Correlation and Regression analysis were also performed in necessary cases to find r, R and p values. Sensitivity, specificity and a suitable cut-off point for Ct values of N gene and ORF-1ab gene with respect were determined using Receiver Operator Characteristic (ROC) curve. Area under curve (AUC) was calculated for each ROC curve to find the suitable curve to get a proper cut-off point. The upper cut off values was taken for this set of experiment were 20, 23, 25, 30, 35 and 40 for each gene.

The average cut-off point (Ct value) obtained from RNAs of each pool extracted from both spin column and magnetic based extraction, upon which, all values became false positive was considered as discriminative point of evaluating negative pools devoid of the positive sample. To calculate the expression in fold change, first average of positive Ct values of both gene (*avgNp, avgOp*) of interest were taken as well as Internal Control (*avgIc*), average Negative Ct values (*avgNn, avgOn)* .

Hence**, ΔCt for N positive** = *avgNp –avgIc,* ΔCt for Ngene negative = *avgNn –avgIc*

and, **ΔCt for ORF1ab positive** =*avgOp-avgIc,* ΔCt for ORF1ab gene negative =*avgOn-avgIc*

∴ **ΔΔCt N gene** = (ΔCt for Ngene negative - ΔCt for Ngene positive) = Fold change of N gene

and,

∴ **ΔΔCt ORF1ab gene** = (ΔCt for ORF1ab negative - ΔCt for ORF1ab positive) = Fold change of ORF1ab gene (Rao et al., 2013).

Results

During the chosen time period (April 2020 to December 2020) the real time PCR based detection of SARS-COV-2 doubted samples resulted in 2,600 positive samples among total 111379 samples received at VRDL, TMCH. Out of the positive samples 2480 samples were from only Assam and 120 were linked with travel from outside the state or resident of other state. So to get rid of probable anomalies could be created during shorting and segregating such a huge number of samples, the necessity of proper pooling system arose at that moment of crisis as it was required to generate the data too as soon as possible.

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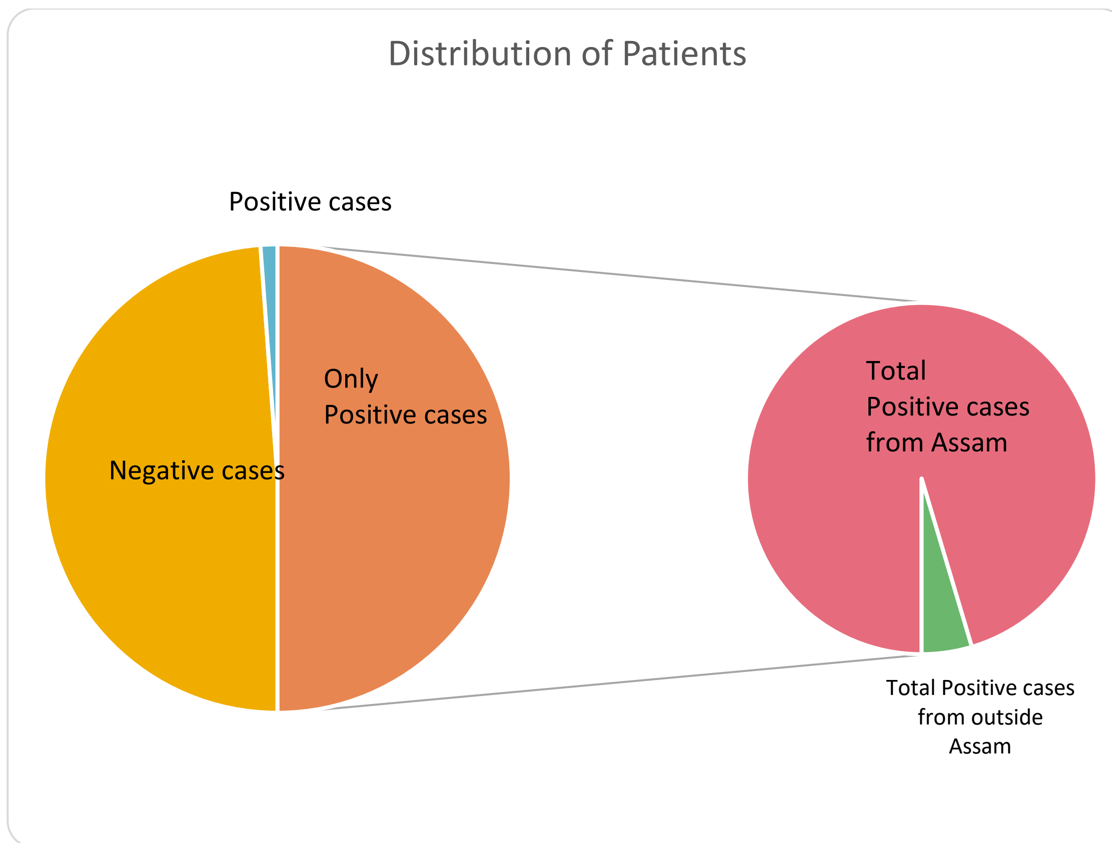
Fig.1 . Distribution of sample of patients visiting Tezpur Medical College & Hospital tested in VRDL. Out of positive cases (2,600) there were (2480) cases found from Assam and (120) from outside Assam



On development of pools S1(only the positive sample) to S10 (one positive + 9 negative samples), when the purity of extracted RNA was checked at 260/280 nm ratio, the ratios were found as 2.3, 2.22 ,2.19,2.21,2.3,2.7,2.81,2.88, 3.44, 3.46 and 1.98, 2.06,2.13,2.11,2.23,2.5, 2.78, 2.94,3.22, 3.28 for the pools in case of Spin column based extraction and Magnetic bead based extraction methods respectively. The correlation coefficient **r** value and $\mathbf{R^2}$ value was found to be 0.91 and 0.847 for column based method and 0.96 and 0.93 for magnetic bead based method. Again, when the pool samples were checked for its impurities at 260/230 nm ratio after two kinds of extraction methods, the pools resulted 1.98, 2, 2.08,1.99,1.96,1.76,1.2,1.18,1.0,0.86 and 2.21, 2.2,1.95,1.91,1.77,1.54,1.23,1.07,1.0,1.0 in case of Spin column based extraction and Magnetic bead based extraction methods respectively(Fig 2. A & B). The regression coefficient **r** value and $\mathbf{R^2}$ value was found to be -0.92 and 0.83 for column based method and -0.98 and 0.96 for magnetic bead based method (Fig 2. C & D).

