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Sensitivity Comparison of Different Methods Used for RNA Extraction with Increasing Capacity for the Detection of COVID-19

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ABSTRACT

The emergence of a novel corona virus was seen in the human population since December 2019. The Covid-19 is posing a major burden on society. Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) has since spread across the globe. Thus, early and correct diagnosis is very crucial to reduce its spread. So, most sensitive and specific method available to diagnose the Covid-19 is real-time reverse transcriptase PCR (qRT-PCR). A better qRT-PCR result depends on the quality of the extracted RNA.

Recently, many commercial kits have become available for extraction of RNA from specimens. The goal of this study was to compare robustness or clinical performance of manual kits available to us (i) MDI Viral RNA Extraction Mini-prep Kit and 2 automated extraction systems such as (i) MagNA Pure 24 Roche (ii) Nextactor NX-48 Genolution. We used 48 pre-examined nasopharyngeal and oropharyngeal swab samples with low, medium and high cycle threshold (Ct) values for this study and extraction was done in pooling of 1:5, 1:6, 1:7, 1:8, 1:9, 1:10.

Through this work, we conclude that MagNA Pure 24 Roche and MDI Viral RNA Extraction Mini-prep kit showed better result as compared to Nextactor NX-48 Genolution.

***Keywords*:-** SARS-CoV-2, qRT-PCR, Extraction, Diagnosis.

INTRODUCTION

In 1960s, first human corona virus was identified and it showed characteristic feature like flu that were responsible for respiratory tract infection. In 2001 according to Canadian study report, about 500 patients were identified with Flu like symptoms out of which 17-18 were confirmed through polymerase chain reaction and found infected with corona virus [(1,2)]. In Dec. 2019 Corona virus was notified again and isolated from the pneumonia patients of Wuhan city in China. It has emerged as biggest pandemic across the world in 2020 and named as Covid-19 disease by World Health Organization [(3,4)]. It is a positive sense single stranded RNA virus.

It is spherical, pleomorphic, with glycoprotein envelope and has four sub-types such as alpha, beta, gamma, delta and many serotype that affect humans and other animals such as birds, dogs, cats, pigs [(5,6,7,8)]. New corona virus belongs to coronaviridae family, it has generated health emergency all over the world [(9,10)]. Because of rapid spreading of SARS-COV-2 all over world it has prompted for fast diagnostic testing based on RT-qPCR following RNA extraction [(11,12,13)]. All qPCR methods require RNA extraction for diagnosis after the swab samples are collected from the oropharyngeal and nasopharyngeal route [(14,15,16,17)]. The diagnosis of COVID -19 in virology laboratory, involves commonly these steps – viral inactivation, lysis, extraction of viral RNA and qRT-PCR amplification. Because of rapid spreading of

virus the demand of molecular testing has increased [18,19,20]. So, to cater to more number of samples efficiently in less time, pooling of samples is an alternative especially when positivity in community is low [21,22,23]. In our lab pooling in numbers from 5 to 10 were tried by various RNA extraction methods to validate the results.

MATERIALS AND METHODS

Sampling:- Total numbers of 48 (8 positive and 40 negative) pre-examined samples were used during this study and extraction was done in the pools of 1:5, 1:6, 1:7, 1:8, 1:9 and 1:10 each of these samples. The samples were selected with Cts varying from high to low [22-30].

RNA Extractions

In this study we compared three RNA extraction methods, (A) Manual Extraction (Column based) MDI Viral RNA Extraction Mini-prep kit (B) automated systems such as MagNA Pure 24 Roche (C) Nextactor NX-48 Genolution. RNA extraction was done by the following procedures using different modes:-

(A) Manual method by MDI Viral RNA Extraction Mini-prep Kit:-

Pipetted 560µl of buffer VRL-Carrier RNA solution into a 1.5 ml micro-centrifuge tube and added 200µl (for single) of sample in it. After vortex Incubated at room temperature for 5 to 10 min. Added 700µl ethanol in mixture then vortex and transferred the solution in spin columns, centrifuged it at 10000 rpm for 1 min. and discarded the flow through. Then washed with 500 µl of VW1, and centrifuged at 10000 rpm for 1 min. Second time washed with 400 µl VW2 for 2 min at 10000 rpm for 1 min. Placed spin column in RNase free 1.5 ml micro-centrifuge tube and added 40µl of buffer VE directly on the centre of column membrane. Left it for 4 to 5 min then spin at same speed for 1 min and stored eluted RNA at -20^{0}C till further use.

