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ORIGINAL ARTICLE

Analysis of Internal Controls in the matrix of HCV RT-PCR assays showed analytical and pre-analytical influences independent of template concentrations

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ABSTRACT
Internal control (IC) made up of the reference gene PCR amplification system with fixed concentration of template and fluorescent probe, and present in the assay matrix of all HCV RT-PCR assay systems was evaluated for detection of the preanalytical and analytical influences on the amplification systems. IC was added to patients' plasma RNA, calibrators, quality control (QC)RNA and non-template control (NTC). All IC additions were from the same lot.The IC fluorescence emission of ROX dye at 610±5 nm, was independent of the HCV template amplification with FAM dye having emission at 510±5 nm. The average IC threshold cycle (Cq) was lowest for NTC (30.346), followed by the calibrators (31.425), quality control samples (33.767) and the HCV infected patients' plasma RNA sample (33.017). The average %CV was the same for NTC, calibrators and QC sample. But was increased for patients' sample. The lowest average Cq in NTC might due to absence of HCV RT-PCR products, which were present in calibrators. The QC and patients' samples contained the PCR products, and in addition it contained the RNA from plasma which included the non-specific RNA. These PCR products might be the causes for the influence on IC Cq. The patients 'samples were from different patients', unlike that of QC sample and calibrators which were a single sample, contributing to higher average patients' %CV. The mean Cq difference between NTC and calibrators might be attributed to analytical influence, while that between the calibrator and patients' sample might be attributed to pre-analytical influences.
Keywords: RT-PCR, Analytical variation, Levey-Jennings plots, Internal Controls

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INTRODUCTION

The plasma viral load in hepatitis C virus (HCV) infection was often found to be very low in a large number of patients [1, 2], much below the validated calibration plots for quantitative PCR (RT-qPCR) [3]. As large number of patients with HCV infection have no symptoms [4], diagnosis of HCV infection was done by screening for anti-HCV antibody, followed by selecting the patients with reactive levels of antibody [5] for confirmation of diagnosis by HCV RT-PCR of RNA isolated from plasma [6, 7]. Most patients with HCV have very low anti-HCV antibody levels and low viral loads [2, 8]. In practice, preanalytical influences are prominent when template concentrations are low, as in HCV RT-PCR, and these influences, whenever possible, may be decreased by selecting patients without tissue damaging conditions such are surgery, crush injury, abscess, chronic ulcers, such as diabetic foot and in autoimmune diseases. The pre-analytical and analytical influence are least evident when the HCV template concentrations were high, especially at Cq <33.

The negative RT-PCR results should truly represent the absence of PCR diagnostic targets. But false-negative results could occur from failure of one of the test steps of nucleic acid extraction, reverse transcription reaction and RT-PCR set up, or inhibitory substances in the samples. Efforts to control false-negative results were based on the addition of exogenous nucleic acids amplification system (the IC) to

the RT-PCR reaction, so that the presence of any inhibitory substances (eg. heparin) in the samples or errors in test steps would also affect the amplification of the exogenous material [9, 10].Therefore, the research problem in the present report was that the IC might be used to detect influences on the RT-PCR assay systems independent of HCV template concentrations.

As HCV RT-PCR Cq value was varying in patients' sample according to viral load, it could not be used for studying the analytical and preanalytical influences. Internal Control (IC) RT-PCR amplification system with fixed concentration of reference gene template, with the ROX dye, present in the assay matrix, could be used to analyse the various influencesindependent of the HCV template amplification. ICs were added to patients' sample, calibrators, quality control (QC) samples and into the non-template control (NTC). Due to these reasons, IC could be used to study the preanalytical and analytical influences on the RT-PCR amplification. This was the research hypothesis of this report.

MATERIAL AND METHODS

The study design was observational, case-control study of ICs in real-time RT-PCR for HCV of RNA isolated from plasma of individuals who were reactive for anti-HCV antibody or suspected to have contacted HCV infection. Study population was the patients from whom EDTA-treated blood samples were obtained for RT-PCR at Amala Institute of Medical Sciences, Thrissur, Kerala state, India. The study was conducted from June 2017 to November 2021.

The Institutional Research and Ethics Committees had approved the study for the research project proposal to DST-SERB, New Delhi from Krishna K Yathi with Jose Jacob as Mentor (Ref. AIMSIEC/37/2017 latter dated 18-05-2017), and for the research proposal for PhD registration of Ann Mary Joseph with Jose Jacob as Guide to Calicut University (AIMSIEC/16/2017 dated 17-01-2017). The DST-SERB project was also approved by IBSC (Institutional Biosafety Committee) under Department of Biotechnology (DBT) guidelines dated 27th October 2017. Participants were approached directly by the investigators. The research program and the method of blood sample collection and assays were described to the participants. After including the participants by clinical and preanalytical evaluation, informed written consents were obtained and blood samples were collected. Samples were also obtained from the data available at the Clinical Molecular Biology Laboratory.

