Research Article

Quality Control in RT-PCR Viral Load Assays: Evaluation of Analytical Performance for HIV, HBV, and HCV

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Article Info

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Keywords
Internal Quality Control; External Quality Control; RT-PCR (Reverse Transcription Polymerase Chain Reaction); HIV (Human Immunodeficiency Virus); HBV (Hepatitis B Virus); HCV (Hepatitis C Virus); Coefficient of Variation; Random Error; Systematic Error; Total Error; Westgard Rules; Levey-Jennings Chart.

Abstract

Introduction
Quality Control Management (QCM) in clinical laboratories is crucial for ensuring reliable results in analytical measurements, with biological variation being a key factor. The study focuses on assessing the analytical performance of the Reverse Transcription Polymerase Chain Reaction (RT-PCR) system for Human Immunodeficiency Virus (HIV), Hepatitis B (HBV), and Hepatitis C (HCV). Five models proposed between 1999 and 2014 offer different approaches to evaluating analytical quality, with Model 2 based on biological variation and Model 5 considering the current state of the art. The study evaluates the RT-PCR system's analytical performance through Internal Quality Control (IQC) and External Quality Control (EQC).

Materials and Methods
The Laboratório Central de Saúde Pública do Estado do Ceará (LACEN-CE) conducted daily IQC using commercial kits, and EQC was performed through proficiency testing rounds. Random error, systematic error, and total error were determined for each analyte.

Results
Analytical performance, assessed through CV and random error, met specifications, with HIV and HBV classified as "desirable" and "optimal." EQC results indicated low systematic error, contributing to total errors considered clinically insignificant.

Conclusion
The study highlights the challenge of defining analytical specifications without sufficient biological variability data. Model 5 is deemed the most suitable. The analytical performance of the RT-PCR system for HIV, HBV, and HCV at LACEN-CE demonstrated satisfactory, emphasizing the importance of continuous quality control in molecular biology methodologies.

Introduction

Quality Control

The analytical measurement of a given human biological parameter is subject to a range of variations due to laboratory and physiological causes. Among the laboratory ones, those related to the pre-analytical phase stand out, that is, posture at the time of collection, sample transport time and conditions, centrifugation time, collection method, order of tubes used, among others; Examples of the analytical phase are: methodology required for the test, particulars and maintenance of the equipment used, technical team that will carry out the test, climatic conditions, validity and batch change of reagents, among others. Biological variability, in turn, is associated with physiological factors, resulting from diet, circadian cycle, menstrual cycle, stress and emotional, diseases, psychological use of medications, sex, age, etc., causes to the patient and the analyte of interest to be assessed [1-3]. For Health Care Establishments (HCE), as well as clinical laboratories, ensuring the reliability of the results issued through Quality Control Management (QCM) is a requirement provided for by the Resolução da Diretoria Colegiada (RDC), which translates to Directors' Collegiate Resolution in English, Nº 786 of 5 May 2023. "The HCE that performs the Clinical Analysis Examination (EAC) must guarantee the reliability of the results through the QCM". The QCM consists of the routine evaluation of the analytical system according to each assay analyzed, considering the internal and external performance of the HCE [4]. The International Organization for Standardization (ISO), through standard 15189:2015, which deals with the quality and competence requirements of clinical laboratories, recommends that laboratories implement analytical procedures to verify the achievement of the desired quality in the results in addition to transporting the resulting variability the imprecision and inaccuracy of analytical methods [5]. To meet the requirements of ISO 15189:2015 and RDC No. 786, the HCE must provide a clinical result with an analytical measurement error lower than the limit allowed after sample processing in all analytical phases (pre-analytical, analytical and post-analytical). Therefore, the correspondence and clinical quality of this result must be guaranteed for medical and therapeutic management [4,5]. To monitor the accuracy of the analytical system, Internal Quality Control (IQC) is used. It is a sample, normally commercial, with an already determined analytical value, whose processing is carried out before the beginning of the laboratory routine in order to evaluate the precision, that is, the agreement between the results of the control sample among themselves, as well as the result that must be within the range recommended by the manufacturer [6]. The Coefficient of Variation (CV) measures the relative variability of the data in relation to the average. This is the statistical parameter most used to evaluate the precision of the analytical method. The CV classification can be according to the following description: CV within the recommended reference, "Minimum" CV, when the value is within 75% of the reference, "Desirable" CV, when the value is within 50% of the reference, "Optimal" CV, when the value is within 25% of the reference [7]. The standard statistical model for monitoring IQC data was proposed in 1950 by researchers Stanley Levey and E. R. Jennings, which is based on a graphical representation of the results participating in the mathematical model of the Gaussian distribution. initially in the industrial sector, until the 1980s, the use of the Levey-Jennings chart aimed to keep the results of the measurand within the range of two standard deviations. In 1981, researcher James O. Westgard proposed a series of rules for evaluating random and systematic errors according to the graphical behavior of IQC results. Levey-Jennings graphs and Westgard rules were then consolidated in the laboratory [8-10].