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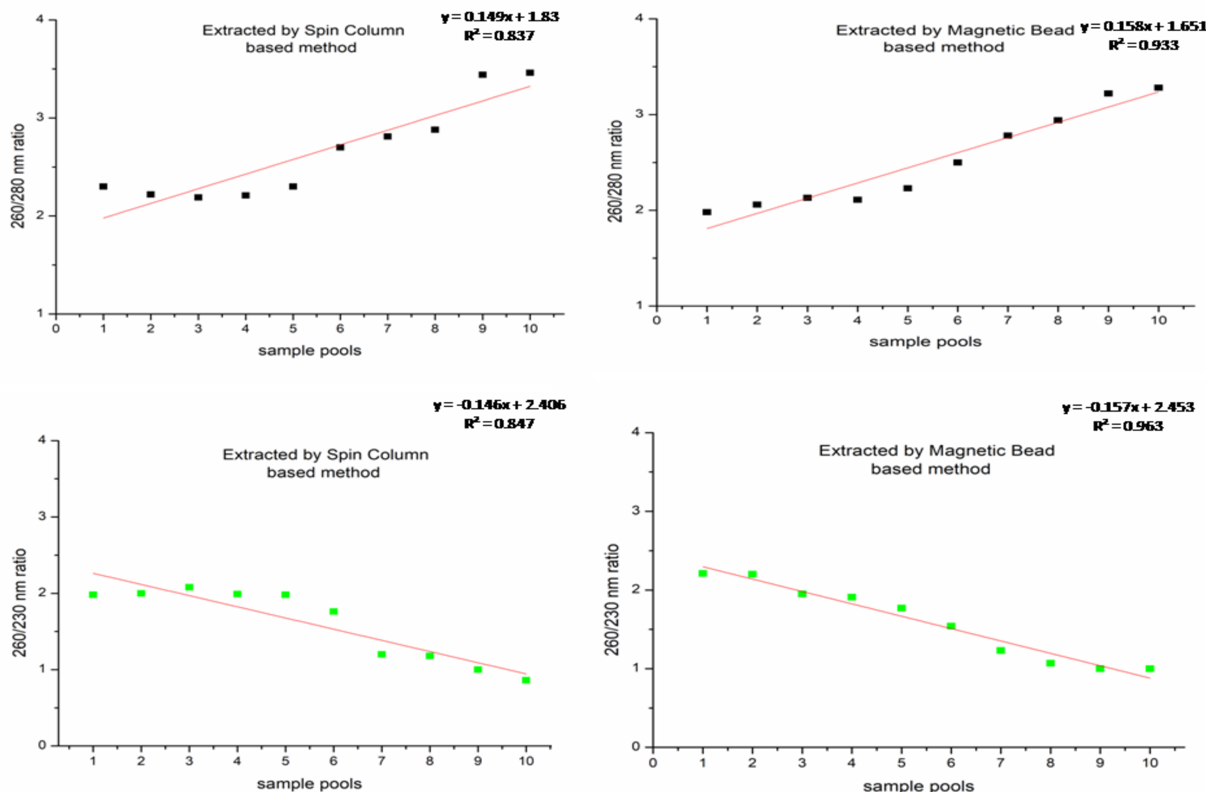
Fig.2. A, B and C,D showing remarkable changes in optical density ratio at 260/280 nm and 260/230 nm for the pools S1 to S10 extracted by Spin column based and Magnetic Bead Based separation methods respectively.



When, the RNAs extracted from the aforementioned 10 pools, the RT-PCR resulted presence of N and ORF1ab gene in all two type of extraction sets (viz; spin column based and magnetic bead based extraction) including in Positive Control. In case of N gene and ORF1ab gene, mean Ct values were tabulated in Table 2 for the pools **S1 to S10** for both the type of extraction strategies. When linear fit was applied to the data corresponding to detection of N gene the correlation coefficient **r** value and $\mathbf{R^2}$value was found to be 0.95 & 0.90 and 0.97& 0.94 for both type of extraction method (Fig 3 A and B). In both cases the mean Ct value for N gene in PC was <35 and NC was undetectable. Again If linear fit was applied to the data corresponding to ORF1ab gene, the correlation coefficient **r** value and $\mathbf{R^2}$ value was found to be 0.95 & 0.91 and 0.92 & 0.85 for both type of extraction method (Fig. 3 C and D). **P** value for all these cases were found to be <0.001.

Table1. List of Ct mean values of sample pools against different types of extraction method for identification of N and ORF1ab gene.

Pool Nos	Ct mean value for N gene (spin column based extraction	Ct mean value for N gene (magnetic bead based extraction	Ct mean value for ORF1ab gene (spin column based extraction	Ct mean value for ORF1ab gene (magnetic bead based extraction
S1	22.4	23	20.5	23
S2	22.3	23.2	21.2	23
S3	23.3	23.3	23.5	23.26
S4	23.4	25	23.6	23.49

