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ORIGINAL ARTICLE

Regenerated silica-based RNA purification columns to address the short supply of RNA purification kits for COVID-19 diagnosis

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Abstract

Background RT-qPCR technique is the current world-wide method used for the early detection of SARS-CoV2 RNA in the suspected clinical samples. Viral RNA extraction is the key pre-analytical step for SARS-CoV2 detection which often achieved using commercial RNA-extraction kits. However, due to the COVID-19 pandemic, bulk production and the supply chains for the commercial RNA-extraction kit have been seriously compromised. The shortage of commercial RNA-extraction kit is even more acute in developing country. Furthermore, use of one-off design RNA-columns can generate plastic wastes that have an environmental pollution effect.

Methods and results To address these issues, in this study, we used warm alkaline solution containing Triton X-100 for the complete removal of the residual SARS-CoV2 RNA from the used RNA-binding silica column. Columns regenerated using the alkaline solution have the viral RNA purification capability that is comparable to the fresh silica columns. We also demonstrated that RNA-binding silica columns can be regenerated and reused for a minimum of five-times.

Conclusions Therefore, the use of the RNA-column regeneration method may benefits several SARS-CoV2 diagnostic laboratories throughout the world by cutting down the requirement of commercial RNA-purification column.

Keywords SARS-CoV2 · COVID-19 · RT-qPCR · RNA-extraction · Regenerated RNA-purification column

Introduction

The coronavirus disease 2019 (COVID-19) pandemic which is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV2) has emerged as a serious threat to the human health globally [1]. The elderly people and those with pre-existing medical conditions are prone to have severe or deadly COVID-19 infection [2], thereby leading to a high burden on global health care system. Detecting the presence of SARS-CoV2 genome and/or antigen is of extreme importance for the containment strategies aiming to reduce the spread of the virus [3, 4]. However, due to several bottlenecks imposed by the cost involvement in timely and quality virus diagnosis, understanding and managing the COVID-19 outbreak has remained a challenge for several countries world-wide [5].

Early diagnosis of COVID-19 relies on the efficient detection of SARS-CoV2 genome using RT-qPCR [6–8]. Several RT-qPCR assays have been used for the virus diagnosis and novel technologies for the detection of the viral RNA are constantly evolving with time [9, 10]. All these nucleic acid recognition methods require an RNA-extraction step to isolate the SARS-CoV2 RNA before its detection. Therefore, the availability of RNA-extraction kits have become a serious limitation for COVID-19 diagnosis due to the world-wide demand-driven shortage of commercial RNA-extraction kits [11]. This is particularly difficult in countries lacking suitable infrastructure and capacity to produce in bulk good-quality RNA extraction kit locally. Furthermore, the commercial RNA-extraction kits recommended for single-use are expensive, and they generate plastic wastes which have a negative implication on the environment [11]. Therefore, the objective of this study is to re-use the RNA purification columns, thereby curtailing the utilization of commercial RNA-extraction kits while not compromising the diagnosis of SARS-CoV2.

Silica-resin columns are the key ingredient in the commercial RNA-purification kits and the columns allow the

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recovery of high-quality RNA. However, because of the retention of substantial amount of nucleic acid in the silica-matrix even after the elution step, the columns can only be used once [12, 13]. Nevertheless, earlier study demonstrated that silica columns from commercial RNA extraction kits can be quickly regenerated and utilized for the isolation of high quality RNA without the risk of carry-over contamination [14]. Since, this method of regenerated RNA-purification columns was not studied earlier for purification and subsequent use for RT-qPCR-based detection of viral genome, in our current endeavour, we applied the method of Tagliavia et al., (2010), for the detection of SARS-CoV2 genome through RT-qPCR without compromising the diagnostic efficiency and simultaneously precluding the chance of carry-over contamination from previous use.