In addition to IQC, External Quality Control (EQC) is intended to measure the accuracy of the analytical method. To this end, the laboratory must regularly participate in proficiency testing or interlaboratory comparison programs, in order to receive samples from an institution and process them in its routine, providing similar treatment to patient samples. The result is reported and subsequently published by the evaluation group. Unlike the IQC, the EQC provides a qualitative-quantitative assessment of accuracy, as it allows the assessment of systematic errors, such as analytical method biases (bias) [6-11]. Sending precision by IQC and accuracy by EQC enables GCQ to mathematically calculate the random error caused by inaccuracy, and the systematic error resulting from inaccuracy, respectively, and, by adding them together, determine the total measurement error of the method which if you are following.

Models of Analytical Specifications

In 1999, during the first "Strategies to set Global Quality Specifications in Laboratory Medicine" in Stockholm, five hierarchical models were defined for the specification of analytical quality for clinical laboratories [12]. Leading the initiative at the Conference was a group of researchers headed by Carmen Ricos, who also published a study in the same year titled "Current databases on biologic variation: pros, cons and progress," containing data for 350 analytes. Until 2019, this group compiled information in partnership with the Westgard Q.C. website. Subsequently, the maintenance of the database and its updates would be the responsibility of the European Federation of Clinical Chemistry and Laboratory Medicine (EFML) [13]. The EFML provides in its biological variation database the records of the intraindividual Coefficient of Biological Variation (CVbi) and intergroup or interindividual Coefficient of Biological Variation (CVBg) for 2716 analytes, with data based on metadata analysis and estimates corroborated by scientific research [14]. The project began after the first conference organized by the same institution, titled "Defining analytical performance goals 15 years after the Stockholm Conference on Quality Specifications in Laboratory Medicine." The central objective of the EFML is to assess the quality of biological variation data to enable users, including clinical laboratories, to make critical analyses of their processes regarding inherent variations in specific analytes [14]. Differences between the models determined at the two conferences can be observed in Table 1. In table 1, it is possible

to observe the advancements between the years 1999 and 2014 regarding models for monitoring the performance of the analytical system [12-15].

Table 1: Models proposed at the Stockholm and Milan conferences.

	Comparative Models	
Model	**Stockholm 1999**	**Milan 2014**
Model 1	Evaluation of the impact of analytical performance on clinical restults in specific clinical settings.	Evaluation of the impact of analytical performance on clinical results. To develop quality specifications using results, one of the following procedures must be followed: A. A results study investigating the impact of analytical performance on the probability of clincal outcomes; C. A survey of opnions from physicians and/or specialists investigating the impact of analytical performance on medical decisions.
Model 2	Evaluation of the impact of analytical performance on overall clinical decisions: A. Data based on components of biological variation; B. Data based on the analysis of physicians' opinions.	Based on components of biological variation: The goal is to ensure that "analytical niose" does not drown out the biological signal. In the new project, it was emphasized that there are indeed significant limitations to this approach, including the relevance and validity of biological data.
Model 3	Published professional recommendations: A. From national and international sepcialized bodies; B. From local specialized groups or individuals.	
Model 4	Performance goals defined by: A. Regulatory bodies; B. From Organizers of External Quality Assessment (EQA) schemes.	
Model 5	Goals based on the current state of the art. A. As demonstrated by data from EQA schemes of Proficiency Tests; B. As found in current publications on methodology.	Based on the current state of the art: It is based on the realistic performance "as-is" in the market. If the best laboratories can only achieve a certain quality but cannot meet the quality required by models 1 and 2, then the current performance is accepted (for now).

Following the implementation in 1999, Model 2 became the most sought after by clinical laboratories due to its provision of a tangible numerical parameter for statistical analysis for HCE. However, after the publication of the Milan conference report, the limitations of the model became evident, including the lack of data for a variety of analytes measured in routine laboratory practice, as well as the validity of the then-available data [13-15]. In the following years, international standards such as ISO and the Clinical and Laboratory Standards Institute (CLSI), along with national regulations developed by regulatory bodies in various countries, based on Model 2, sought to determine desirable limits of imprecision, bias, and total error using CVBi and CVBg data [13].