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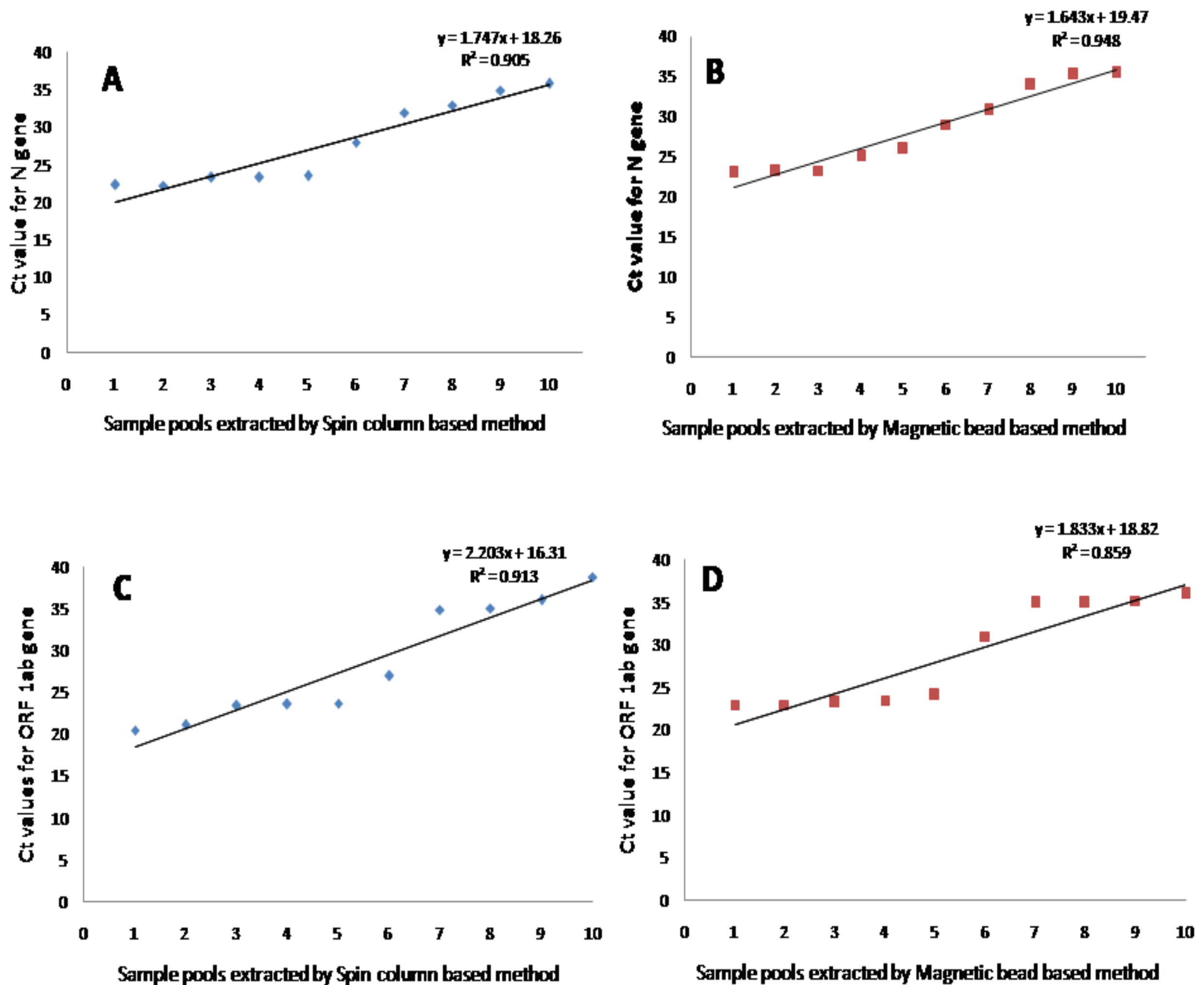
S5	23.5	26	23.7	24.1
S6	28	29.04	27	31
S7	32	33	34.9	35
S8	33	35.7	35.1	35
S9	34	36.8	36	35.2
S10	35.8	38	38.8	36

Fig 3. A and B depicted significant changes in Ct values obtained after RT-PCR based analysis for presence of N gene in respective pool RNA samples (1 to 10), C and D depicted significant changes in Ct values obtained after RT-PCR based analysis for presence of ORF1ab gene in respective pool RNA samples (1 to 10)



For understanding which pools could be selected as better one on the basis of their Ct values to corresponding genes indicating the positive nature of a sample an upper cut off value was taken, i.e., 35 as per RT-PCR kit manual and as no individual positive cases had shown higher Ct value for any gene more than 35. So, the central scattered plot keeping Ct value as a cut off depicted that in spin column based and magnetic bead based

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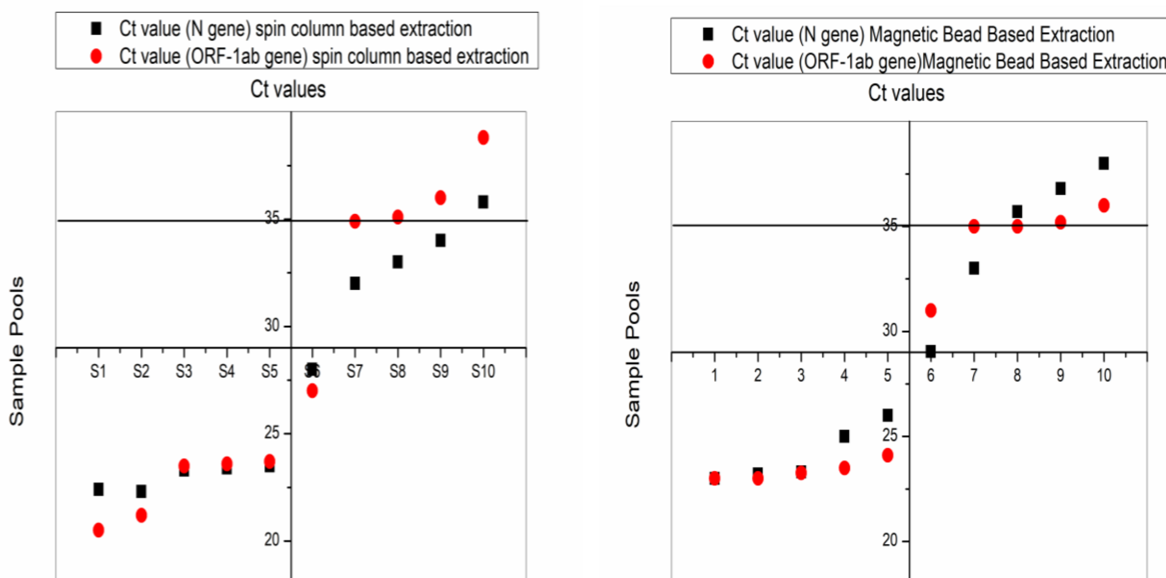
extraction methods Ct values of N genes remained ≤ 35 till S8 and S7th pool and for ORF-1ab those remained on S6 and S6th pools in both the procedures respectively (Fig 4). Other pools beyond these shown Ct values more than 35 which could be considered as positive nature.

