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

ABSTRACT

Introduction: Laboratory diagnosis of Corona Virus Disease-2019 (COVID-19) plays a major role in diagnosing and treating infection. The cornerstone 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.

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

Keywords: Corona virus disease-2019, Internal control, Quality control, RT-PCR test
Observation
Ruled out test contamination
Not amplified
Requires Root Cause Analysis (RCA)
Valid
Ruled out amplification failures
Not valid
Probable problems
Outcome
Positive- Exponential amplification
remedial action needed
Valid
Below the threshold level- no amplification
Valid
Probable problems
• Calibration
Positive-Exponential amplification as per the kit insert Ct value
2
2
541
28
553
28
611
28
623
28
669
28
681
28
692
28
704
28
718
28
730
28
741
28
764
28
776
28
Improper storage and repeated freezing and thawing can cause degradation of reagents problems as described above. Improper storage and repeated freezing (thawing can cause degradation of exogenous IC)

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 post-analytical 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 [6]. 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].

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

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

<table>
<thead>
<tr>
<th>Parameters in PCR testing</th>
<th>Probable problems</th>
<th>Remedial action needed</th>
</tr>
</thead>
</table>
| 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. 
(Problems checklist to analyse the invalid Internal Control (IC) (prepared in house by the authors))

<table>
<thead>
<tr>
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<th>Probable problems</th>
<th>Remedial action needed</th>
</tr>
</thead>
</table>
| 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 
                          | • Proper dilution of kit reagents according to the manufacturer’s instructions 
                          | • Aliquoting of the kit contents according to the daily needs, 
                          | • Avoiding repeated freezing and thawing 
                          | • 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 
                          | • Calibration | • 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 |

[Table/Fig-2b]: Analytical 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 test result. Majority of the samples 15416 (65.6%) were collected in the age group 20 to 40 [Table/Fig-3]. Out of the total samples tested (23500) for COVID-19 RT-PCR during the study period, 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.

Factors associated with 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].

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%) followed by errors in cold chain maintenance 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.

Factors associated with invalid Internal Control (IC) in COVID-19 testing.

Factors in postanalytical phase identified as the most common factor in 21% of samples (84 samples) followed by errors in cold chain maintenance in 37% (297 samples). 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.
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, not enough washing of 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 un Consumed 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 [8]. 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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REFERENCES
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