Sample Collection and Anti-HCV antibody Screening assay

Blood samples were collected in vacuum tubes with clot activator (red capped, 4 ml), mixed, allowed to clot for 10 minutes, centrifuged at 3000 rpm for 5 minutes in a table top centrifuge, serum was separated, and clear serum samples without haemolysis, jaundice, cloudiness and clot particles were screened for the anti-HCV antibody with third generation enzyme immunoassays, against recombinant epitopes of NS5, c22-3, c200 antigens, having high sero-conversion detection rates [5, 11]. The assay was performed in Vitros ECi immunochemistry autoanalyser (Ortho Clinical Diagnostics, USA) using enhanced chemiluminescence method. When screening assay showed reactivity above the cut off limit, RT-PCR was done for confirming the diagnosis of HCV infection. HCV RT-PCR was done directly, without reactivity to antibody screening assay, in individuals frequently exposed to HCV infection, such as patients on dialysis or repeated blood transfusion. This was done to cover the long window period of HCV infection.

Inclusion and Exclusion criteria, Preanalytical influences, Sample collection for RT-PCR

Inclusion criteria were reactivity to anti-HCV antibody and exposure to HCV infection. Exclusion criteria were related to the preanalytical condition of the patient, such as acute tissue damaging disease conditions (acute and chronic inflammatory changes, autoimmune disorders, abscesses, ulcers, crush injury, surgery), patients on chemotherapy and who were heparinised (dialysis and cardiology patients) were found to influence the RT-PCR [12 - 17]. These conditions were avoided as far as possible. Sample collection was delayed till the condition subsides or was overcome, up to a day or two for heparinised patients, or sample was collected before the next intravenous injection [18,19]. In all emergency and other unavoidable circumstances, all the above preanalytical criteria were overlooked, but the results of RT-PCR were analysed for influences.

Blood sample was taken with 4 ml EDTA-vacutainers, centrifuged at 3000 rpm for 15 minutes in a table top centrifuge. Plasma sample without haemolysis, jaundice, cloudiness and without clot particles was separated [20, 21] and used for RNA isolation and HCV RT-PCR. Plasma and RNA samples were stored immediately at -20^{o}C and -80^{0}C, respectively, in aliquots.

Organisation of study: A. Types of assays and the assay matrix components

The study was organized with reference to the four types of assays, NTC, calibrators, QCs and patients' sample (Table 1). HCV and IC templates with their respective fluorescent probes were in the same assay matrix but with different excitation and emission wavelengths, and detection channels. Total volume of 128 µl of reagents were prepared and 30 µl was distributed into the four types of assay tubes so that

concentration of the fluorescent probes was same in each assay tube. If there were more than one of each assay type then the total volume of reagents were increased in multiples of 30µl.

Table 1. Organisation of the study: the four types of assays and ICs in the assay matrix, total of 128 µl was distributed at 30 µl in each tube.

Reagents	**Different RT-PCR Assays**				**Total volume µl**
	NTC µl	**Calibrators µl**	**QC RNA µl**	**Test RNA µl**	
Reagent A + B, with HCV probe FAM Ex. = 470±10, Em./detection = 510±5	12 +18	12 + 18	12 + 18	12 +18	48 + 72 = 120
IC, with its probe ROX, Ex. = 585±5 nm, Em. = 610±5nm	2	2	2	2	8
Total volume of reagents used	30	30	30	30	128 µl
Template RNA / RNase free water	20 µl water	20 µl RNA	20 µl RNA	20 µl RNA	---

Organisation of study: B. Differentiating Analytical and Preanalytical influences

The components of RT-PCR assay that would influence the amplification system might be considered as the following: (1) RT-PCR amplification products of reference gene in IC; (2) RT-PCR amplification products of HCV template; (3) RT-PCR influences of non-specific RNA and the possible influences of amplification products of non-specific RNA, and (4) varying RNA isolated from plasma of different patients might influence the Cq of IC causing higher variations.

The NTC is influenced by (1); the calibrators by (1+2); the QC samples by (1+2+3); the patients' sample might be influenced by (1+2+3+4).

