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Original Article

Microbiology Section

Root Cause Analysis of Invalid Internal Control in COVID-19 RT-PCR Testing: A Learning Experience

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ABSTRACT

Introduction: Laboratory diagnosis of Corona Virus Disease-2019 (COVID-19) plays a major role in diagnosing and treating infection. The corner stone in strategic public health interventions and breaking the chain of transmission was diagnostic laboratory testing and case-based data. The purpose of the study was to establish quality control standards for RT-PCR testing laboratory by performing Root Cause Analysis (RCA) of invalid Internal Control (IC).

Aim: The aim of the study was to estimate the percentage of invalid IC and to describe the most probable associated factors.

Materials and Methods: This cross-sectional observational study was conducted at the RT-PCR laboratory in Microbiology department of Govt. Kilpauk Medical College and Hospital for a period of one month, October 2021. The nasopharyngeal and oropharyngeal swabs collected for COVID-19 testing were included in the study. A detailed check-list was prepared by the author for evaluating of invalid IC.

Results: All the variables of RT-PCR test were categorised as pre-analytical, analytical and postanalytical phases and each and every component was assessed as per the protocol. Out of 23500 samples included in the study, a positive test result for COVID-19 was obtained in 164 (0.69%) and a negative result in 22533 (95.89%) patients. Around 803 (3.41%) samples included in the study showed an invalid IC.

Conclusion: Current observation showed that an invalid IC could be caused by any factors starting from sample collection to reporting. Major causes for an invalid IC were due to improper extraction and sample collection.

Keywords: Corona virus disease-2019, Internal control, Quality control, RT-PCR test

INTRODUCTION

Since the outbreak of the current pandemic, COVID-19 in December 2019 the disease has been fast spreading to neighboring countries and necessitated all the countries to expedite setting up diagnostics for this new virus [1]. Although numerous molecular laboratories were fully operational within a short span of time, utmost importance had to be given to quality assurance in testing. Reverse Transcription Polymerase Chain Reaction (RT-PCR) testing conducted by laboratories for COVID-19 testing use a wide array of testing kits with variable testing potency and quality due to differences in their target genes. Thus, accuracy, reliability and timeliness of results is of utmost priority for the testing laboratories [2].

As Quality Control (QC) of molecular testing was crucial to build trust and confidence in this vast network of laboratories, this component was inbuilt in the expansion plan of the laboratory network [3]. Our RT-PCR laboratory was started in response to COVID-19 pandemic, constructed according to the ICMR guidelines and inaugurated in April 17th 2020. All the medical officers and lab technicians had undergone RT-PCR training at the ICMR approved Virus Research and Diagnostic Laboratory (VRDL), King Institute of Preventive Medicine and Research, Guindy, Chennai.

Laboratory diagnosis helps in early identification and containment of the disease [4]. Hence, the role of the laboratory is crucial, in strengthening the government strategy "test, trace, track, treat, technology" in the fight against COVID-19 [3]. In order to strengthen the quality control measures, troubleshooting and Root Cause Analysis (RCA) of invalid IC was done. RCA is defined as "an objective, thorough and detailed methodology which is employed to determine the most probable underlying causes of problems, complaints and undesired events which occur within an organisation, with the aim of formulating and agreeing with corrective actions, to atleast mitigate, if not eliminate those causes and to so produce a significant, long-term performance improvement" [5].

IC is necessary to differentiate the true negative results from that due to a failure in some step of the RT-PCR testing [6]. A second target nucleic acid amplification, such as an IC is useful in identifying inhibitory factors present in the sample tested. Validation of a negative result for the primary target requires successful amplification of IC which is also subjected to same testing conditions as the primary target [7]. Thus, the amplification of a successful IC indicates that there are no false negatives associated with the test and ensures confident reporting of the RT-PCR results. Thus, the study was carried out with the objectives to estimate the percentage of invalid IC and to describe the most probable factors for that and to devise an action plan for troubleshooting the problems.

MATERIALS AND METHODS

The present observational cross-sectional study was conducted in the RT-PCR laboratory of Department of Microbiology, Government Kilpauk Medical College and Hospital for a period of 1 month, October 2021. This study was approved by the Institutional Ethics committee (No 624-A/2021).

Sample size: Sample size was calculated with confidence level of 95%, precision 0.5% and a prevalence of 2% of invalid IC in COVID 19 RT-PCR with our past experience and as referred by the Community Medicine department of Govt Kilpauk Medical College.

Inclusion criteria: All the samples referred for COVID-19 testing by RT-PCR during the study period were included.

Exclusion criteria: Samples rejected due to leakage; mismatch labelling were excluded from the study.