Materials and methods

Swab samples and RNA extraction

Samples included in this study consisted of nasal and throat swab samples in viral transport medium (VTM) that has been sent to the ICAR-International Centre for FMD for the diagnosis of SARS-CoV2 virus through RT-qPCR following the guidelines of Indian Council of Medical Research (ICMR). For RNA extraction, 200 µl of swab samples in VTM was mixed with 560 µl of lysis buffer, and RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) as per the manufacturer's instruction.

SARS-CoV2 real-time reverse transcription PCR (RT-qPCR) analyses

The extracted RNA samples were amplified with commercial SARS-CoV2 one-step multiplex RT-qPCR kit (Allplex 2019-nCoV kit). The Allplex 2019-nCoV kit (Allplex; Seegene, Seoul, Korea) targets the Envelop (E), RNA-dependant RNA-polymerase (RdRp) and nucleocapsid protein (N)-coding genes of SARS-CoV2. The kit uses bacteriophage MS2 as an exogenous internal control by spiking the internal control directly into the swab samples prior to RNA extraction [15]. The internal control was used to address the reliability of RNA-extraction procedure and any PCR-inhibition effects by the sample type. The results from the RT-qPCR assay were interpreted as positive when all target genes were detected together (Ct value < 40).

Residual RNA elimination and regeneration of used RNA columns

Elimination of residual RNA (decontamination) from the used columns was performed as per the procedure described earlier [14] with modification. The used QIAamp Viral Mini kit silica-columns were loaded with 600 µl of pre-warmed (80 °C) alkaline solution containing 0.25 M NaOH and 0.1% (v/v) Triton-X-100, the columns were incubated for 10 min at 37 °C, and subsequently centrifuged at 12,000 g for 3 min. The alkaline solution treatment of used-columns was repeated by incubating the columns at 37 °C for 20 min. Then, the used-columns were incubated for 5 min at 37 °C with 600 µl of 50 mM sodium acetate buffer, pH 4.0, and centrifuged at 12,000×*g* for 3 min. Afterwards, the RNA-binding silica columns were incubated for 5 min at room-temperature with 600 µl of RNase-free water and subsequently centrifuged at 12,000×*g* for 1 min.

To evaluate the extent of elimination of the residual RNA bound to the columns from the first use, 40 µl of elution buffer was added to the centre of each regenerated column, and the RNA remaining within the column was eluted by centrifugation and collected in a 1.5 ml micro-centrifuge tube. The eluted solution was subsequently analysed by RT-qPCR to determine the efficacy of elimination of residual RNA from the used RNA column.

Since SARS-CoV2 viral-RNA is potentially infectious in nature, all the plastic wastes and flow-through generated during the RNA-column regeneration procedure should be discarded into recommended disinfectants (e.g. 0.1% Sodium hypochlorite solution).

Statistical analysis

Mean Ct values obtained through both the method of RNA extraction (fresh RNA column or regenerated RNA column) for each SARS-CoV2 target gene were analysed in pairwise comparisons using the paired Student's t-test. The analysis was performed using GraphPad Prism 8 software.

Results

Elimination of residual SARS-CoV2 RNA from the used RNA-binding columns and regeneration for re-use

To optimise the method of elimination of residual SARS-CoV2 RNA and subsequent regeneration of used RNA-binding silica columns, fresh columns from commercial RNA-extraction kit (QIAamp Viral RNA mini kit,Qiagen) were initially used to extract the viral-RNA from suspected COVID-19 swab samples. The residual SARS-CoV2 viral RNA in the used columns was eliminated using the methodology described in the "Residual RNA elimination and regeneration of used RNA columns" section. To evaluate the efficacy of elimination, any residual RNA was recovered by 40 µl elution and the eluate was used as template

in SARS-CoV2 specific RT-qPCR assays. The RT-qPCR assays showed that the methodology used in our study has been effective in the complete elimination of residual viral RNA from the used RNA-binding silica column (Fig. 1). Furthermore, 44 number of used RNA-binding silica columns from known COVID-19 positive sample-extractions were regenerated and analysed by RT-qPCR for the presence of any residual SARS-CoV2 RNA. The analyses suggested a complete removal of residual RNAfrom the used RNA-binding silica columns after the process of RNA-decontamination and column-regeneration (Table 1).