The analytical and pre-analytical influences could be differentiated on the following principles:

a) **The analytical influences** might be considered as the differences in the performance of Cq of ICs in the calibrators and NTC:
   - Analytical Bias = mean Cq of calibrator ICs – mean Cq of NTC ICs
   - Analytical imprecision = %CV of calibrator ICs – %CV of NTC ICs

b) **The preanalytical influences** might be considered as the differences in the performance of ICs in plasma RNA (patients' sample or QC sample) and the calibrators.
   - Pre analytical Bias = mean Cq of ICs in patients' sample – mean Cq of ICs in calibrator
   - Pre analytical imprecision = %CV of ICs in patients' sample – %CV of ICs in calibrator

HCV viral RNA isolation was done by chromatography method from plasma with QIAamp Viral RNA Mini Kit (Qiagen, Germany). Plasma (140 µl) was added to AVL buffer with carrier RNA, precipitated with ethanol, passed through QIAamp mini column. Unbound material was washed out. RNA was eluted with 60 µl elution buffer. Eluted RNA was assayed immediately and stored in aliquots at -80°C [22].

HCV RT-PCR of RNA isolated from patients' plasma

HCV RT-PCR was doneusing RNA isolated from plasma or QC sample, along with one validated calibrator, blank (NTC) andIC with reference gene in the assay matrix (Table 1), according to manufacturer's instructions [3].

Equation for conversion of IU/µl of calibrator to IU/ml of plasma sample

Calculation of plasma viral load from Cq obtained by real time RT-PCR of HCV infected plasma sample, and converted to concentration of the template from the calibration plot.

$$\text{Viral load (IU/ml of plasma)} = \frac{(\text{Patients' result Cq as IU/µl from calibration plot}) \times \text{Elution Volume (60 µl)}}{\text{Sample Volume (0.14 ml)}}$$

Quality Control Reagents

First-party (Qiagen, Germany) or third-party (Qnostics, UK, supplied by Randox, UK) quality control (QC) reagents were used for QC assays with each set of HCV RT-PCR of patients' RNA. The third-party controls were from a quality control reagent supplier and there were of three reagents,plasma samples of QC-1, QC-2 (genotype 1) and QC-3 (genotype 3) samples.The first-party controls were the validated calibrators of the reagent manufacturer. The performance values (mean±SD, 95% CI of mean and %CV) for QC samples were calculated and given under results.NTC contained all the assay mix including IC, except the HCV 5'UTR template. The second party QC samples were prepared by the user laboratory from HCV RT-PCR positive samples, typically immediately after calibrating with fresh calibrators and QC reagents.

Internal Control (IC) with reference genes were added to the assay matrix of NTC, calibrators, QCs and patients' samples.IC fluorescence probe ROX (6-carboxy-X-rhodamine)was with excitation 585±5 nm and

detection 610±5 nm. The hydrolysis probe in the test sample was Fluorescein amidites (FAM) and which has an excitation 470±10 nm and emission wavelength and detection at 510±5nm.
Calibrators, Of the four validated calibrators supplied by the manufacturer (Qiagen) S3 (100 IU/µl) and S4 (10 IU/µl) were used in this study.Traceability of the validated calibrators were to the reference standard at the National Institute for Biological Standards and Control (NIBSC, UK) which coordinated the traceability studies on behalf of WHO [3].

Levey-Jennings Plot

Levey-Jennings (L-J)plot is the common representation of quality control (bias and imprecision) and is done to evaluate the QC results. The QC results from the same lot of QC reagents were added to the plot with time (daily or with varying frequency). When the QC showed a stable performance, the mean value line represented the expected target value and the Standard Deviations (1SD, 2SD, 3SD) lines represented the expected imprecision. Assuming a Gaussian distribution, of imprecision, we expect approximately 99.72% within ±3SD, 0.28% outside ±3SD, 95.44% within ±2SD, 4.56% outside±2SD, 68.26% within±1SD and 31.74% outside±1SD. These data should be differentiated from the QC rules and events data, where $\mathbf{1_{1S}}$represented the single data points between ±1SD and ±2SD, and1_{2S} event represented two consecutive data points between ±1SD and ±2SD.

RESULTS

HCV RT-PCR amplification plots of various assay systems and their internal controls

Amplification plots of calibrators, QC reagents, patient sample and NTC were plotted to evaluate the amplification, Cq and fluorescence intensity. In the QC-1 sample, the Cq was 34.09, the fluorescence intensity and slope at inflection point were decreased when compared with the samples having lower Cq (or higher template concentration). NTC, as expected, was below the threshold line. The validated calibrators S3 (100 IU/µl), S4 (10 IU/µl) and the patient sample (Cq = 25.81) showed amplification plots that had good RT-PCR amplification and slope at inflection point (Fig. 1A).
The ICs of all the five RT-PCR assays in Fig. 1A were evaluated for their Cq and fluorescence intensity. The IC of NTC had the lowest Cq of 30.04.The patient's sample showed the maximum increase in Cq of 32.58 and washigher than that of S3 and S4 calibrators (30.31 to 30.88). The Cq of QC-1 sample which had the same sample matrix of plasma as the patient's sample was intermediate between calibrators and patient sample (Fig. 1B).