Fig. 4. The central scatter plots depicted that in spin column based and magnetic bead based extraction methods Ct values of N genes remained ≤ 35 till S8 and S7th pool and for ORF-1ab those remained on S6 and S6th pools in both the procedures respectively. A Ct value of 35 was initially taken as upper cut off point for consideration of positivity of RNA samples.



As the ROC curves were needed to develop for selecting a suitable upper cut off value of Ct corresponding to the confirmatory set of viral gene to be detected in RT-PCR platform, this set of experiment on different regressive pools of positive/negative samples resulted interesting selection criteria of discrimination. For the 10 pools the RNAs extracted by spin column based method and for magnetic based method, when targeted for expression of gene under cut off value ≤30 and ≤35 are found to be most suitable. The cut off value taken for this purpose were Ct values as 20, 23, 25, 30, 35 & 40. The combination of sensitivity, specificity and AUC for **spin column based method** for N gene were found (0%, 100%, 0.5; 80%, 82%, 0.78; 84%, 76%, 0.99; 90%, 0%,1; 100%, 0%, 0) and for **magnetic bead based method** (0%, 100%, 0.5, 88%, 66%, 0.94, 86%, 75%, 0.98, 84%, 77%, 0.99; 87%, 67%, 0.99; 100%, 0%, 0) for the cut off values selected respectively. Accordingly for sensitivity, specificity and AUC for **spin column based method** for ORF 1ab gene werefound (20%, 100%, 0.6; 90%, 50%, 0.67, 84%, 81%, 0.73; 85%, 75%, 0.95; 88%, 68%, 1.0; 100%, 0%, 0.0) and for **magnetic bead based method** they are (0%, 100%, 0.5; 87%, 50%, 0.71; 81%, 60%, 0.96; 81%, 60%, 0.96; 78%, 50%, 0.84; 100%, 0%, 0). So comparing Cut off values with Ct values obtained from each pool, the set of data depicting highest sensitive but least false positive rate and good AUC could be considered a in pool development. So, for expression of N gene the corresponding suitable pools could be below ≤S6 and ≤S6 in respective extraction methods (ref to Table 1). Again, in case of ORF1ab gene, for expression of it the corresponding suitable pools could be ≤ S8 and ≤S8 in respective extraction methods.

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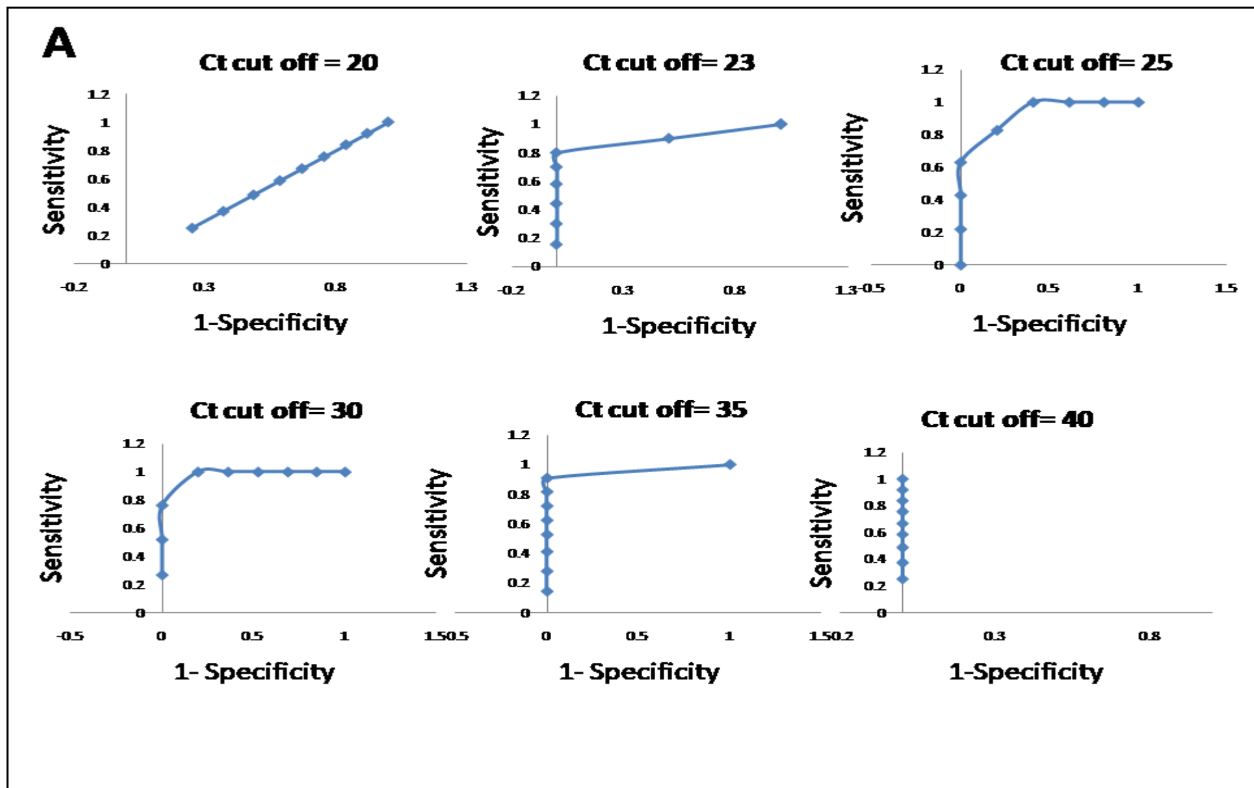
Type of extraction	Suitable pool	Cut off (Ct)	Sensitivity (%)	1- Specificity (%)	AUC
Spin column based (N gene)	≤S6	≤ 30	84.3	76.2	1.0
Magnetic bead based (N gene)	≤S6	≤ 30	84.6	77.0	1.0
Spin column based (ORF 1ab)	≤S8	≤ 35	88.2	68.0	1.0
Magnetic bead based (ORF 1ab)	≤S8	≤ 35	78.8	50.5	1.0

Table 2. ROC data presenting suitable pool to be constructed for better identification of N and ORF1ab gene.