Molecular Biology and Viruses

Polymerase Chain Reaction (PCR) is a molecular biology technique developed and automated since the 1980s [16,17]. The success of this method lies in its heightened sensitivity and accuracy in detecting and identifying genetic material unique to the analyte of interest through genetic material extraction, followed by amplification (composed of denaturation, annealing, and extension of genetic material), culminating in its analysis [18-20]. Subsequently, to maximize the analytical process, Reverse Transcription Polymerase Chain Reaction (RT-PCR) was developed. The main attraction of this modification was the condensation of the amplification and result analysis steps, as well as the reduction of the minimum genetic material required for the reaction, the ability of the method to process RNA template strands, and provide quantitative results according to gene expression [20-21]. Given the various permissible applications of RT-PCR, the quantification of microorganisms such as viruses and bacteria made it unique for monitoring and guiding medical interventions in the management of highly complex conditions [20]. In this context, Human Immunodeficiency Virus (HIV), Hepatitis B and C (HBV and HCV, respectively), conditions of public health interest, once assessed through rapid

and serological tests incapable of providing quantitative results regarding the viral load in the patient, are currently evaluated by molecular biology methods, which have advantages in terms of specificity, sensitivity, and better monitoring of therapies employed for the treatment of such infections.

In the pursuit of continuous improvement, Clinical Laboratories employ new technologies and methods, such as molecular biology, to provide healthcare professionals with more sensitive clinical data to assist in therapeutic decision-making. This effort aims to minimize costs associated with unnecessary or inappropriate therapies, expedite the diagnostic process, and enhance the capacity for short and long-term therapeutic monitoring. To ensure that such methods are under analytical control, i.e., their results are reliable and under stabilized random and systematic errors, is of utmost importance for issuing clinically meaningful reports. QCM, therefore, plays a fundamental role in ensuring this success. The objective of this study was to evaluate the analytical performance of the RT-PCR system used for the assay of determining the viral load of HIV, HBV, and HCV. Thus, random error was calculated according to IQC, systematic error according to EQC, and total error based on the two previous ones.

Materials and Methods

The Laboratório Central de Saúde Pública do Estado do Ceará (LACEN-CE) is the Reference Laboratory for the State of Ceará, with the responsibility of conducting Laboratory Surveillance through analyses of interest to Health Surveillance, acting, among other functions, in monitoring the epidemiological situation in the State of Ceará. It has more than 11 sectors dedicated to monitoring various health issues, such as bacterial and mycobacterial diseases, parasitic diseases, arboviruses, mycoses, and viral diseases, totaling 787,861 assays during the year 2023. To achieve this, it relies on a wide variety of technologies for monitoring the respective analytes. In this context, the Laboratory for HIV and Viral Hepatitis Viral Load (BHH) operates at the forefront of monitoring viral diseases using the molecular biology method, RT-PCR, detecting and quantifying the viral load of previously diagnosed patients undergoing pharmacotherapeutic monitoring. Routine procedures involve processing an internal Roche quality control kit containing three IQC levels for each condition for every 21 samples, with low, high and negative levels [22]. The quantitative data from the low and high levels are manually entered into the Google Sheets© software, which automatically transforms this data into logarithmic values of base 10. They are then evaluated using Levey-Jennings graphs, initially following the knowledge principles of manufacturer. After 100 observations, obtaining mean and standard deviation values, the results are evaluated according to pre-established Westgard rules. The determination of random error, considering 95% reliability, was made from the coefficient of variation mathematically obtained, as demonstrated by equation 1, using data collected during the months of October, November, and December 2023. Only results for low and high levels, within 2 standard deviations, the limit recommended by the manufacturer, were considered valid [23].

Equation 1:

$$Coefficient\ of\ Variation\ (\%) = \left(\frac{Standard\ Deviation}{Mean}\right) * 100$$

To obtain the random error, equation 2 was applied. The evaluation of the random error results was done according to the classification of “minimum,” “desirable,” and “optimal.” The specification criterion used was the maximum limit recommended in the package insert for each analyte.

Equation 2:

$$Random\ error\ (95\%\ confidence) = CV * 1.65$$

Trimestrally, the laboratory participates in external quality control rounds, during which it receives samples with unknown presence and viral load results. The determination of the systematic error of the method was obtained with the results from the two rounds of the year 2023, covering the months of August, September, October, November, and December of the year 2023. For this purpose, equation 3 was employed:

$$Systematic\ error\ (\%) = \left(\frac{Laboratory\ result - Round\ Mean}{Round\ Mean}\right) * 100$$

The total error for each assay was calculated by summing the systematic error and random error. It was classified according to the total error guidelines recommended by the Ministério da Saúde (MS) which translates to Ministry of Health, in English. [24]. As specification criteria for the IQC, reference values from the manufacturer’s instructions were used, and then the corresponding CV and random error limits for analytical performance were mathematically calculated.

