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Original Article

Microbiology Section

Extraction Free Rapid Detection of SARS-CoV-2 from Oropharyngeal/Nasopharyngeal Swabs by Real Time PCR

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ABSTRACT

Introduction: The emergence of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) pandemic has been troublesome particularly for developing countries that lack infrastructure and capacities to produce the kits locally. Simplification of the method can increase diagnostic efficiency which can benefit patients and help in infection control, consequently saving time and lives.

Aim: To evaluate the diagnostic value of four methods (that omit extraction step) for detection of SARS-CoV-2 against the traditional extraction method.

Materials and Methods: This was a cross-sectional analysis for evaluating diagnostic accuracy of four methods for detection of SARS-CoV-2 by real-time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR), conducted in the Department of Microbiology, SMS Medical College, Jaipur, Rajasthan, India, in October 2020. Ninety four SARS-CoV-2 RT-PCR positive samples and 20 negative samples were taken for this study. Automated extraction system was used for Ribonucleic Acid (RNA) extraction and four different approaches were compared to the traditional extraction method for detection of SARS-CoV-2 by RT-PCR. Data was entered and analysed using Statistical Package for the Social Sciences (SPSS) statistical software version 24.0.

Results: The automated RNA extraction method was compared to the method of direct addition of samples with (Heat processed Direct Viral transport medium Sample (HDVS)) and without heating (Direct Viral transport medium Sample (DVS)), directs addition of diluted (1:5) sample with (Heat processed diluted VTM sample (HdVS)) and without heating (Diluted VTM sample (dVS)) as well as after addition of Proteinse K (PK) to the diluted samples that came either negative/invalid. Out of four methods, the HdVS method gave the best results, considering extraction with Perkin Elmer as standard, this method showed sensitivity of 96.74%, specificity of 100%.

Conclusion: In current pandemic, molecular testing is critically challenged by the limited supplies of reagents of nucleic acid extraction alternative method like diluting and heating of Viral Transport Media (VTM) samples and using them directly as elutes serve as an easy, fast and inexpensive alternative.

Keywords: Coronavirus disease-19, Molecular testing, Reverse transcriptase polymerase chain reaction, Viral transport media

INTRODUCTION

Coronavirus Disease-19 (COVID-19) is a respiratory tract infection caused by SARS-CoV-2 which ranges from asymptomatic cases to severe pneumonia requiring Intensive Care Units (ICUs) [1]. This disease was first noticed in Wuhan, China in December, 2019 but because of its high transmission rate and high proportion of asymptomatic infections, it led to massive worldwide spread across the globe within few months [2].

It is of utmost importance to test for the presence of virus to plan for containment strategies and to aim reduce dissemination of the virus and for appropriate and timely treatment of the affected patient. However, it has been a challenge to understand and manage the outbreak for most of the countries due to significant bottlenecks imposed by diagnosis [3].

The current standard assay for diagnosis of COVID-19 is based on extraction of RNA from respiratory samples like Nasopharyngeal (NP) and Oropharyngeal (OP)/nasal swabs collected in VTM and subsequently rRT-PCR targeting several sequences from SARS-CoV-2 genome [4].

The emergence of SARS-CoV-2 pandemic and subsequent lockdowns to contain the spread of infection has led to a situation of supply deficit of critical reagents required for diagnostic tests [5]. This has been troublesome particularly for developing countries that lack infrastructure and capacities to produce the kits locally [6].

Presently in use RNA extraction methods typically involve three general steps: Cell lysis, separation of RNA from other macromolecules such as DNA, proteins and lipids followed by RNA concentration. RNA extraction from clinical samples constitutes a major hold-up in the diagnostic process as it is manually laborious, time consuming and expensive [7]. This is the reason why it is crucial that a new test should not only be efficient, quick and affordable but also that it should keep the use of industrial kits to the minimum [8]. Many different protocols have been published over years that optimise or simplify the RNA extraction process from various types of samples [6].

In the context of current pandemic, our institute has been receiving approximately 6000-7000 samples per day in VTM for testing by rRT-PCR. Procedures that include RNA extraction methods apart from being costly consume a lot of time and do not allow this kind of throughput. Moreover, RNA extraction kits are in limited supply due to high global demand. Simplification of the method can increase diagnostic efficiency which can benefit patients and help in infection control, consequently saving time and lives [8].