Comparative efficiency of the regenerated and fresh RNA-binding columns for viral RNA purification

To compare the RNA-extraction ability of used-regenerated columns with that of the fresh-RNA binding columns, 44 clinical samples with various Ct values were analysed. Viral RNA was extracted using QIAamp Viral RNA min kit, and the Taqman probe-based RT-qPCR assay was carried using the Allplex 2019-nCoV kit. We analysed the efficiency between the fresh and used-regenerated RNA-binding columns in three different groups of positive samples: samples with low Ct value (Ct < 25; $n = 20$), samples with moderate Ct value (Ct = 26 to 34; $n = 14$), and samples with high Ct value (Ct = 35 to 40; $n = 10$). The detailed results for these 44 samples are shown in Table 1. The mean Ct values obtained through both the methods for each target gene of SARS-CoV2 were also analysed in pair-wisecomparison (Fig. 2). As illustrated in Fig. 2, there were no significant differences in Ct values for RdRp, N and E genes obtained using either fresh RNA columns or used-regenerated columns. However, for some samples with high Ct values, SARS-CoV2 target genes could not be detected using the RNA extracted through the regenerated RNA-columns (Table 1).

Use-regeneration-reuse of RNA-binding columns for a minimum of five-times

To determine whether the RNA-binding disposable silica columns from the viral RNA extraction kit could be repeatedly regenerated and reused for RNA purification, SARS-CoV2 RNA was extracted using a fresh QIAamp Viral RNA mini kit column (Qiagen) and analysed for the presence of viral genes by RT-qPCR. The same used column was repeatedly regenerated and used for the extraction