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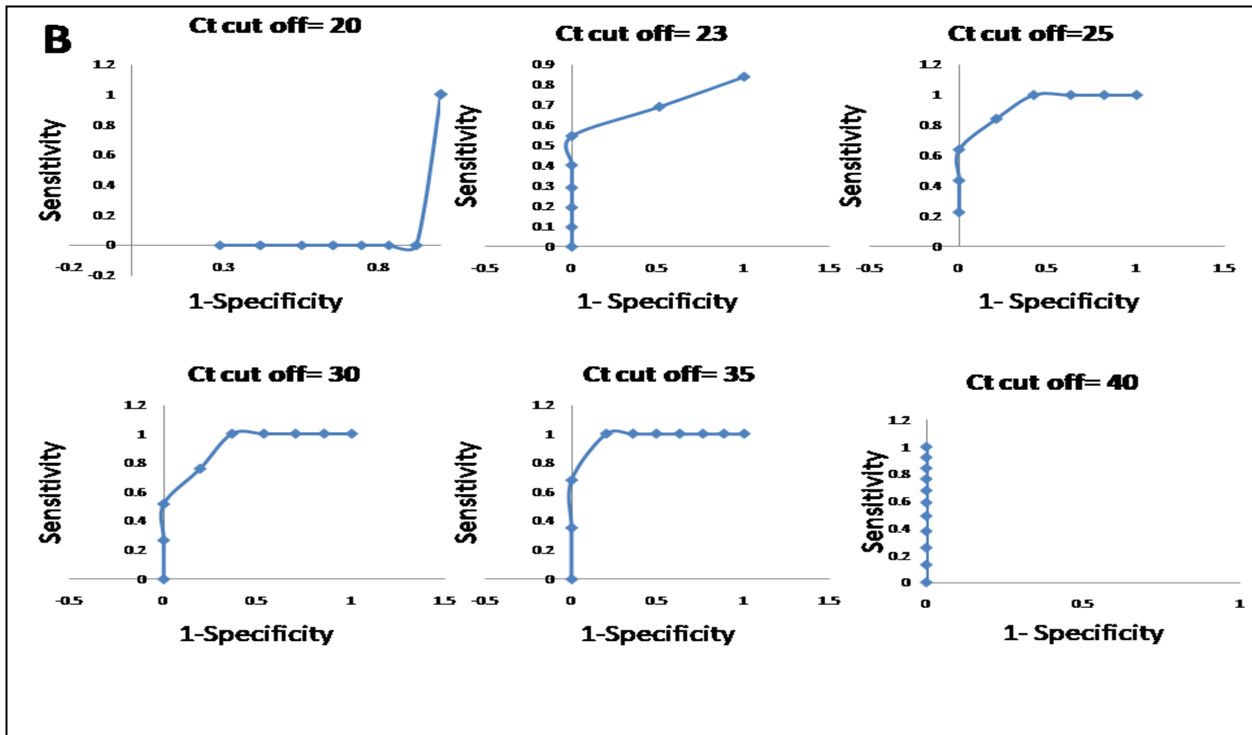