(B) Automated method (I) by Nextractor NX-48 Genolution:-

NX-48s viral NA kit contains four preloaded deep well plates (24 well each) with strips. Sample volume of 200 µl required for each well as per the protocol provided by the company. The volume used for pools as 1:5, 1:6, 1:7, 1:8, 1:9 and 1:10 shown below in the table 1. It works on the principle of using magnetic beads and extraction procedure followed the steps such as- (i) beads capturing (ii) lysis (iii) binding (iv) washing (v) evaporation and (vi) elution which took about 27 mins. Eluted RNA collected in RNase free micro-centrifuge tube and stored at -20^{0}C till further use.

(C) Automated method (II) by MagNA Pure 24 Roche:-

We used external lysis protocol for the extraction with Magna Pure 24 Total isolation kit and used all the accessories and reagents provided by the Roche Diagnostics. We standardized this protocol by making some changes in the volume of reagents and samples as shown below in the table 1. It took about 1 hour 20 min in the completion of extraction by following steps as lysis, binding, washing and elution. Eluted RNA stored it at -20^{0}C till further use. For all above methods protocol provided by the company was properly followed. Sample volume used for each pool ratio is given below in table 1.

Table 1

S.NO	Sample Id.	Pools Ratio with volume (µl) for single sample by MDI Viral RNA Extraction Mini-prep kit					
		1:5	1:6	1:7	1:8	1:9	1:10
1	R 1512	40	33	28	25	22	20
2	R 1420	40	33	28	25	22	20
3	R1419	40	33	28	25	22	20
4	R1165	40	33	28	25	22	20
5	R1373	40	33	28	25	22	20
6	R1423	40	33	28	25	22	20
7	R1345	40	33	28	25	22	20
8	R 1371	40	33	28	25	22	20

Table 1 Continued...							
		Pools Ratio with volume (µl) for single sample by Nextactor NX-48 Genolution Machine					
		1:5	1:6	1:7	1:8	1:9	1:10
1	R 1512	40	33	28	25	22	20
2	R 1420	40	33	28	25	22	20
3	R1419	40	33	28	25	22	20
4	R1165	40	33	28	25	22	20
5	R1373	40	33	28	25	22	20
6	R1423	40	33	28	25	22	20
7	R1345	40	33	28	25	22	20
8	R 1371	40	33	28	25	22	20
		Pools Ratio with volume (µl) for single sample by MagNA Pure 24 Roche Machine					
		1:5	1:6	1:7	1:8	1:9	1:10
1	R 1512	125	104.1	89.2	78.1	69.4	62.5
2	R 1420	125	104.1	89.2	78.1	69.4	62.5
3	R 1419	125	104.1	89.2	78.1	69.4	62.5
4	R 1165	125	104.1	89.2	78.1	69.4	62.5
5	R 1373	125	104.1	89.2	78.1	69.4	62.5
6	R 1423	125	104.1	89.2	78.1	69.4	62.5
7	R 1345	125	104.1	89.2	78.1	69.4	62.5
8	R 1371	125	104.1	89.2	78.1	69.4	62.5