Here, authors describe a simplified workflow for molecular detection of SARS-CoV-2 omitting different steps of RNA extraction. This could serve as an alternative in diagnostic laboratories to overcome kits shortage as well saving time in testing. This study was aimed to evaluate the accuracy of four methods (that omit extraction step) for detection of SARS-CoV-2 from NP/OP samples against the traditional automated extraction method so that one gets same results in lesser time and cost.

MATERIALS AND METHODS

The present cross-sectional pilot study conducted in Department of Microbiology, SMS Medical College, Jaipur, Rajasthan, India in October 2020, for evaluating diagnostic value of four methods for

direct detection of SARS CoV-2 from NP/OP samples in VTM by rRT-PCR without the need for nucleic acid extraction.

Inclusion criteria: Total 94 stored nCoV RT-PCR positive samples (Nasal and OP swabs collected in VTM) (Vitromed healthcare, Biotech Park, Jaipur, Rajasthan) with varied cycle threshold (Ct) values and 20 negative samples were included in this study which was stored samples from 2019.

Exclusion criteria: Positive samples with Ct value >37 were excluded from the study.

Sample size calculation: A sample size of 114 VTM samples was adequate at 95% confidence interval and 10% absolute error to verify the assumed with atleast 50% accuracy of different methods for detection of SARS-CoV-2 (50% proportion was taken as it gives maximum sample size at any defined error).

Automated extraction system (Perkin Elmer ChemagicTM 360) was used for RNA extraction followed by TruPCR master mix for SARS-CoV-2 detection by rRT-PCR.

Extraction of Sample

After thorough vortexing, followed by brief centrifugation of VTM samples, 300 µL of the sample was transferred to a 96 deep well processing plate to which 4 µL Poly (A) RNA, 10 µL of proteinase K, 300 µL lysis buffer along with 150 µL magnetic beads and 900 µL of RNA binding buffer were already been added. The beads/RNA mixture was washed with washing buffer and elutes were obtained in elution buffer in the automated system (Perkins Elmer ChemagicTM 360), as per the manufacturer's instructions.

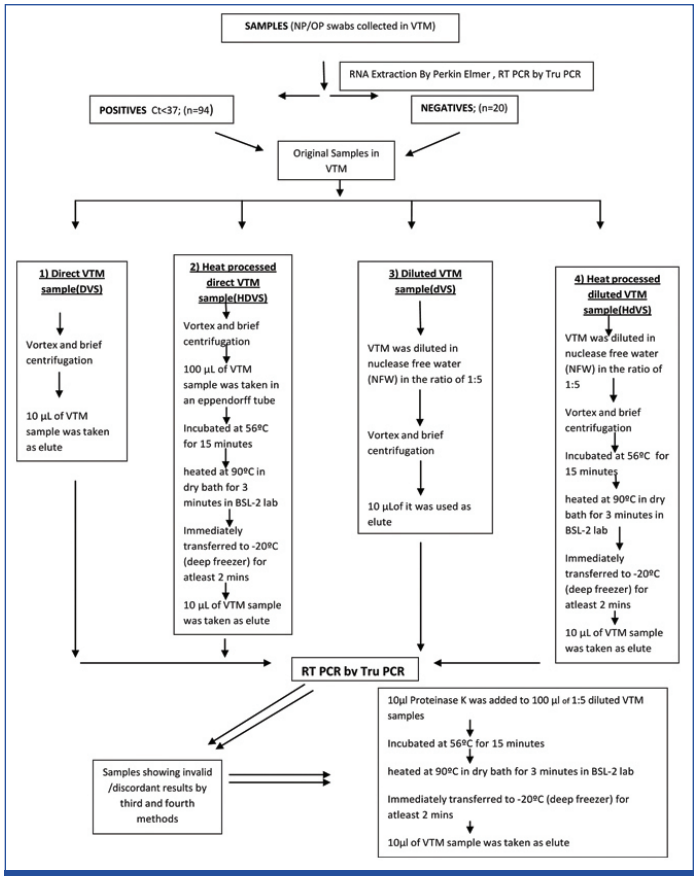
Real Time PCR

The primers used in TruPCR rRT-PCR kit are designed to target Envelope (E) gene, nucleocapsid (N)/RNA-dependent RNA polymerase (RdRp) and Ribonuclease P (RNase P) genes. For PCR, 10 µL RNA and 15 µL PCR master mix solution containing 10 µL master mix reagent, 0.35 µL enzyme mix and 4.65 µL of primer probe mix. Cyclic conditions used as per the manufacturer's instructions were 50°C for 15 minutes, 95°C for 5 minutes, then 38 repeat cycles of 95°C for 5 seconds, 60°C for 40 seconds and 72°C for 15 seconds, using Biorad CFx 96 platform.