The following kits and machines were used for COVID-19 testing in the laboratory. Hi Media RNA Extraction kit was used for manual

extraction and Helini magnetic beads extraction kit for automated extraction. RT-PCR thermal cyclers used were Qiagen rotor gene q and CFX 96 Biorad system. PCR kits used for COVID-19 detection were Labgun Genomics, Labgun Exofast and Biosensor. The Labgun genomics and Biosensor kits, targets the detection of E gene and RdRp genes and the Labgun Exofast targets the detection of N gene and RdRp. Each kit was validated as per the kit protocol and Cycle threshold (Ct) for the corresponding kit.

Validation criteria of RT-PCR testing require appropriate controls for correct interpretation of results. The controls used in RT-PCR kits are PC, Negative Control (NC), No Template Control (NTC) and IC as per kit protocol. The PC is designed to check on the amplification of the RNA and to detect any inhibitions of amplification. Internal Positive Control (IPC) is usually an in-house positive sample which was included in each run to validate extraction, amplification and to detect PCR inhibition. The INC (in house negative sample) was used to check on contamination, both in extraction and PCR amplification. NTC is usually RNAse free water added to the PCR mix which helps to detect contamination in the reaction mixture. As per the kit procedure, the PC, NC and IC were checked during each run for validation of results before reporting. Batches with invalid PC or NC were subjected to retesting. Samples with valid PC and NC as per the described criteria and with invalid IC were included in the study. Validation criteria of RT-PCR test (check-list prepared for result interpretation in the laboratory) is given in [Table/Fig-1].

A detailed check-list as given in [Table/Fig-2a,b,c] was prepared by the authors including all the components of RT-PCR testing from collection of samples to reporting. All these factors were categorised as pre-analytical, analytical and postanalytical phases and each and every protocol was assessed. IC in RT-PCR is of two types, exogenous and endogenous ICs. Endogenous IC is naturally occurring host genome sequence like RNase P, B actin etc., Exogenous IC is a synthetic sequence of DNA that is spiked into the patient's sample before amplification [8]. It usually checks whether the amplification process had occurred uninterrupted.

The RT-PCR run validation requires that all the above controls used have been run according to the kit manufacturer's instructions and that the controls have attained the defined criteria and value limits as mentioned in the kit protocol. In the majority of RT-PCR methods, a positive-result decision is based on the presence of an exponential amplification curve with a Ct value above a given cut-off threshold and depends on the total number of cycles programmed for the test [9,10]. However, as with any diagnostic method, the RT-PCR must meet strict performance criteria, which contribute to the reliability of the test results provided by the laboratory. These criteria validate the analytical and diagnostic sensitivity (detection limit or Limit of Detection), analytical and diagnostic specificity, amplification efficiency, repeatability and reproducibility of results, etc., [11].

Parameter	Observation	Outcome	Interpretation
Positive control (PC)	Positive-Exponential amplification as per the kit insert Ct value	Valid	Ruled out amplification failures
Negative control (NC)	Below the threshold level- no amplification	Valid	Ruled out test contamination
NTC	Below the threshold level- no amplification	Valid	Reagents are not contaminated
IPC	Positive- Exponential amplification	Valid	Extraction and amplification proper
Internal control (IC)	Not amplified	Not valid	Requires Root Cause Analysis (RCA)

[Table/Fig-1]: Validation criteria of RT-PCR test (check-list prepared for result interpretation in the laboratory).

Variables in PCR testing	Probable problems	Remedial action needed
Sample collection	• VTM contamination • Improper and inadequate sample collection	• Proper storage and visual inspection of VTM for indicator change • Adequate training of Lab technicians on proper collection, transport and storage.
Sample transportation	• Cold chain • Sample leakage on transportation • Improper storage of sample	• Maintenance of cold chain during transportation • Store it at 2-8°C

[Table/Fig-2a]: Pre-analytical phase.
Check-list to analyse the invalid Internal Control (IC) (prepared in house by the authors)

Variables in PCR testing	Probable problems	Remedial action needed
RNA Extraction and PCR- Kits and reagents	• Breach in storage specification • Possible contamination	• Extraction kit to be stored at room temperature and PCR kit in deep freezer -20°C • Avoiding repeated freezing and thawing • Proper dilution of kit reagents according to the manufacturer's instructions • Aliquoting of the kit contents according to the daily needs. • Avoiding storage of kits along with samples • Running Internal quality controls to check reagent potency for each new batch of kits [10]
Equipment for storage of kits and samples	• Calibration	• Equipment to be calibrated (Deep freezer, Refrigerators) once in a year • Temperature chart maintenance
Pipettes	• Calibration	• Calibration of pipettes once in 6 months • Using appropriate volume filter tips, • Cleaning and autoclaving of pipettes, • Vertical positioning and proper handling techniques of pipettes • Dispensing of appropriate volume by the pipette must also be monitored
Extraction failures (This can happen because of kit and reagents problems as described above. Improper storage and repeated freezing thawing can cause degradation of exogenous IC)	Problems in kits with exogenous IC can be due to • Inadequate amount added to each sample or improper or inadequate mixing of VTM or the extraction reagents Improper centrifuging of samples, • Inadequate washing steps, • Inadequate duration missed steps in extraction protocols, • Ethanol residues present in sample after washing	• To monitor the extraction steps, addition of sample, buffers and other reagents during manual extraction • To monitor steps in semi-automated extraction. • To evaluate issues in automated extractor. • To check centrifugation, vertexing of samples and elutes to ensure proper mixing

[Table/Fig-2b]: Analytical phase.