PCR Amplification

Amplification has been done by using COVIDsure Multiplex Realtime RT-PCR Kit (LABSYSTEMS Diagnostics) with Roche LightCycler 480. SARS-CoV-2 specific primers and fluorescence labeled hydrolysis probes were used in this study according to the instructions of the kit. The specific primers are labeled with FAM specific to Orf1ab and HEX specific to E gene of SARS-CoV genome. The ROX specific dye was labeled to RPP30 human gene and serves as an internal control. Reaction mixture was prepared and used 15 µl/ reaction as per instructions. 5 µl of RNA/reaction was used as template. Negative and positive controls were also run in every PCR plate. PCR plate was rotated in mini-spin at 2500 RPM for 20 secs. The parameters for PCR cycling included reverse transcription (1 cycle) at 46^0C for 15 min, initial activation (1 cycle) at 95^0C for 2 mins. Amplification cycles as Annealing, Extension and signal acquisition run for 40 cycles at 58^0C for 30 secs.

RESULT

There was no false positive and negative amplification observed during this study. Results were analyzed under the following conditions:-

Table 2

Sample	FAM (Orf1ab)	HEX (E gene)	ROX (RPP30)	Interpretation
Test sample	Ct ≤ 36	Ct ≤ 36	Ct ≤ 36	Viral RNA detected
Test sample	Ct ≤ 36	No amplification	Ct ≤ 36	Viral RNA detected
Test sample	No amplification	Ct ≤ 36	Ct ≤ 36	Presumptive positive (retested)
Test sample	No amplification	No amplification	Ct ≤ 36	Viral RNA not detected
Test sample	No amplification	No amplification	No amplification	Invalid result, re-perform RNA extraction

Through this interpretation all three RNA extraction methodologies were checked. Ct value of the test samples was used earlier by RT- PCR was taken as control value. Results were shown as given table below:-

Table 3.

S No.	Sample Id		Ct value of pools by MDI Viral RNA Extraction Mini-prep Kit					
		Control value	1:5	1:6	1:7	1:8	1:9	1:10
1	R 1512	29	29.5	29.6	29.7	30.0	30.1	30.2
2	R 1420	30	30.8	30.9	31.0	31.5	31.6	31.6
3	R 1419	30	30.7	30.8	31.2	31.3	31.4	31.4
4	R 1165	24	23.9	24.1	24.4	24.7	25.1	25.7
5	R 1373	24	24.8	26.4	26.5	27.1	27.2	27.2
6	R 1423	28	29.0	29.1	29.2	29.6	30.1	30.2
7	R 1345	22	22.1	22.5	23.6	23.7	23.8	23.9
8	R 1371	21	20.8	21.0	21.4	21.5	21.5	22.6

Table 3 Continued...								
			Ct value of pools by Nextractor NX-48 Genolution					
		Control value	1:5	1:6	1:7	1:8	1:9	1:10
1	R 1512	29	35	35	35	32	32	35
2	R 1420	30	35	35	35	32	31	35
3	R1419	30	34	36.4	35	32	31	31
4	R1165	24	28	30	30	28	28	26
5	R1373	24	27	29	29	27	27	29
6	R1423	28	34	34	32	29	36.6	36.7
7	R1345	22	26	26	28	25	24	28
8	R 1371	21	26	26	27	21	22	26
			Ct value of pools by MagNA Pure 24 Roche					
		Control value	1:5	1:6	1:7	1:8	1:9	1:10
1	R 1512	29	28.7	28.9	29.9	30.8	30.9	31.4
2	R 1420	30	28.5	28.8	30.1	30.3	30.3	31.0
3	R 1419	30	28.3	28.8	28.8	29.4	29.7	30.7
4	R 1165	24	26.0	26.7	26.8	28.3	29.3	29.3
5	R 1373	24	25.3	25.4	25.4	25.5	25.9	27.7
6	R 1423	28	28.2	30.1	30.2	30.2	30.2	31.0
7	R 1345	22	23.1	23.2	23.2	23.9	24.0	24.0
8	R 1371	21	22.5	22.5	22.6	22.7	22.8	23.8