Four simplified approaches omitting/bypassing RNA extraction step were performed before RT-PCR for SARS-CoV-2 after doing some modifications in the protocols followed by Grant PR et al., Fomsgaard AS, Rosenstierne MW and Freppel W et al., [Table/Fig-1] [1,9,10].

- Direct VTM sample (DVS): Directly VTM samples were used as elutes for PCR reaction. After thorough vortexing and brief centrifugation 10 µL of VTM sample was added as elute for rRT-PCR in Biosafety Level-2 (BSL-2) lab.
- Heat processed direct VTM sample (HDVS): Approximately, 100 µL of VTM sample was taken in eppendorff tube and incubated for 15 minutes in incubator at 56ºC followed by heating in dry bath for three minutes at 90ºC in BSL-2 laboratory. After that these were immediately transferred to -20ºC (deep freezer) taking them in box of ice. These were used as elutes after two minutes for RT-PCR reaction.
- Diluted VTM sample (dVS): VTM was diluted in Nuclease Free Water (NFW) in the ratio of 1:5 and 10 µL of it was used as elute for RT-PCR reaction after thorough vortexing and brief centrifugation in BSL-2 lab.
- Heat processed diluted VTM sample (HdVS): Diluted VTM samples in NFW (1:5) were heat processed in same way as described above.

Use of proteinase K: All the samples showing invalid/discordant results by third and fourth methods were processed after addition of proteinase k(PK) into them considering PK to reduce the interfering substances.



[Table/Fig-1]: Flow chart showing the steps of the four extraction free protocols followed in the present study.

A total of 10 µL PK was added to 100 µL of the 1:5 diluted samples before heating step. Then the samples were heated and immediately transferred to deep freezer for two minutes.

The rRT-PCR results of all the samples were processed in all of the above ways were noted along with their Ct values and were compared with the results of these samples processed via Perkin Extraction system and rRT-PCR via Tru-PCR master mix kit.

STATISTICAL ANALYSIS

Data was entered and analysed using Statistical Package for the Social Sciences (SPSS) statistical software version 24.0. The sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) were calculated. Cohen's' kappa coefficient was used to estimate the agreement between conventional extraction method used routinely and four simplified approaches as mentioned previously.

RESULTS

The automated RNA extraction method (Perkin Elmer SARS-CoV-2 testing workflow) was compared to the method of direct addition of samples (DVS) with and without heating, direct addition of diluted (1:5) sample (dVS) with and without heating as well as after addition of PK to the diluted samples that came either negative/invalid after testing with rRT-PCR (using commercially supplied Tru-PCR kit). No Template Control (NTC) did not show amplification in any of the protocols followed and the positive control of all the genes yielded positive results.

Furthermore, all the samples gave positive results for Rnase P when extraction was done using the automated extraction system. All the 20 negative samples gave negative results by dVS and HDVS methods while 6/20 samples were invalid by DVS method and 4/20 by HdVS method.

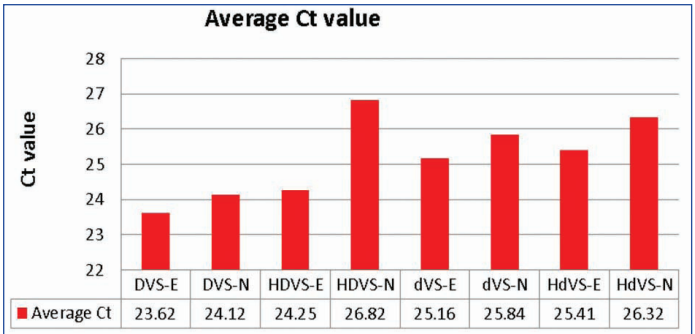
As shown in [Table/Fig-2], 49 (52.12%) samples yielded positive results for both E and RdRp genes together by DVS method. A 49 (52.12%) samples were positive for *E* gene and 55 (58.51%) for

N/RdRp genes. A 24 (25.53%) samples came negative whereas 15 (15.95%) samples gave invalid results i.e., without any amplification of RNase P. ΔCT (Difference in Average Ct values in comparison with that of extraction by Perkin Elmer) for *E* genes was 7.87 and ΔCT for N/RdRp was 3.59.