Influence of various assay systems on the Internal Controlthreshold cycle, Cq

The performance characteristics of Cq ofIC of various assay systems (Table 2) were evaluated. The IC of NTC (n = 6) showed the lowest mean Cq of 30.346 and the variation, as expressed by %CV 1.3, was low. The mean Cq of calibrators, which were used as first party control reagents, was found to be 31.425(S4) and 30.92 (S3), and were higher than that obtained by IC in NTC. The ICs in QC-1 was even higher with mean Cq of 33.767 with %CV of 1.52 (Table 2). IC of QC-2 and QC-3 were also increased at 31.563 and 31.663, respectively. Similarly, the mean Cq of IC was also increased in the second party quality control reagents. These results indicated that the assay matrix of the plasma sample can influence the Cq and cause an increase in the mean Cq value.
The influence of RT-PCR products and patient RNA on Cq of IC were analysed by plotting the mean Cq of IC from various HCV RT-PCR assays and controls. The mean IC Cq of patient sample with Cq 30 – 36(n=6)was 32.42,patient sample with Cq >36 (n=6)was 33.053, and that in patient sample with Cq <30 (n=6) was 32.2 (Fig. 2A). The Cq was lowest for IC of NTC and the Cq increased sequentially for IC of calibrator, QC reagents and inpatients' sample (Fig. 2A).
Similarly, the average %CV of Cq from IC of NTC, calibrators, QC reagents and patient samples were found to be similar, unlike that seen with mean Cq. But the %CV was markedly increased in the patient's sample (Fig. 2B). The results indicated that, even though the mean Cq increased sequentially, the variation was increased only in the different patients' sample.

Levey-Jennings Plot of Internal Controls from various assay systems

When the L-J plot of ICs of various samples were analysed, there were large number single events between ±1SD and ±2SD. The number decreased to 3 events between ±2SD and ±3SD, and further decreased to 1 event outside ±3SD (Table 3).
The total number of events outside ±1SD for NTC was 10 (8+2) and the total number of samples were 30. Therefore, 33.3 % of the events were outside ±1SD, which was very close to the expected events, 31.74%. Similarly, the IC Cq values for calibrator S4, QC-1 and patient RNA were calculated.
a)Percentage of events outside the ±1SD for NTC, calibrator S4 and QC-1 are 33.33%, 36.67% and 40%, respectively. There were no events outside ±2SD for the above.

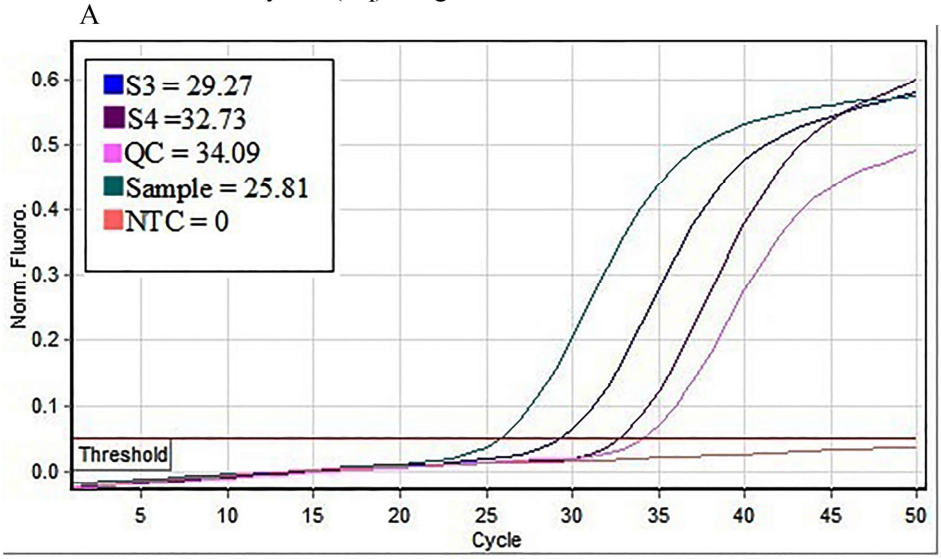
b) Percentage of events outside the ±1SD for HCV patients' RNA with Cq<30, Cq 30 – 36, Cq >36 were22.22%, 16.66% and 20.69%, respectively. Percentage of events outside the ±2SDfor patients' samples with Cq<30, Cq 30 – 36, Cq >36 were 3.7%, 8.33% and 0%, respectively. But percentage of events outside ±3SD for Cq >36 was 3.45%.