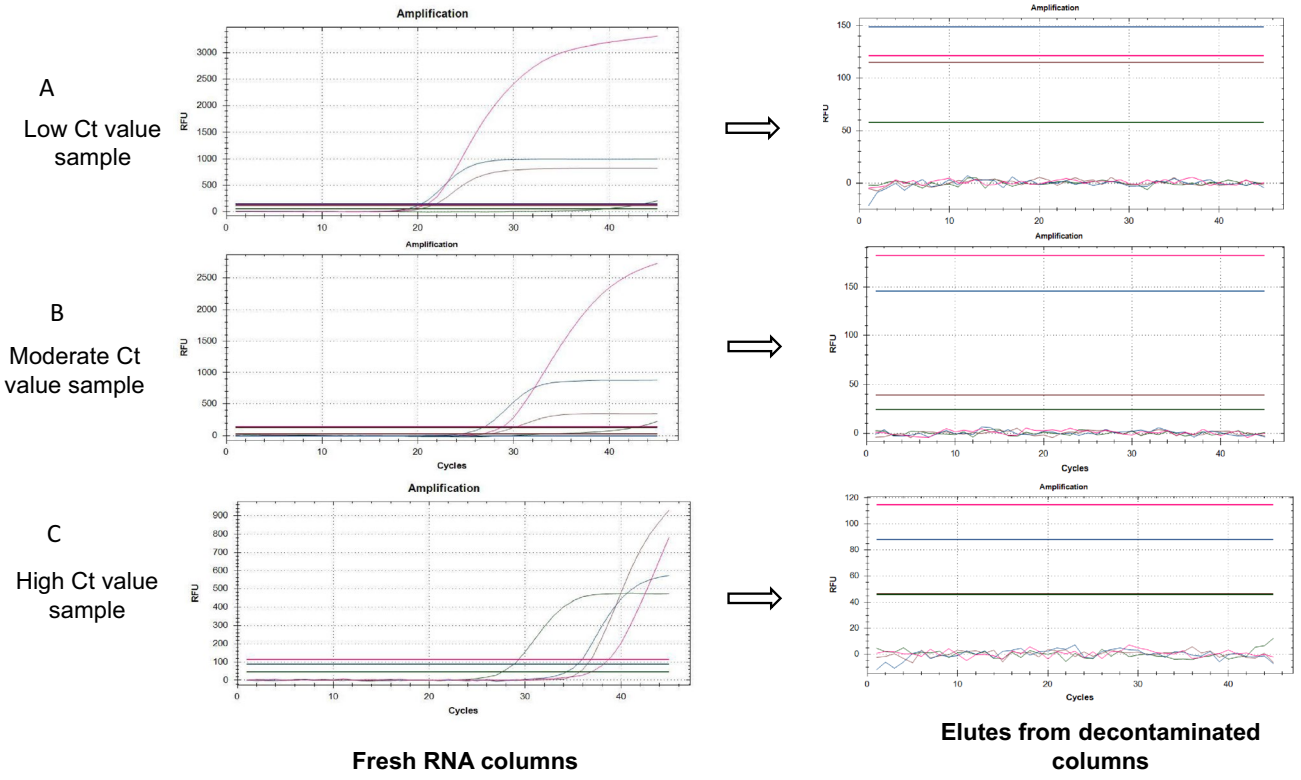

Fig. 1 Elimination of residual SARS-CoV2 RNA from the used QIAamp Viral RNA mini kit column by heated alkaline solution treatment. RT-qPCR analysis showed the complete removal of residual viral RNA in the elute from the used RNA-binding columns that has been treated with heated alkaline solution as per the methodology described in "Materials and methods" section. Columns used for RNA-extraction of SARS-CoV2 positive samples with low Ct-value (**A**), moderate Ct value (**B**) and high Ct value (**C**)

Table 1 Comparative Ct value data for SARS-CoV2 RNA extracted using the fresh RNA-binding silica column (QIAamp Viral RNA Mini Kit) and regenerated RNA-column for the known COVID-19 swab samples ($n=44$)