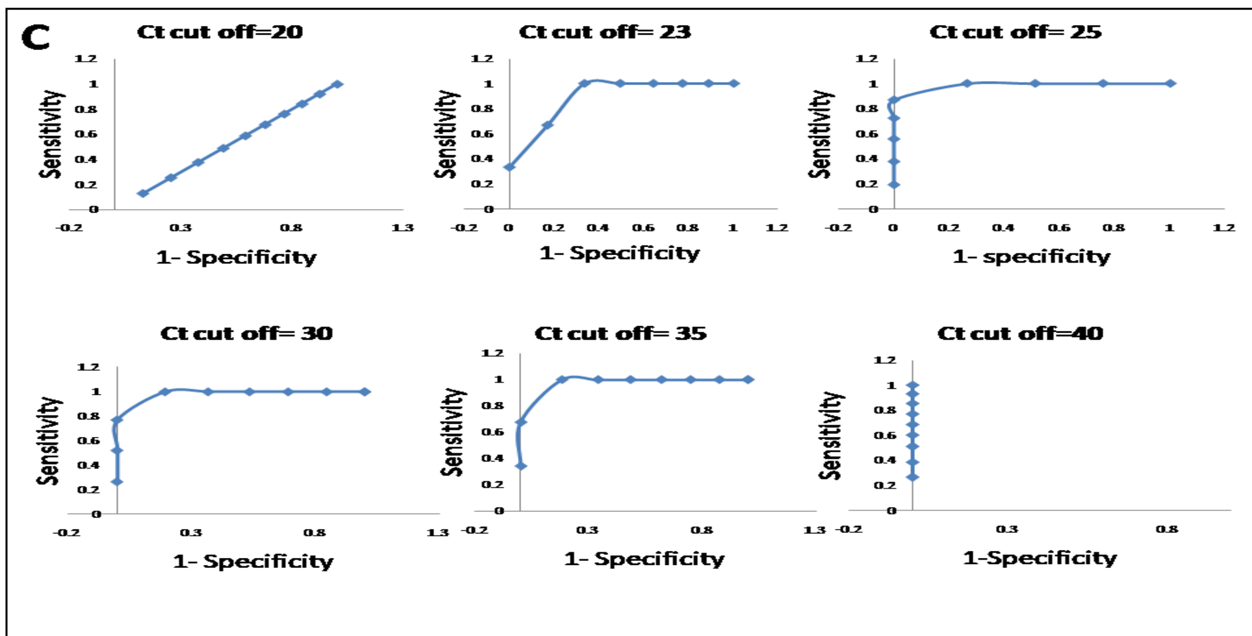

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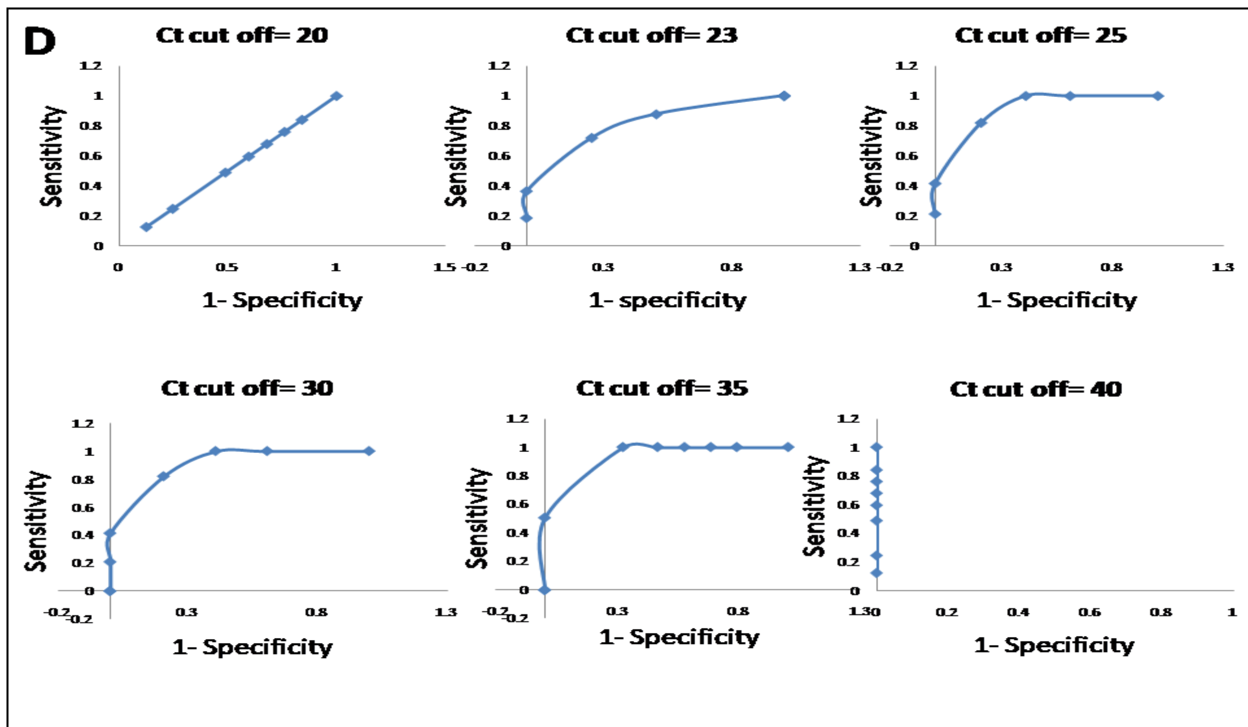

Fig 5. Graph A and B represented ROC curves for different cut off value taken for Ct values of N gene identified from RT-PCR results of different pools extracted by spin column and magnetic bead based extraction method. Graph C and D represented ROC curves for different cut off value taken for Ct values of ORF1ab gene identified from RT-PCR results of different pools extracted by spin column and magnetic bead based extraction method.

When, fold change study was done on the Ct mean values of corresponding genes of various pools (S1 to S10) RNA samples, for confirmation of presence of SARS-CoV-2 in human sample, it showed interesting outcome. The fold change value ΔΔCt N gene for spin column based and magnetic bead based extraction procedureswas found to be 9.95 and 10.37 times. Again, fold change value ΔΔCt ORF1ab gene for spin column based and magnetic bead based extraction procedures was found to be 11.56 and 11.41 times. This showed a very little change of gene expression of both gene in the respective kinds of extraction procedure, but confirmed higher expression of ORF1ab than that of N gene throughout this study which indicated presence of a true COVID +ve case in the pools.

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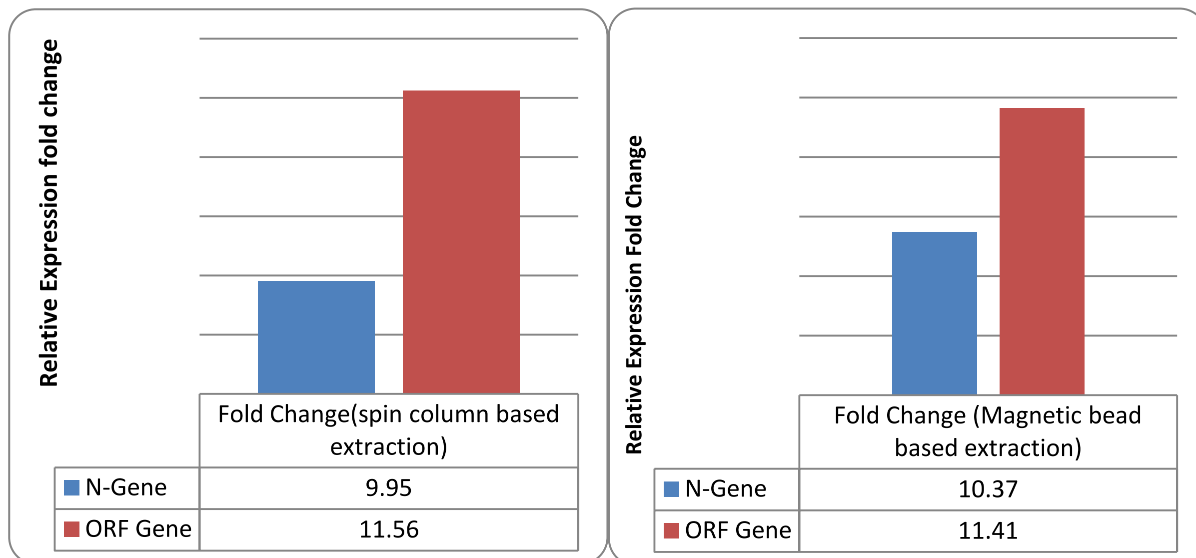
Fig 6. The comparative Fold scale study on ΔΔCt changes for N and ORF1ab gene throughout the experiment



Discussion

The purity of RNA sample is considered to be the most acceptable if the optical density of the sample checked within 260 and 280 nm wavelength gives a ratio around 2.1. As dsDNA could be detected within 260 nm, a 260/280 nm ratio higher than 1.8 means less presence of DNA in sample. However, wavelength ratio of 260/230 of which a range within 230 nm indicates micro-contaminants in the process of extraction of nucleic acid, the purity of any RNA sample indicates the ratio around or above 2.0. (Koetsier et al., 2019 ; Wilfinger et al., 1997). In the present study, when the viral nucleic acid samples extracted from pools of swab loaded VTM was checked for purity of RNA, a gradual increase in 260/280 nm ratio observed until 3.48 at 10th pool indicated lesser RNA quantity in final pools. Again, gradual decrease in 260/230 nm ratio in consecutive pools indicated possible presence of microcontaminants in the tested samples and lack of sufficient viral RNA in them. Such micropollutants could be present in buffers that contains Guanidinium Chloride etc. This is used to denature proteins in the sample but aiding a little bit acidity to the column may change in the yield of nucleic acid. Again, Viral transport liquid media are composed of buffers to control pH, protein to stabilize the virus, and often other substances to control osmolality or onto which the viruses can adsorb. Proteins, such as bovine serum albumin, gelatin, skimmed milk, normal serum or even some antibiotics are added as protective agents in them (Johnson, 1990). So, in preparation of a large pool such elements may decrease in 260/230 nm ratio. However, in both extraction platforms we followed, viz, spin column based and magnetic bead based extraction method, no highly significant changes in 260/280 and 260/230 ratio results.