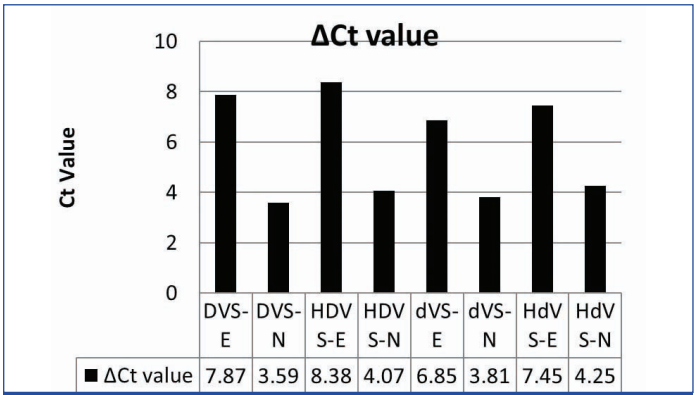
Methods	E gene Positivity n (%)	N/RdRp gene positivity n (%)	Both genes positivity n (%)
DVS	49 (52.12%)	55 (58.51%)	49 (52.12%)
HDVS	66 (70.21%)	75 (79.78%)	65 (69.14%)
dVS	86 (91.48%)	87 (92.55%)	87 (92.55%)
HdVS	90 (95.74%)	90 (95.74%)	90 (95.74%)

[Table/Fig-2]: Positivity rate of the samples by all the methods.

Around 65 (69.14%) samples yielded positive results for both the genes together by HDVS. 66 (70.21%) were positive for E gene and 75 (79.78%) for N/RdRp [Table/Fig-2] 14 (14.89%) samples came negative whereas 5 (5.31%) samples came invalid. ΔCT for E genes was 8.38 and ΔCT for N/RdRp was 4.07 [Table/Fig-3,4].



[Table/Fig-3]: Average Ct value comparison to the traditional extraction method as standard.



[Table/Fig-4]: Difference in Ct values by each method in comparison to the traditional extraction method as standard.

In present study, 87 (92.55%) samples showed amplification of all genes together by dVS, 86 (91.48%) showed amplification of E gene and 87 (92.55%) showed amplification of N/RdRp gene [Table/Fig-2], 4 (4.25%) came negative and 3 (3.19%) samples gave invalid results. ΔCT for E genes was 6.85 and ΔCT for N/RdRp was 3.81 [Table/Fig-3,4]. In this study, 90 (95.74%) samples showed amplification of both the genes together by HdVS, 90 (95.74%) samples showed amplification of E gene and same percentage of samples showed amplification of N/RdRp [Table/Fig-2]. A 3 (3.19%) came invalid and only 1 (1.06%) came negative. ΔCT for E gene was 7.45 and ΔCT for N/RdRp was 4.25 [Table/Fig-3,4].

Finally, PK was added to all those diluted 7 (7.44%) samples that came either negative or invalid by dVS and HdVS method, thermal lysis was done after addition of PK. A 6 (85.71%) out of seven came positive and 1 (14.28%) sample remained negative which was negative by all the methods.

Sensitivity, specificity, PPV and NPV of each method are shown in [Table/Fig-5], considering extraction by Perkin Elmer as standard. After addition of PK to all the diluted samples that came negative/invalid, Sensitivity and NPV further increased from 90 (96.74%) to 93 (98.9%) and 83.33% (20/24) to 95.2% (20/21), respectively. The [Table/Fig-6] depicts a notable agreement between the positivity by original extraction method and other four simplified approaches mentioned before. Substantial and almost perfect agreement was observed with dVS (K coefficient- 0.768, 95% CI-0.627-0.910) and HdVS method (K coefficient- 0.862, 95% CI- 0.745-0.979), respectively.