	Sample	RNA extracted using fresh column				Residual RNA in regenerated column				RNA extracted using regenerated column			
		RdRp	E	N	IC	RdRp	E	N	IC	RdRp	E	N	IC
Low Ct value samples	S1	17.91	16.15	15.06	38.43	nd	nd	nd	nd	19.10	17.52	18.21	38.60
	S2	15.87	17.32	19.37	36.62	nd	nd	nd	nd	14.74	15.97	15.65	40.20
	S3	19.26	19.93	15.54	33.72	nd	nd	nd	nd	18.11	19.10	18.65	38.65
	S4	15.43	15.13	14.69	38.12	nd	nd	nd	nd	15.21	15.91	17.73	35.56
	S5	17.32	19.37	15.87	36.62	nd	nd	nd	nd	19.10	17.96	17.39	38.32
	S6	19.26	15.23	16.82	36.98	nd	nd	nd	nd	15.23	18.50	16.88	39.10
	S7	18.81	17.95	15.64	39.19	nd	nd	nd	nd	16.21	15.58	15.87	36.45
	S8	18.83	18.94	16.13	30.07	nd	nd	nd	nd	14.16	17.87	14.32	40.24
	S9	19.23	18.88	19.49	36.32	nd	nd	nd	nd	19.88	16.42	17.07	38.90
	S10	18.52	18.06	14.42	30.78	nd	nd	nd	nd	16.88	16.12	18.10	35.56
	S11	19.55	17.92	19.16	37.12	nd	nd	nd	nd	18.91	17.87	17.10	39.56
	S12	23.95	21.40	23.00	36.72	nd	nd	nd	nd	24.12	21.85	20.33	38.18
	S13	24.09	20.94	22.31	33.94	nd	nd	nd	nd	26.15	22.41	24.22	37.99
	S14	24.43	21.83	23.93	40.27	nd	nd	nd	nd	24.14	21.35	24.32	36.66
	S15	22.29	20.11	22.12	37.47	nd	nd	nd	nd	20.42	18.87	19.39	38.81
	S16	20.23	20.64	18.88	38.90	nd	nd	nd	nd	18.10	16.60	18.34	36.92
	S17	24.51	22.05	23.05	36.72	nd	nd	nd	nd	26.66	23.06	24.03	33.08
	S18	23.15	22.41	24.22	35.99	nd	nd	nd	nd	24.37	24.64	24.84	32.61
	S19	23.14	21.35	22.32	36.66	nd	nd	nd	nd	24.32	21.74	23.35	38.20
	S20	24.26	22.72	24.16	32.29	nd	nd	nd	nd	22.10	24.59	23.48	33.05
Moderate Ct value samples	S21	30.60	27.90	29.84	26.69	nd	nd	nd	nd	32.49	29.33	29.08	32.98
	S22	31.10	28.34	29.67	25.82	nd	nd	nd	nd	32.08	31.58	30.06	32.37
	S23	33.56	33.86	33.69	31.56	nd	nd	nd	nd	34.62	32.59	32.14	27.33
	S24	29.47	28.19	28.04	26.60	nd	nd	nd	nd	29.39	27.34	26.80	30.12
	S25	26.95	21.40	23.00	36.72	nd	nd	nd	nd	28.15	22.41	24.22	40.91
	S26	29.39	26.80	27.34	29.18	nd	nd	nd	nd	30.18	28.14	28.80	31.61
	S27	31.20	28.96	28.35	29.03	nd	nd	nd	nd	29.63	26.87	27.01	31.01
	S28	26.45	25.91	29.69	28.53	nd	nd	nd	nd	24.43	23.95	28.83	30.12
	S29	31.57	29.64	32.03	32.66	nd	nd	nd	nd	33.26	28.89	29.35	31.61
	S30	26.32	21.74	23.35	38.20	nd	nd	nd	nd	24.14	21.35	22.32	36.66
	S31	26.08	27.61	28.51	35.95	nd	nd	nd	nd	29.54	27.99	26.31	31.30
	S32	28.10	25.51	27.03	36.61	nd	nd	nd	nd	30.18	27.11	28.14	38.43
	S33	33.29	30.68	31.24	32.90	nd	nd	nd	nd	32.69	30.33	32.55	32.32
	S34	28.80	28.09	27.17	42.37	nd	nd	nd	nd	26.08	29.41	29.17	39.93
High Ct value samples	S35	38.65	35.15	38.62	32.49	nd	nd	nd	nd	34.14	31.69	33.78	31.09
	S36	36.32	39.29	37.89	32.87	nd	nd	nd	nd	40.02	nd	36.46	29.10
	S37	37.06	38.42	41.43	27.30	nd	nd	nd	nd	nd	nd	nd	28.65
	S38	36.96	37.11	38.30	28.49	nd	nd	nd	nd	30.02	35.35	35.87	27.10
	S39	38.85	37.47	33.41	30.41	nd	nd	nd	nd	39.61	41.20	37.35	29.91
	S40	36.19	36.02	36.99	32.44	nd	nd	nd	nd	32.02	30.48	31.91	29.47
	S41	38.68	35.43	35.65	29.19	nd	nd	nd	nd	40.19	36.66	39.02	26.43
	S42	38.37	37.82	38.57	28.35	nd	nd	nd	nd	nd	40.0	nd	25.56
	S43	37.15	36.02	35.68	30.00	nd	nd	nd	nd	31.92	33.45	33.76	29.36
	S44	36.71	33.58	34.21	32.27	nd	nd	nd	nd	40.10	35.43	36.73	31.10