Amplification failure (Can be caused, due to improper storage of kits or repeated thawing)	**Problem with the reaction plates, strip tubes (tubes or plates non compatible with the thermal cycler)**	**Run with different manufacture. Test with different batch or new kit**
	• Errors in calculation and addition of PCR mix, exogenous IC to the sample, • Competitive inhibition of IC in positive sample due to inadequate nucleotides for amplification. • Improper labeling and numbering of the strip tubes, • Improper mixing of sample and PCR mix, • Presence of air bubbles in the strip tubes or wells due to improper positioning of pipette tips while adding and mixing of sample • Improper closure or sealing of tubes leading to spillage of samples, using inappropriate volume of the reaction mixture.	• To monitor the calculation and addition of PCR reaction mix. • Proper labeling and numbering • To evaluate the strip tubes for air bubbles, proper closure and sealing of tubes and plates, attachment of locking ring in Rotor
	• Problems with the Thermal cycler, • errors in programming, • temperature issues	• Test to be run in a different thermal cycler • Check programming • Thermal cycler to be kept in air-conditioned room with doors closed.

[Table/Fig-2c]: Postanalytical phase.

RESULTS

Total number of samples analysed during the study period was 23500. Majority of the samples 15416 (65.6%) were collected from patients in the age group 20 to 40 [Table/Fig-3]. Out of 23500 samples included in the study, a positive test result for COVID-19 was obtained in 164 (0.69%) and a negative result in 22533 (95.89%). Around 803 (3.41%) samples included in the study showed a failed IC [Table/Fig-4].

Age (in years)	Number of patients	Percantage (%)
20-39	15416	65.6%
40-59	5405	23%
60 and above	2679	11.4%
Total	23500	100%

[Table/Fig-3]: Age distribution of samples included in the study.

Total number of samples	Total positives (%)		Total negatives (%)		Samples with invalid IC (%)	
23500	164	0.69	22533	95.89	803	3.41

[Table/Fig-4]: Results of the samples included in the study.

Factors associated with invalid IC are depicted in [Table/Fig-5a-c]. The most common pre-analytical factor associated in the table was identified as improper and inadequate sample collection in 169 samples (21%) followed by errors in cold chain maintenance

Factors associated	Number of samples with invalid IC in Pre-analytical phase	Percentage (%)
VTM contamination	0	0
Improper and inadequate sample collection	169	21
Sample leakage on transportation	0	0
Cold chain	84	10.5
Improper storage of sample	84	10.5

[Table/Fig-5a]: Factors associated with invalid Internal Control (IC)- pre-analytical N=337.

Factors associated	Number of samples with invalid IC in analytical phase	Percentage (%)
RNA extraction and PCR-kits and reagents Breach in storage specification Possible contamination	0	0
Calibration equipment for storage of kits and samples	0	0
Pipettes calibration	0	0
Extraction failures Due to technical faults	297	37%

[Table/Fig-5b]: Factors associated with invalid Internal Control (IC)- analytical (N=297).

Factors associated	No. of samples with invalid IC in Postanalytical phase	%
Problem with the reaction Plates, strip tubes (tubes or plates non compatible with the thermal cycler)	169	21%
Problems with the thermal cycler, errors in programming, temperature issues	0	0

[Table/Fig-5c]: Factors associated with invalid Internal Control (IC)- postanalytical (N=169).

in about 84 samples (10.5%) and improper sample storage in about 84 samples (10.5%). The common factor associated in analytical phase was due to technical errors and improper extraction identified in around 297 samples (37%). Problems with mismatching of reaction plates and tubes with the thermal cycler were identified in 169 samples (21%) as the most common cause in postanalytical phase.

DISCUSSION

The quality conscious RT-PCR testing would ensure accurate, reliable reporting of COVID-19 samples. In spite of the enormous sample overload and overburdened working hours we tried to maintain the quality assurance by performing the RCA of trouble shooting factors. We had evaluated the cause and reasons for the invalid IC and managed to maintain the quality of testing and reporting through various measures outlined. A result "invalid" reflects the failure to amplify the IC and is likely related to poor sampling or inadequate RNA extraction usually due to high viscosity of the sample. Samples showing invalid test results should always be repeated with a new sample due to unpredictability of the result [12].