These results indicate that the number of events outside ±2SD and ±3SD were more in patients' samples.

The assays given in the L-J plot were analysed for their bias and imprecision by calculating the mean and SD.The mean and %CV of performance of Cq of ICs in the environment of assay matrix of NTC, calibrators (first party QC), QC-1 and patient sample were analysed. The NTC had the lowest mean Cq, followed by the calibrator. The patients' sample and QC-1 showed the higher mean Cq. The patients' sample had high %CV. The %CV was much lower for NTC, calibrator and QC-1.

Fig. 1. HCV RT-PCR amplification plots (A) and IC amplification plots (B) of calibrators, QC RNA, patient's plasma RNA and no template control (NTC). The threshold cycles (Cq) are given in the box.

A



B

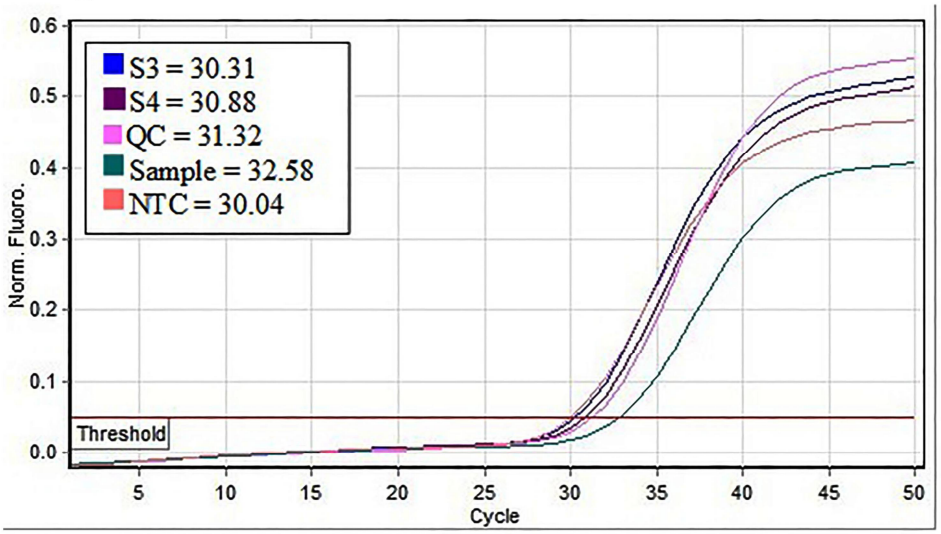


Table 2. Performance of the IC Cq in different assay systems.

Control samples	Mean±SD	95% CI	%CV
IC in NTC	30.346±0.395	29.932 – 30.761	1.3
IC in third party HCV control plasma, n = 6 (Qnostics, Randox, QC provider)			
IC of QC-1	33.767±0.498	32.31 – 33.23	1.52
IC of QC-2	31.563±0.334	31.212 – 31.914	1.05
IC of QC-3	31.663±0.355	31.290 – 32.036	1.12
IC in second party control plasma, n = 6 (user defined)			
IC in control plasma with Cq 36	33.017±0.816	32.16 – 33.873	2.47
IC in control plasma with Cq 31	32.303±1.05	31.19 – 33.41	3.27
IC in first party controls, n = 6 (calibrators from Qiagen, reagent manufacturer)			
IC in S4 (4280 IU/ml)	31.425±0.485	30.92 – 31.93	1.54
IC in S3 (42800 IU/ml)	30.92±0.38	30.52 – 31.32	1.23
IC in patients' sample, n = 6 (from 6 different patients)			
Patients' sample with Cq <30	32.2±1.373	30.76 – 33.64	4.26
Patients' sample with Cq 30 – 36	32.42±0.833	31.54 – 33.29	2.57
Patients' sample with Cq >36	33.053±1.051	31.95 – 34.16	3.18

Fig. 2. IC mean Cq (A) and average %CV (B) of NTC, calibrator, QC samples and patients' samples (n = 6).