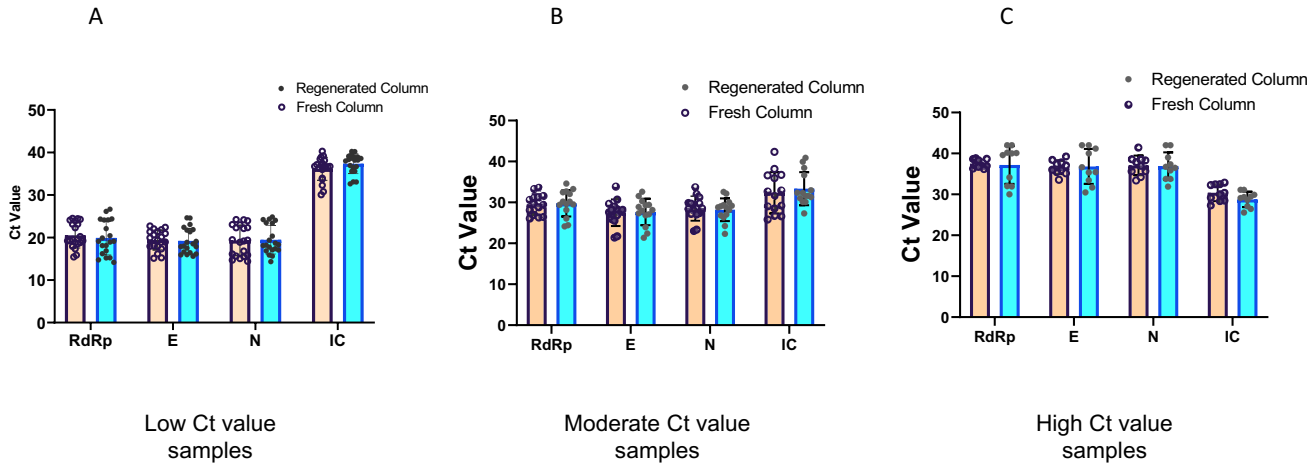

Fig. 2 Regenerated Viral-RNA purification kit columns have a comparable capacity for SARS-CoV2 RNA purification as the fresh columns. Bars represent the mean ± standard deviation Ct values for each SARS-CoV2 RT-qPCR target genes RdRp, E, N, and IC, for swab samples with Ct value ≤ 25 (**A**), Ct value = 25–35 (**B**), and Ct value = 36–40 (**C**). Each dot represents one sample. Pairwise comparisons of mean Ct values for each SARS-CoV2 target gene were done using a two-tailed paired Student's t-test, with a confidence level of 95%. For low Ct value samples p value for RdRp, E and N genes were 0.18, 0.59 and 0.75 respectively, for moderate Ct value samples p value for RdRp, E and N genes were 0.57, 0.65 and 0.47 respectively, and for high Ct value samples p value for RdRp, E and N genes were 0.84, 0.85 and 0.86 respectively

of SARS-CoV2 RNA from the above known COVID-19 positive swab sample for four additional times.The concentration of viral RNA after each round was determined through SARS-CoV2 RT-qPCR. Figure 3 illustrated that the RNA-binding silica columns regenerated for the fourth time exhibited a comparable viral-RNA yield as the fresh column. Therefore, the disposable viral RNA extraction