In this study, 65.6% of patients were in the age group of 20-39 whereas in another study by Mardani R et al., 40.5% of patients were in the age group of 30 to 49 years [13]. This may be due to active mobilisation resulting in exposure of the younger and middle age group compared to other age groups.

Out of the total samples tested (23500) for COVID-19 RT-PCR during the study period, 803 (3.41%) samples did not show amplification curve in the IC channel. This data could not be compared as there were no similar studies involving invalid IC in COVID-19 testing with the best of literature search done. The positivity rate of COVID-19 in our study was 0.63% which was well in concordance with the state positivity rate during that time (0.92%) [14].

Factors in Pre-analytical phase: Among the pre-analytical variables analysed for invalid IC [Table/Fig-5a], improper and inadequate sample collection was identified as the most common factor in 169 samples (21%). This might be due to the fresh recruits of lab technicians without prior work experience and the high inflow of patients for testing during the pandemic. The method of collection was evaluated as per CDC guidelines [15-17]. After RCA, this was rectified with proper streamlining of the patient's sample collection and improvising the training protocol of collection technicians for COVID-19 samples.

The other factors associated were identified as flaws in cold chain maintenance (10.5%) and sample storage (10.5%). All these steps can influence the integrity of samples and thus affect the results of analyses. Viral Transport Medium (VTM) procurement, noting down manufacturing batch, expiry dates to follow "first expiry first out principle" of inventory management and adhering storage specification all are crucial as it would have an adverse effect in extraction of nucleic acids.

Factors in analytical phase: The main factor associated with invalid IC during the analytical phase [Table/Fig-5b] was problems in extraction and technical errors (37%). The checklist was analysed and the following measures were taken. All the steps of extraction, like addition of sample, lysis buffer, carrier RNA and vortexing of samples were closely monitored. Usage of appropriate tips and pipettes was checked during extraction. Inadequate incubation time if any was also monitored.

During the semi-automated extraction, we found that the addition of magnetic beads was not done adequately. This may be due to the fact that the consistency of the bead's suspension was not uniform. We also found out that use of inappropriate tips also accounted for uneven dispensing of the beads in the semi-automated extraction unit. Improper mixing and vortexing of samples were found to play a major role in IC failure during manual extraction [18].

Each step-in master mix preparation was also evaluated. As per the study by Liu H-B et al., air bubbles produced during the thermocycling procedure in PCR tubes has been identified as one of the major causes for PCR failure [19]. In this experiential learning, authors found that presence of air bubbles in the strip tubes of Qiagen was found to be one of the simple causes for IC failure in RT-PCR. Repeated freeze-thawing of IC [20] and using unconsumed previous batch IC for new batch of kits also were found to have adverse effects on testing. All these factors might have accounted for failure of IC during the RT-PCR run.

Factors in postanalytical phase: Postanalytical phase factors [Table/Fig-5c] were identified as problem with the reaction Plates and strip tubes (21%). This may be due to non compatibility of the tube with the thermal cycler and it was rectified using the checklist prepared. The RT-PCR plates and tubes were evaluated for compatibility with the thermocycler before starting a new batch of consumables.

Inhibition of IC in positive sample was found to be common issue in all the kits due to competitive inhibition and usage of nucleotides by the positive sample. The reason identified was due to a common pool of oligonucleotides and polymerase used for amplification of IC and target RNA. This can result in suppression of IC amplification due to large amount of target RNA present in the sample [6]. However, a positive amplification in green channel/red channel without IC amplification can be reported as positive as per the kit protocol but a negative sample without IC amplification cannot be reported as negative.

Limitation(s)

The prevalence of Invalid IC data obtained from the study could not be compared with similar such studies due to lack of studies in literature. Hence, the check-list for RCA for the invalid IC was entirely prepared in house based on the experience of troubleshooting done by the authors for the RT-PCR COVID-19 testing. The major limitation of the study was lack of correlation with clinical cases for Invalid Internal Control. We could not trace the patient associated factors for Invalid Internal Control.

CONCLUSION(S)

The above observation showed that an invalid IC could be caused by any issues starting from sample collection to RT-PCR reporting. Each RT-PCR run has to be validated before reporting and an invalid IC pose problems such as delay in reporting due to retesting. Thus, this has to be looked upon seriously and necessary actions have to be taken to evaluate its cause, thereby preventing the occurrence of problems related to processing and reporting of COVID-19 samples.

By preparing a checklist and by following proper SOP for COVID-19 testing, will ensure timely dispatching of the reports and help in breaking of the chain of transmission in pandemic situation. Furthermore, this practice will strengthen the quality assurance of RT-PCR testing. Such quality measures of RCA will ensure proper utilisation of valuable resources like PCR kits, consumables and manpower thereby reducing the cost and time involved in testing of COVID-19 samples.

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