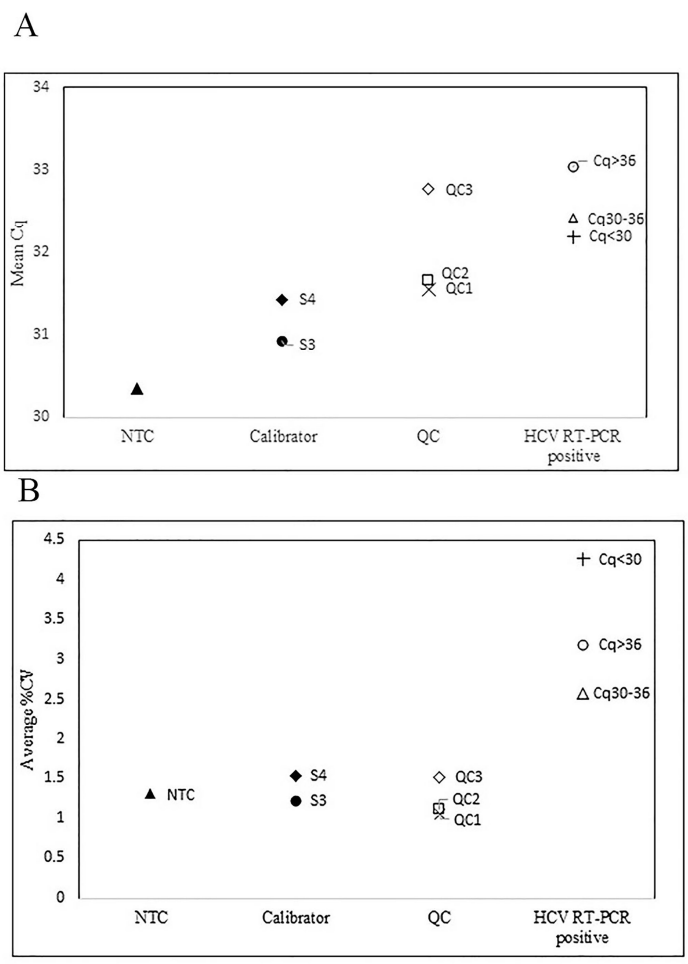
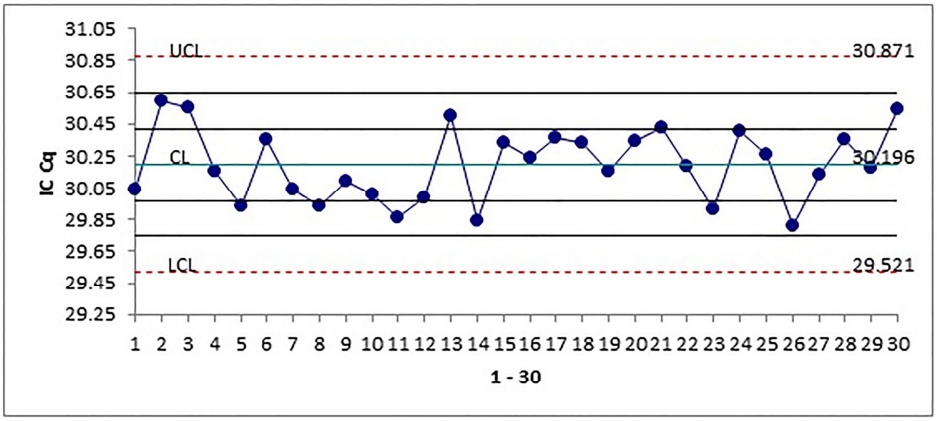


Table 3. The summary of the Levey Jennings plot data of IC Cq in NTC, calibrators, QC and patient samples

	NTC (n = 30)	Calibrator S4 (n = 30)	QC 1 (n = 15)	Patient RNA, Cq<30 (n = 27)	Patient RNA, Cq,30-36 (n = 24)	Patient sample, Cq>36 (n = 29)
Mean±SD	30.19±0.23	31.71±0.74	33.17±0.61	32.87±2.10	31.97±0.74	33.14±1.41
95% CI	30.11 – 30.28	31.43 – 31.98	32.83 – 33.51	32.041 – 33.71	31.66 – 32.29	32.60 – 33.67
%CV	0.75	2.33	1.85	6.39	2.33	4.24
QC rules	**QC events**					
1_{1S}	8	7	4	5	2	5
1_{2S}	-	-	-	1	2	-
1_{3S}	-	-	-	-	-	1
2_{1S}	1	2	1	-	-	-
2_{2S}	-	-	-	-	-	-
2_{3S}	-	-	-	-	-	-

Fig. 3. Levey Jennings Plot of IC Cq from NTC (A) and IC Cq from S4 (B) with n = 30. UCL and LCL are upper and lower control limits at ±3SD.