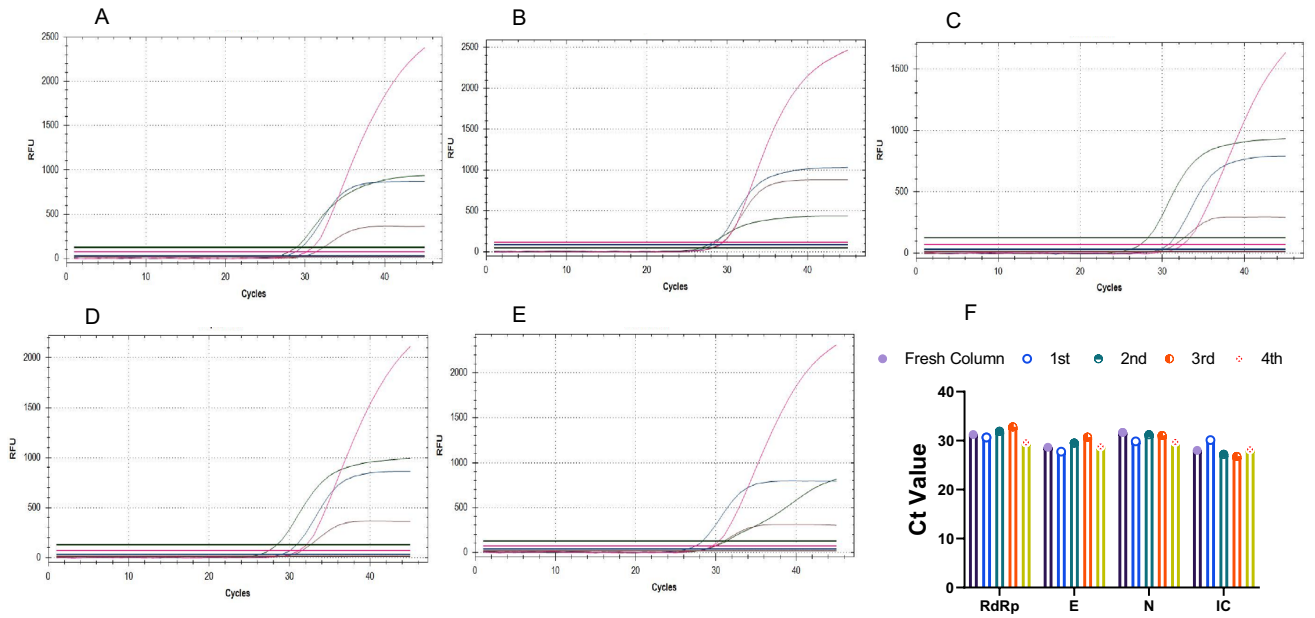

Fig. 3 RNA-binding silica columns can be repeatedly regenerated and reused for a minimum of five cycles. RNA was extracted from the SARS-CoV2 suspected swab samples using fresh silica column and analysed for the presence of viral genes by multiplex RT-qPCR (**A**). The same column was regenerated and reused for extraction of viral RNA from the above known COVID-19 positive samples for four more times (**B**–**E**). RT-qPCR analyses suggested that RNA-binding silica columns regenerated for the fourth time exhibited a comparable viral-RNA yield as the fresh column (**F**)

column could be regenerated and reused for a minimum of five cycles without hampering the RNA-binding ability.

Discussion

RNA binding silica columns in the RNA-extraction kits allow the recovery of high-quality RNA without any organic extraction step. However, the major disadvantage of silica column-based RNA-extraction kit is the cost and their availability during world-wide infectious disease pandemic as in the case of COVID-19. Therefore, techniques need to be developed and evaluated for the recycling of RNA-binding silica columns in order to reduce the cost of RNA extraction and to ensure continuity of diagnostic service during short supply of kits in COVID-19 clinical diagnostic laboratories.

However, the challenge in the viral RNA-binding silica columns regeneration procedure is not only the complete removal of viral RNA, but also the requirement of a quick protocol making the used columns ready again for the purification of good quality viral RNA without compromising the sensitivity and specificity of the viral diagnostic assay. Owing to the presence of 2′ hydroxyl group (OH), RNA is very unstable in alkaline condition as compared to DNA [16]. The 2′-OH group of RNA becomes de-protonated under high-alkaline condition leading to nucleophilic attack on the 5′-PO_4 group of the adjacent nucleotide, resulting in the cleavage of the phospho-pentose backbone of RNA. Therefore, alkaline pH solution along with heat treatment has been used earlier to selectively degrade RNA in RNA–DNA hybrids [17, 18].So, the silica column-bound residual RNA in the used columns can be effectively hydrolysed and removed by heated alkaline solution. Furthermore, alkaline solution can inactivate RNase, leaving the used silica columns both RNA and RNase-free [19].