Method	Sensitivity		Specificity		PPV		NPV	
	E gene	N/RdRp gene	E gene	N/RdRp gene	E gene	N/RdRp gene	E gene	N/RdRp
DVS	53.19%	57.46%	100%	100%	100%	100%	31.25%	33.30%
HDVS	70.21%	79.78%	100%	100%	100%	100%	41.66%	51.28%
dVS	91.48%	92.55%	100%	100%	100%	100%	71.4%	74.07%
HdVS	96.74%	96.74%	100%	100%	100%	100%	83.33%	83.33%

[Table/Fig-5]: Comprehensive assessment of diagnostic value of four methods in comparison to standard extraction method.
PPV: Positive predictive value; NPV: Negative predictive value
When PK was added in the diluted samples sensitivity was increased to 98.9% and NPV was increased to 95.2%

Tests	Gene	Cohen's Kappa (K)	Association
Traditional	E gene	0.276	Fair agreement
Extraction vs DVS	N/RdRp gene	0.312	Fair agreement
Traditional	E gene	0.49	Fair agreement
Extraction vs HDVS	N/RdRp gene	0.565	Moderate agreement
Traditional	E gene	0.768	Substantial agreement
Extraction vs dVS	N/RdRp gene	0.790	Substantial agreement
Traditional	E gene	0.862	Almost perfect agreement
Extraction vs HdVS	N/RdRp gene	0.862	Almost perfect agreement

[Table/Fig-6]: Agreement Level of four simplified approaches with traditional extraction method as standard.

DISCUSSION

During pandemics, molecular diagnostics are very crucial to obtain accurate and timely data to influence public health policy decisions in real time as well as saving lives [11]. As the clinical symptoms of COVID-19 are very non specific (cough, fever, sore throat, fatigue) similar to other respiratory diseases or even absent despite infection, molecular testing is necessary for a more correct diagnosis [9].

The newly emerged SARS-CoV-2 virus has challenged the global health system in all the aspects including the ability to provide sufficient reagents for molecular diagnostics [12]. To overcome the shortening of supplies as well as to save time alternative dependable methods are always in demand that can further improve clinical care and surveillance efforts.

In this trial, four procedures to circumvent extraction step were explored. Samples collected in VTM were directly used as elutes for rRT-PCR with minor modifications as mentioned. Results showed that RT-PCR based testing of SARS-CoV-2 virus can be done by using simpler protocol without the use of RNA extraction kits, at the same time without compromising the accuracy of detecting positive and negative cases.

Out of four methods, the HdVS method of diluting the samples followed by thermal lysis gave the best results, considering extraction with Perkin Elmer as standard, this method showed sensitivity of 96.74%, specificity and PPV of 100% and NPV of 83.33%. These figures were in concordance with the studies done by others [1,7,10]. Grant PR et al., concluded that after diluting sample in the ratio of 1:5 and heating at 95ºC for 10 minutes, sensitivity came 98.8%, specificity and PPV 100% each and NPV 97.3% [1]. Freppel W et

al., inferred that 94.4% sensitivity was achieved when the samples were diluted in the ratio of 1:5 and heated to 50ºC for 15 minutes followed by heating at 90ºC for three minutes and rapid cooling at 4ºC [10]. Alcoba-Florez J et al., analysed that heating the samples without diluting at 70ºC for 10 minutes gave sensitivity of 87.8% and specificity of 100% [7]. Smyrlaki I et al., deduced sensitivity of 96% and specificity of 99.8% when heating was done at 95ºC for five minutes without diluting the samples [8]. In the present study, lower sensitivity (79.7%) was noted. This can be due to difference in VTMs used as media with lesser inhibitors can give better results.

In the current study, direct addition of VTM samples as elutes showed sensitivity of 53.19% for E gene and 57.46% for N/RdRp gene, which improved to 91.48% (E) and 92.55% (N/RdRp) on dilution. Same concordance was noted in the study done by Freppel W et al., who has reported 50% sensitivity rate on adding VTM samples directly for rRT-PCR which further improved to 93.3% on diluting (1:5) the samples [10]. This shows that dilution of samples is required to obtain reliable detection [11]. Also, it depicts that transport media itself might be inhibitory for rRT-PCR reaction as suggested by requirement of stronger dilutions (1:5) for optimal detection on specimen stored in transport media [10].