A



B

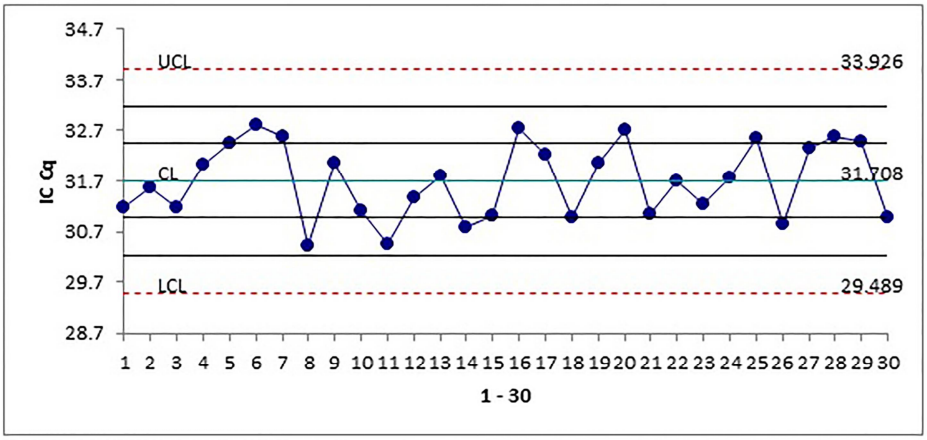


Fig. 4. Levey Jennings plot of IC Cq from HCV QC-1 sample (n = 15).

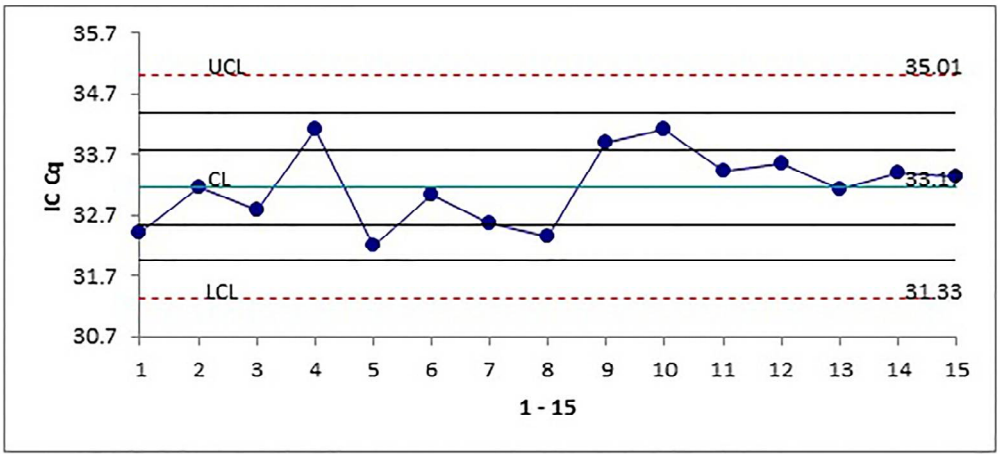
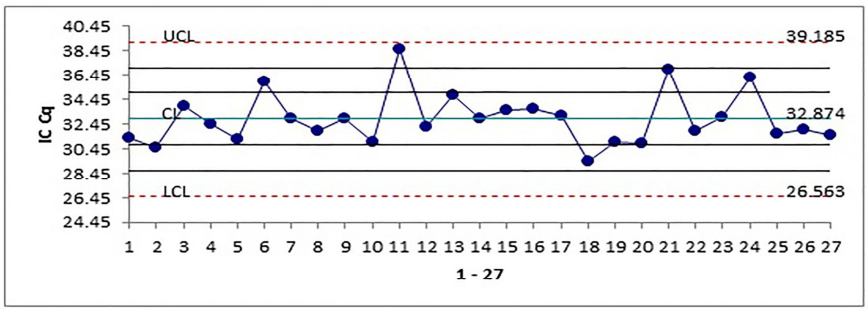
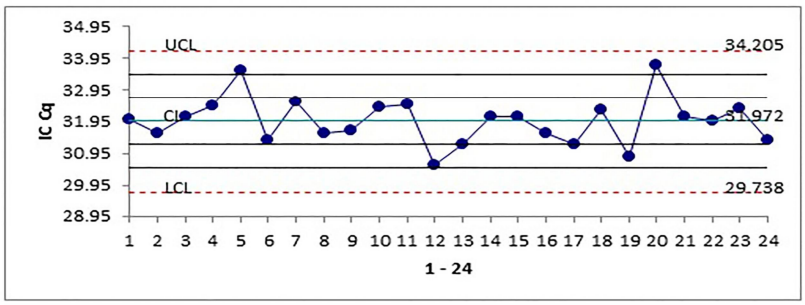


Fig. 5. Levey Jennings plot of IC Cq from patient's plasma with Cq < 30, n = 27 (A), Cq 30 - 36, n = 24 (B) and Cq > 36.0, n = 29 (C).