Earlier through agarose-gel electrophoresis-based PCR assay it has been shown that disposable columns from RNA-extraction kit can be decontaminated using heated alkaline solution [14]. However, until now, decontaminated and regenerated RNA-binding silica column has not been used for virus detection assays through the TaqMan probe-based RT-qPCR assay. Since, RT-qPCR PCR assay is more sensitive to detect the PCR-amplicons as compared to the agarose-gel electrophoresis-based PCR; we had modified the earlier procedure for purification of used-RNA columns, so that the complete degradation of left-over RNA from earlier run could be possible. In addition, during the initial development and validation of decontamination of RNA columns as per the earlier reported procedures [14], we had detected left-over RNA for certain swab samples with low Ct values (Ct value < 20), however, the observations was not uniform for all the clinical samples with low Ct values (data not shown). Nevertheless, to ensure complete degradation of left-over RNA we had modified the earlier reported protocol.

In our study, through real time RT-PCR assay we confirmed that regenerated disposable RNA columns can be used for the detection of COVID-19 viral genome without any risk of carryover contaminated viral RNA from previous experiment. Furthermore, no significant difference in Ct-values for the SARS-CoV2 target genes was observed using the viral RNA either from the regenerated or fresh RNA-binding columns. However, it is worth mentioning that fluctuation has been observed in the Ct value between the two methods of RNA extraction for the samples with viral load around the cut-off value of the RT-qPCR assay (Ct = 40). Although, samples with higher Ct value may be associated with low viral load and low risk of infection transmission [20, 21], a swab-sample collected at a single time point does not provide details about the trajectory of SARS-CoV2 illness status [22]. Therefore, the regenerated RNA-columns may only be used for urgent conditions during the short supply of RNA-purification kit. In our study, we also established that the used disposable RNA-binding columns could be stripped of bound-RNA, regenerated, and re-used at least for five-times without compromising either the quality of viral RNA or the binding properties of silica columns. In our study, the method of regeneration and re-use of used RNA-purification columns for COVID-19 diagnosis was also extended for RNA-binding silica columns from two commercial sources (HiPurA™ Viral RNA Purification Kit, HIMEDIA, and PureLink RNA Mini Kit, Invitrogen, Thermo). The results were comparable to the columns from the QIAamp Viral RNA Mini Kit (data not shown). Therefore, the methodology adopted in this manuscript can be applied to any used RNA-purification column from other commercial sources apart from Qiagen.

In conclusion, we showed that the used disposable viral-RNA binding columns can be decontaminated using warm alkaline buffer and regenerated using acidic sodium acetate buffer. The regeneration process could be completed in approximately 25 min for a set of 10-columns. The treatment process does not impair the column's RNA-binding efficacy, thereby, making it possible to use the same column for COVID-19 diagnosis for at least five times. The use of regenerated RNA-binding silica columns can reduce the production of laboratory plastic-wastes and could help to save fund and ensure continuity of diagnostic service during the COVID-19 pandemic.

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Author contributions Study concept and design was done by JKB. Experiments, data collection and analyses were performed by JKB, RR, SSD, SM, & JKM. The first draft of the manuscript was written by JKB

and all authors commented on the previous version of the manuscript. All the authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval The study described in this manuscript was conducted as part of the diagnosis of COVID-19 suspected human swab samples submitted at the authors' laboratory. Since there was no disclosure regarding the names or the physical, economic, cultural, and social status of the patients, individual patient consent or ethical approval is not required.

Informed consent Authors approve for submitting the publication.

Research involved in human or animal rights No experiment was conducted on animals in this study.

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