When thermal lysis of diluted samples was done, sensitivity further increased from 91.48% to 96.74% for E gene and from 92.55% to 96.74% for N/RdRp. This increase was also noted by the study done by Freppel W et al., in which increase in sensitivity from 93% to 97% for *N* gene was reported [10]. This shows that thermal lysis could contribute more to specimen inhibitor denaturation rather than a direct effect on viral template release. SARS-CoV-2 lose infectivity above 56ºC within a short period of time without any significant effect on number of viral gene copies below 92ºC even after 30-60 minutes as detected by RT-PCR [10,13]. Therefore, authors postulate that heat shock treatment at 90ºC for three minutes after 15 minutes at 56ºC may help in diminishing the infectiveness of the samples by lysis of viral particles without viral RNA degradation during the process.

With use of PK in this study, sensitivity of heated diluted samples method was increased further from 96.74% to 98.9% and NPV from 83.3% to 95.2%. The increase in detection rate was also noted by Freppel W et al., who has inferred increase in sensitivity from 94.4% to 98.9% on adding PK [10]. PK is a well known enzyme that has several activities such as protein denaturation and nuclease inhibition and whose impact is greater when RNA levels are limited [10].

Sensitivity of detection of N/RdRp gene was higher than that of E gene in all the four methods used for RT-PCR in this study. Also, the difference in Ct values (ΔCT) for N/RdRp gene is very less in comparison to E gene. This was in agreement with the studies done by Smyrlaki I et al., and Freppel W et al., [8,10]. The reasons may be attributed to the presence of N gene sequence in all the sub genomic m RNAs whereas E/RdRp is less represented or higher sensitivity of *N* gene primer [10]. Heat inactivation cleaves RNA into shorter fragments, so the primer probe set with shortest amplicon N gene (72bp) performed better in comparison to E gene (long amplicon 113bp) [8]. That's why in case of heat inactivation, primer probe consideration carry more importance for its sensitivity than for extraction based RT-PCR of more intact RNA strands [8].

It was also observed in the present work, that Ct values of all the samples on RT-PCR by all four mentioned methods were higher in comparison to extracted RNA of the samples (by Perkin Elmer automated extraction method). This was in agreement with the study done by Smyrlaki I et al., [8]. The reason could be because RNA concentration in the elute obtained by the extraction system is much higher than in the direct/diluted sample as elute. Also, RNA extraction was performed on fresh samples, while the aliquots that were kept frozen at -80ºC were used for the four methods, in this study. Besides these, heating may degrade RNA in the presence of RNases and/or metal ions present in the media [8]. Looking at the increase in Ct value, it can be assumed that by these methods, positive samples with CT values >35 might be missed. Only (4/94) samples came negative/invalid by the HdVS method. But three of them came positive after addition of PK. All of these were of higher Ct values (E and N/RdRp=32-36). It has already been proposed that detected n gene with Ct >30 could be either non infectious viral particles or fragmented viral genomes [14]. So, it can be concluded from this study that using dilution and heating method, missing positive samples will not be very significant.

It is also suggested that effective surveillance depends on testing and the speed of reporting was even more than high test sensitivity [15]. With this method, the positive cases can be reported within few hours of receiving the samples. The direct method is attractive in settings where repeated cheaper and quicker testing is desirable for example in frequent testing of healthcare personnel. This method would also be compatible with sequencing based detection [8].

Limitation(s)

Choice of rRT-PCR kit might have an impact on the sensitivity of the direct protocol. So, results with different RT-PCR kits may vary and need separate studies. The study was done on stored samples at -80ºC and sample size was also small. Therefore a prospective study using fresh and more number of samples and with different PCR kits should be done for validation of heat processing workflow, before being implemented in diagnostics.

CONCLUSION(S)

During a time when spread of SARS-CoV-2 is immense and molecular testing is critically challenged by the limited supplies of reagents of nucleic acid extraction, alternative method like diluting and heating of VTM samples and using them directly as elutes serve as an easy, fast, reliable and inexpensive alternative to chemical extraction kits. It was recommended that basis of rRT-PCR inhibition should be included in the standard specifications made for commercial transport media so as to assist direct testing in forthcoming epidemics. Focus on downstream viral culturing is not much meaningful for vast majority of samples during epidemics so shouldn't be given prime concern.

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