As per present global scenario of COVID expansion, extensive testing for SARS-CoV-2 is one of the most important components of COVID-19 control strategy at present (Li, 2016). Timely and accurate reporting can lead to proper contact tracing and effective containment measures. As we found a tertiary health center situated at state level medical college received sample size of 111379 in last wave of pandemic in our country, the RT-PCR laboratories would have no choice other than making proper pool from swab collected VTMs to extract mixture of RNAs from which RT-PCR platform can easily detect screening genes and confirmatory genes. In a previous study (Praharaj et al, 2020), researchers checked for getting a perfect pool beyond that RT-PCR results may be vague. But they considered only one gene E corresponding to structural protein, that may present due many respiratory viral infections in human body, hence it is being considered as “screening” gene too. The genome structure of the single-stranded non-segmented positive-sense RNA of the CoVs includes two large nonstructural polyproteins that are not involved in host response modulation, called as open reading frames (**ORF1ab**). On studying the whole genome sequence of SARS-CoV-2 virus it was found

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that there are seven conserved replicate domains in the ORF1ab SARS-CoV-2 gene that share a 94.4% sequence identity with SARS-CoV (Sheikhzadeh et al., 2020) So, for confirmation it is better to identify one structural gene (E or N etc) and one non-structural genes such as RdRP / and or ORF1ab. 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 (Giri et al., 2020, Chu et al., 2020). Based on their detection performances, the N gene RT-PCR is recommended as a screening assay, and the Orf1b assay is recommended as a confirmatory one. Using a diagnostic algorithm similar to MERS, an N gene positive/Orf1b negative result should be regarded as indeterminate and the case is recommended to be referred to a WHO reference lab for further testing (Chu et al., 2020). In our study the already an upper cut off Ct value of 35 was taken as per kit-book and single positive sample's Ct mean value corresponding to N gene and ORF1ab never observed to occur beyond. As increasing Ct value reflects lesser amount of RNA present as template, this supports the centered scattered plot of which Ct value for N gene in both cases of spin-column and magnetic bead based extraction limited up to 35 till S8 and S7th pool and for ORF1ab, they were up to S6 th pool for both cases. Again, in present case of spin column and magnetic bead based method, it resulted 260/280 nm ratio as 2.88, 2.5 and 2.3 for poolS8, S7 and S6. Hence, these experiments actually do not allow to construct more than a pool containing 1 positive sample in 5 negative samples mostly (S6). However, for understanding expression of N and ORF1ab gene and getting a Ct mean Cut off value ROC curve and Fold change study was conducted.

The ROC curve experiment conducted by taking various cut off values which were obtained from RT-PCR analysis of RNA sample extracted through the pools (from S1 to S10). This declared that the best cut off value for spin column and magnetic beadbased extraction responding to N gene found to be ≤ 30, that means the $S6^{th}$ pool. The selection of a good cut off value was dependent on high sensitivity and (1-specificity) which should have an excellent AUC value. The analytical sensitivity of SARS-CoV-2 RT-PCR assays is still up for debate, but it is generally thought to be high. However, the clinical sensitivity of RT-PCR for SARS-CoV-2 is only around 70-80% at best. In our study we were able to obtain more than 80% sensitivity in selected pools for both the genes (Wiesbauer, 2020; Arnaout et al., 2020). Again, for ORF1ab gene in both type of extraction method, it showed bestcut off ≤ 35, the $S8^{th}$ pool. This indicated that in a pool if the positive sample should be discriminated on the basis of detection of N gene it would be difficult to separate false positive samples beyond 6th pool and in case of ORF1ab the limit should be 8th pool. Thus highest Ct cut off could be 35 only in this set of experiment. Such data could be supported by work from other institutes (Rao et al., 2013) where 5 pools system came out with highest concordance for giving Ct value ≤ 35, however they tested only 3 kinds of pools and considered only E gene in their study. So for getting better result lesser than 6th pool can also work to discriminate false positive if one screening gene is selected. Existence of such results present too globally. Again, as fold change data had shown more expression of ORF1ab gene in all the cases rather than N gene, it indicated the reason to get a higher limit of pool (S8) to get could cut off value. As the gene expression is higher so the higher presence of RNA and so the higher limit of exclusion for a positive case. The availability of higher Ct value and gene expression in case of magnetic bead based extraction might occur due some advantage of this kind of extraction process which is able to extract unfragmented or longer nucleic acid molecules (Ali et al., 2017). Some study states it superiority than spin column based extraction method (which is mostly one time used) because of its robust and user friendly nature or high automation potential (Koo et al., 1998), however in our study we found very less difference in quality in both system, whether in terms of purity or Ct values.

Conclusion

This set of experiment was actually imagined and executed not only to scrutinize the myth behind perfect pool or finding a way to get more positive samples in a single set but also to observe the expression pattern of screening as well as confirmatory genes in an arbitrary lot of samples. The results showed that for proper identification of a true positive SARS-CoV-2 infected case if a pool may be constructed less than adding 6 samples altogether it would be effective if single-plex primer-probe set up

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is done for screening genes, but in the other hand multiplexing two or more set of genes (both screening or confirmatory) it could be expandable up to conjugation of 8 unknown samples to make a pool. This small piece of work may help the scientific community associated with viral diagnosis to re-think and re-arrange strategy to cut short time, optimized in their own laboratory environment to face if any new wave of pandemic is on march in near future. However, our studies may moderately differ in other laboratories as RT-PCR is a very delicate platform of quantitating RNA and even a minute operational change may play crucial role in change in results.