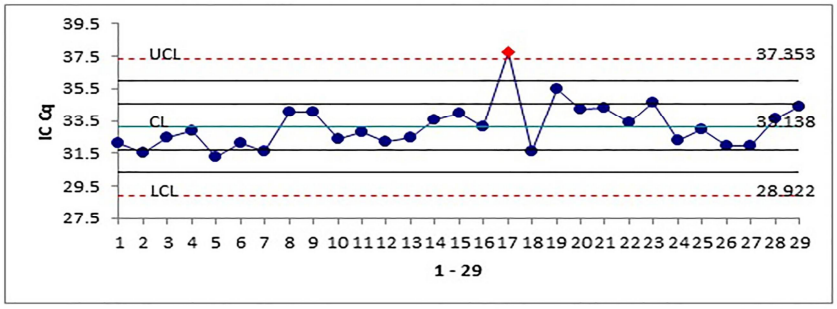
A



B



C



DISCUSSION

RT-PCR influences were typically observed in the test sample amplification when the template or viral load was very low. But it might be assumed that there were preanalytical and analytical influences even when the viral loads were high. When the template or viral loads were high, the early rise in fluorescence intensity and the lower Cq value may not make the analytical and preanalytical influences prominent enough for observation. The influences were visible only when the Cq values were>33 or the template concentrations was <10 IU/µl (Fig. 1A). These observations might be due to (a) the larger number of cycles required for the low concentration of non-specific RNA from plasma to influence the RT-PCR amplification plots, and b) the influences of PCR products on the RT-PCR amplifications. The influence of PCR products from template amplification should be seen in all amplification plots at all concentrations of the template, except in NTC.

In the case of patient sample, Cq varies from sample to sample from high to low Cq and, therefore, Cq of patients' sample cannot be used for studying the influences on Cq. But IC concentrations were same in all assays for a particular lot of reagents.Due to these reasons, the variations in Cq of IC were evaluated to study the analytical and preanalytical influences. All data given in the present report were reproduced with a single lot of IC reagent.

We might consider analytical influences as the ones caused by the PCR amplification products of HCV template RNA. The preanalytical influences may be considered as the influences of components in the plasma sample that were isolated along with HCV RNA. These co-isolates of HCV RNA might probably be contaminating RNA or might be DNA. Such contaminating nucleic acids were increased in tissue damaging conditions which were exclusion criteria for sample collection, except in emergencies. If such samples were not excluded, the influences of contaminating RNA might be much more. Drugs commonly affected enzymes of RT-PCR [17]. Non-specific RNA and their non-specific amplification products also might consume the primers and affect amplification[23].

The most analytical and preanalytical influences might be considered to be least in NTC, where the only amplification product was that from the reference gene. Therefore, as expected, the analytical influences were the lowest leading to the lowest IC mean Cq and low %CV (Table 2 and Fig. 2). In the first party control samples were the calibrators of HCV, commonly the S4 or 10 IU/µl calibrator. Therefore, the preanalytical influence of plasma samples were absent. But in the third-party and second-party control RNA was isolated from plasma and preanalytical influences would be more evident.

The influences on the Cq were seen as an increase in mean Cq value (Fig. 2A). Patients' sample showed greater %CV, as the plasma RNA samples were isolated from different patients (Table 2 and Fig. 2).NTC which has no preanalytical and least analytical influence has least mean Cq, followed by first party controls (calibrators), then third party controls and then followed by highest mean Cq of IC in patient sample. But the Cq variation, represented as %CV, remained fairly constant in all samples, except in the patients' sample where the variation markedly increased (Fig. 3). The high %CV of patients' RNA might be due the sample collection from different patients and there was no repetition with the same sample as with calibrator and QC samples.

CONCLUSIONS

IC was added to RT-PCR assays with patients' plasma RNA, calibrators, QC RNA and NTC. All IC additions were from the same lot. The IC fluorescence emission of ROX dye at 610±5 nm, was independent of the HCV template amplification with FAM dye having emission at 510±5 nm. The average IC Cq was lowest for NTC, followed by the calibrators, QC samples and the HCV infected patients' plasma RNA sample. The average %CV was the same for NTC, calibrators and QC sample, but was increased for patients' sample. The results showed that the analytical influences might be due to HCV RT-PCR products which could be attributed to the IC Cq difference between the calibrator and NTC. The preanalytical influences might be contributed by IC Cq difference between patients'(or QC) sample, and the calibrator. QC and patients' sample contained non-specific RNA from plasma which might contribute to the pre-analytical influences. The patients' samples were from different patients' contributing to higher average patients' %CV, unlike that of QC sample and calibrators which were a single sample.

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molecular diagnostic reporting of HCV infection by data analysis, test validation, quality control and evaluation of preanalytical influences (Ref. No. U.O.No-1244/2017/Admn dated 02.02.2017).

COMPETING INTERESTS

The authors have declared that no competing interest exists.

ETHICAL STANDARDS AND INFORMED CONSENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study

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