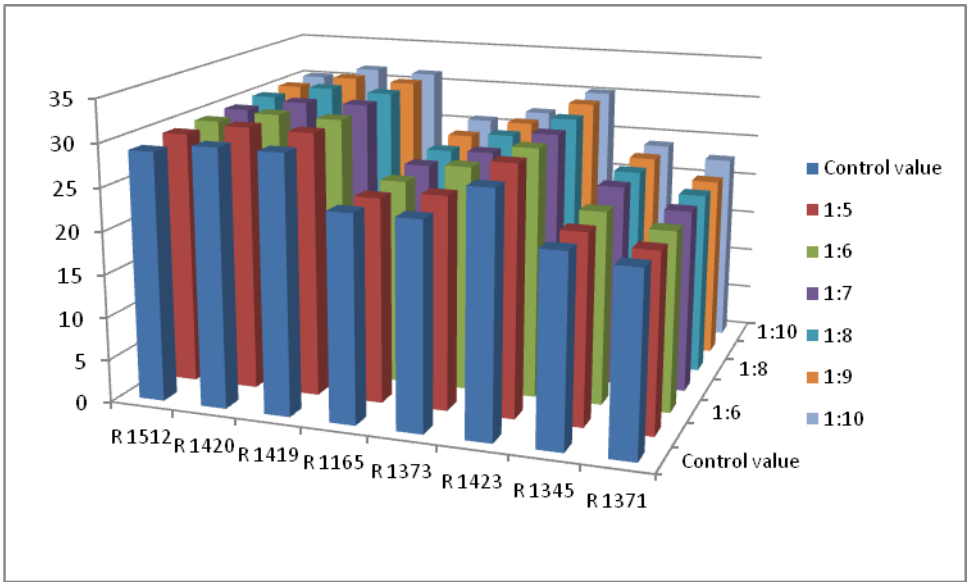

Fig 1 Vertical axis represents Ct values (pools ratios) and horizontal axis represents sample id. (MDI Viral RNA Extraction Mini-prep Kit)

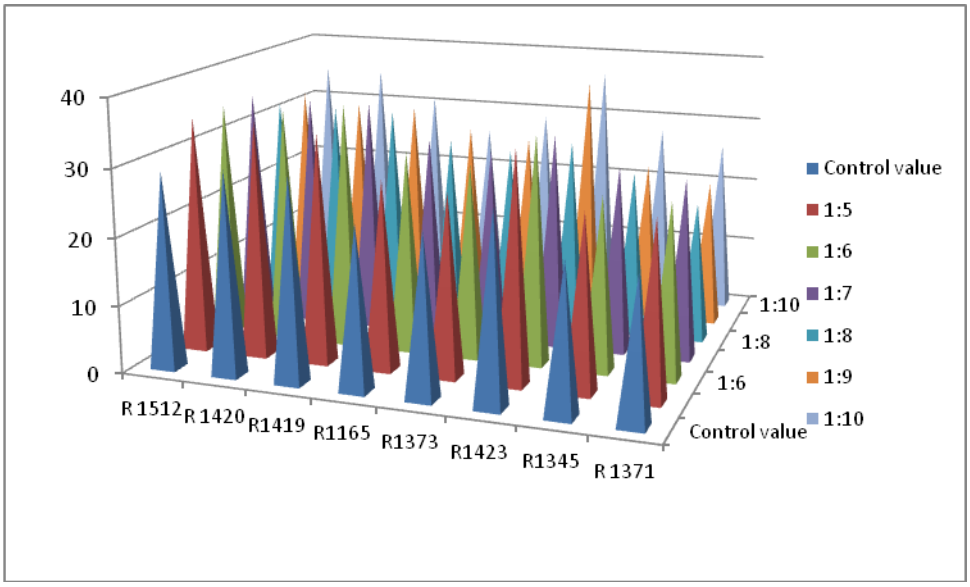

Fig2. Vertical axis represents Ct values (pools ratios) and horizontal axis represents sample id. (Nextractor NX-48 Genolution)