Author Contributions:

All the authors substantially contributed to the conception, design, analysis and interpretation of data, checking and approving the final version of the manuscript and agree to be accountable for its contents.

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References

1. Ali, N., Rampazzo, R.C.P., Costa , A.D.T., Krieger, M.A., 2017. Current nucleic acid extraction methods and their implications to point-of-care diagnostics, BioMed Res. Int., 2017, 1-13. https://doi.org/10.1155/2017/9306564

2. Arnaout, R., Lee, R.A., Lee, G.R., Callahan, C., Yen, C. F., Smith, K.P., Arora, R., Kirby, J.E., 2020. SARS-CoV2 Testing: The Limit of Detection Matters, BioRxiv , https://doi.org/10.1101/2020.06.02.131144

3. Chang, M.C., Hur J., Park , D., 2020. Interpreting the COVID-19 test results: a guide for 247 physiatrists. Am. J. Phys. Med. Rehabil., 99(7), 583-585. https://doi:10.1097/PHM.0000000000001471

4. Chu, D., Pan, K.W.Y., Cheng, S.M.S., Hui, K.P.Y., Krishnan, P., Liu, Y., Ng, D.Y.M., Wan, C.K.C., Yang, P., Wang, Q., Malik, P., Poon, L.L.M., 2020. Molecular Diagnosis of a Novel Coronavirus (2019-nCoV) Causing an Outbreak of Pneumonia , Clin. Chem. 66 (4), 549–555, https://doi.org/10.1093/clinchem/hvaa029

5. COVID-19 Map - Johns Hopkins Coronavirus Resource Center (jhu.edu)

6. Deckert, A., Bärnighausen, T., Kyei, N., 2020. Pooled-sample analysis strategies for COVID-19 mass testing: A simulation study. Bull. World Health Organ. doi: http://dx.doi.org/10.2471/BLT.20.257188.

7. Giri, R., Bhardwaj, T., Shegane, M., Gehi, B.R., Kumar, P., Godhave, K., Oldfield, C.J., Uversky, V.N., 2020. Understanding COVID-19 via comparative analysis of dark proteomes of SARS-CoV-2, human SARS and bat SARS-like coronaviruses. Cell. Mol. Life. Sci. 25, 1–34. https://doi.org/10.1007/s00018-020-03603-x

8. Johnson, F. B., 1990. Transport of Viral Specimens, Cilin. Microbiol. Rev.,120-131

9. Koetsier, G., Cantor, E., 2019. A practical guide to analyzing nucleic acid concentration and purity with microvolume spectrophotometers. *Technical Note, New England Bioalbs,.*

10. Koo, K., Foegeding, P. M., Swaisgood, H. E., 1998. Isolation of RNA and DNA fragments using diatomaceous earth, Biotechnol. Tech. 12 (7),549–552. https://doi.org/10.1023/A:1008859632378

11. Lee, C.Y., Lin, R.T.P., Renia, L., Ng, L.F.P., 2020. Serological approaches for COVID-19: epidemiologic perspective on surveillance and control, Front. Immunol. 11 , 879. https://doi.org/10.3389/fimmu.2020.00879

12. Li, F. , 2016. Structure, function, and evolution of coronavirus spike proteins. Annu. Re. Virol. 3, 237–261 https://doi.org/10.1146/annrev-virology-110615-042301 .

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13. Praharaj, I., Jain, A, Singh, M., Balakrishnan, A et al., 2020. Pooled testing for COVID-19 diagnosis by real-time RT-PCR: A multi-site comparative evaluation of 5 & 10 sample pooling. Indian J.Med.Res.152, 88-94. https://doi.org/10.4103/ijmr.ijmr_2304_20

14. Rao, S.N., Manissero, D., Steele V.R., Pareja, Josep., 2020. A systematic review of the clinical utility of cycle threshold values in the context of COVID-19. Infect. Dis. Ther,. 9(3), 573-586. https://doi.org/10.1007/40121-020-00324-3

15. Rao , X., Huang, X., Zhou, Z., Lin, X. 2013., An improvement of the 2ˆ(–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis**,** Biostat. Bioinforma. Biomath. 3(3), 71–85. https://pubmed.ncbi.nlm.nih.gov/25558171

16. Sahin, A.E., Erdogan, P., Mutlu Agaoglu, Y., Dineri, A.Y., Cakirci, M., Egemen Senel, R. A., Okyay, A., Tasdogan, M., 2020. Novel coronavirus (COVID-19) outbreak: a review of the current literature, Eurasian J. Med. Oncol. 4, 1–7, https://doi.org/10.14744/ejmo.2020.12220.

17. Shahi, S., Zununi Vahed , S., Fathi , N., Sharifi, S., 2018. Polymerase chain reaction (PCR)-based methods: promising molecular tools in dentistry, Int. J. Biol. Macromol. 117, 983–992. https://doi.org/10.1016/j.ijbiomac.2018.05.085 .

18. Sheikhzadeh, E. , Eissa, S., Ismail, A., Zourob, M., 2020. Diagnostic techniques for COVID-19 and new developments. Talanta, 220, 121392. https://doi.org/10.1016/j.talanta.2020.121392

19. Wiesbauer , F., 2020. https://www.medmastery.com/guide/covid-19-clinical-guide

20. Wilfinger, W.W., Mackey, K., Chomczynski, P, 1997. BioTechniques. 22, 474–481, https://doi.org/10.2144/97223st01

21. Wishaupt, J.O., Ploeg, T. , Smeets, L.C. , de Groot, R., Versteegh, F. G . A , Hartwig, N. G ., 2017. Pitfalls in interpretation of CT-values of RT-PCR in children with acute respiratory tract infections. J. Clin. Virol., 90, 1-6, https://doi:10.1016/j.jcv.2017.02.010

22. Younes, N., D.W., Al-Sadeq , H., Al-Jighefee, Younes ,S., Al-Jamal , O., Daas , H.I., Yassine, H. M., Nasrallah , G.K., 2020. Challenges in Laboratory Diagnosis of the Novel Coronavirus SARS-CoV-2, Viruses,1-12. https://doi:10.3390/v12060582

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