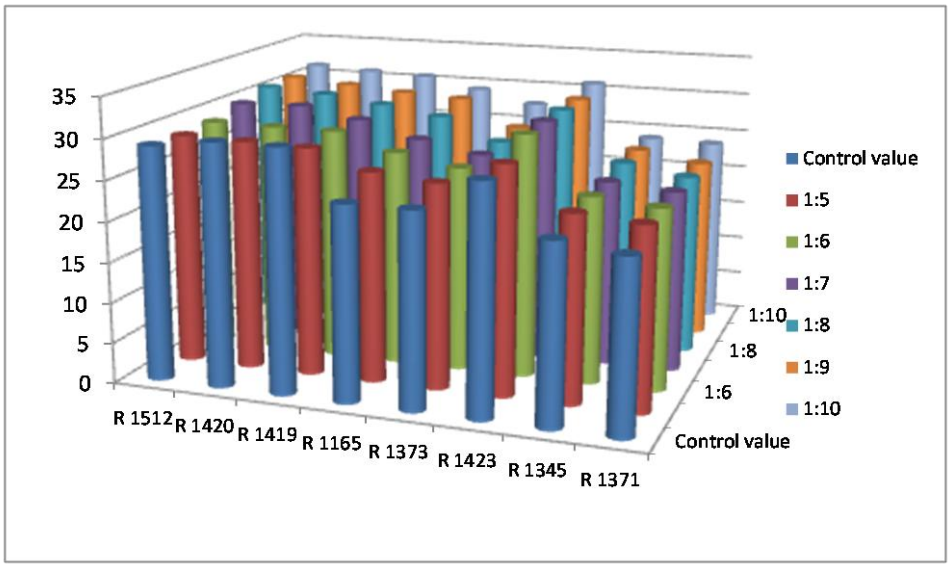

Fig3. Vertical axis represents Ct values (pools ratios) and horizontal axis represents sample id. (MagNA Pure 24 Roche)

Same samples were used during the manual and automated extraction. Sample R 1512, R 1420, R 1419 and R 1371 showed the better results in case of MagNA Pure 24 Roche and MDI Viral RNA Extraction Mini-prep Kit. All other samples also showed good results and extraction efficiency and sensitivity was good in these two extraction methodologies. Extraction result was analyzed through qPCR and given in table 2 and graphical presentation in fig1 and fig 3. Samples checked through Nextractor NX-48 Genolution showed higher differences in cycle threshold (Ct) values as dilution of pools increased. Samples R 1419 and R 1423 showed negative (≥ 36) with the dilutions of 1:6, 1:9 and 1:10 respectively. It showed less sensitivity in extraction when result was compared with control values table 2 and fig.2. in case of Nextractor NX-48 Genolution results were not satisfactory towards sensitivity and efficiency of extraction in our study. In case of MagNA Pure 24 Roche and MDI Viral RNA Extraction Mini-prep Kit extraction showed high efficiency and sensitivity in approximately all samples. Kit based manual extraction results were better than Nextractor NX-48 Genolution.

DISCUSSION & CONCLUSION

In this study, we found that the results of MagNa Pure 24 Roche and MDI Viral RNA Extraction Mini-prep Kit extraction are better as compared to Nextractor NX-48 Genolution. As regards the duration of the time of provided manual extraction is the shortest method while MagNa Pure 24 Roche took the longest time. Some modifications in the manual method could further shorten the duration. Results of Nextractor NX-48 Genolution gave higher Ct values than control. The results of the pools in 1: 5 however comparable with the other two methods.

As per interpretation of results pools of (1:5 – 1:10) can be recommended for listing in the time of high load of samples. The purpose of this study is to introduce that the increased size of pooling samples protocol will be helpful to save time, labor and logistics along with the reducing cost per test which is important in this crisis time. This will also reduce the plastic biohazard waste generated due to large scale testing in this COVID crisis. Manual method involves less expenditure as compared to automated nucleic extraction machines and their costly consumables. One caution to prevent false negativity in pools is proper standardization of sample and chemical volume. Also the size of the pools

depends largely on the load of positivity in the community.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the Publication of this research article.

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Ethical Approval: Approved

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