Results

During the last quarter of 2023, 7,119, 231, and 336 samples of HIV, HCV, HBV, respectively, from different healthcare units in the State of Ceará, were processed, totaling 7,686 assays during the period. This represents 22.4% of the analyses conducted by the sector throughout the year. Table 2 shows the quantity of controls processed during this interval. In table 2, it is possible to observe the number of samples processed during the year 2023, in the last quarter of 2023, and the number of controls processed for the same period.

Table 2: Historical series of analyses and IQC processed in 2023.

	Samples		Control Kits
Virus	**Number of Samples in 2023**	**Number of Samples in the Last Quarter**	**Number of Control Kits in the Last Quarter**
HIV	32699	7119	339
HBV	980	336	16
HCV	606	231	11

The results of the IQC were categorized based on the data from the IQC kit manufacturer, meeting the terms "minimum," "desirable," and "optimal," as shown in Table 2. The analytical performance, by month, can be seen in Table 3. Considering the cumulative results for the quarter, it is observed that HIV maintained a "desirable" result, HBV an "optimal" result, and HCV maintained a "desirable" result in two months. All evaluated analytes remained within specifications for both CV and random error. In table 3, the manufacturer's data regarding the analytical performance of the IQC is presented. The CV and Random Error Specification were calculated based on the Mean and SD values.

Table 3: Parameters of the manufacturer's IQC kit.

	Manufacturer's IQC Parameters					
	HIV		HBV		HCV	
Control level of analytes	**HIV Low**	**HIV High**	**HBV Low**	**HBV High**	**HVC Low**	**HVC High**
Mean (Log)	2.57	5.31	2.17	6.30	2.15	6.24
SD (Log)	0.32	0.32	0.32	0.32	0.32	0.32
Reference CV (%)	9.38	9.38	10.08	10.08	10.16	10.16
Minimun (%)	7.04	7.04	7.62	7.62	7.56	7.56
Desirable (%)	4.69	4,69	5.08	5.08	5.04	5.04
Optimal (%)	2.35	2.35	2.54	2.54	2.52	2.52
Random Error Specification (95% CI%)	15.48	15.48	16.63	16.63	16.76	16.76
Minumum (%)	11.62	11.62	12.57	12.57	12.47	12.47
Desirable (%)	7.74	7.74	8.38	8.38	8.32	8.32
Optimal (%)	3.88	3.88	4.19	4.19	4.16	4.16

In table 4, you can observe the results of Coefficient of Variation (CV) and random error for each analyte in each observed month, along with their averages. The classification values are based on the parameters from Table 3.

Table 4: IQC Performance Results

Analyte	CV (%)	Random Error (%)	Classification
October			
HIV	2.74	4.52	Desirable
HBV	1.41	2.32	Optimal
HCV	2.99		Desirable
November			
HIV	2.60	4.30	Desirable
HBV	2.48	4.09	Optimal
HCV	2.32	3.83	Optimal
December			
HIV	2.68	4.43	Desirable
HBV	1.86	3.07	Optimal
HCV	0.80	1.32	Optimal
Quarterly Comulative Average			
HIV	2.68	4.42	Desirable
HBV	1.86	3.16	Optimal
HCV	2.04	3.36	Optimal

EQC was assessed based on the results of the last two rounds of the year 2023. The average percentage of systematic error was calculated for each condition from the obtained results, as shown in Table 3.

In table 5, it is possible to evaluate the results of the EQC according to the rounds in August and November, as well as the total systematic error per analyte during the assessed period. The systematic error was calculated in absolute value.

Table 5: EQC Results.

	External Quality Control			
Round	**Group Round Mean**	**LACEN-CE Mean**	**System Error (%)**	**Average Systematic Error (%)**
HIV				
August	4.16	4.00	3.89	3.37
November	2.79	2.66	2.85	
HBV				
August	3.34	3.32	0.51	0.65
November	2.54	2.52	0.79	
HCV				
August	5.82	5.73	1.58	1.75
November	4.48	4.39	1.92	

The total error was determined by summing the average systematic and random errors, as shown in Table 6.

Table 6: The table presents the sum of IQC and EQC

	Total Error		
Analyte	**Average Random Error (%)**	**Average Systematic Error (%)**	**Total Error (%)**
HIV	4.42	3.37	7.79
HBV	3.16	0.65	3.81
HCV	3.36	1.75	5.11

In Table 7, the simulation involved calculating the deviation of the result from the total error value, derived from the average value in the leaflet, for each control level. This was done to assess whether the variation between the average result and the result considering the error is clinically significant, based on the recommendation of the MS, which determines a variation of up to 0.5 Log as a limit [24].

Table 7: Mathematical simulations of the clinical impact of total error on system-released results.

		Total Error	
Control level of Analyte	**LACEN-CE EQC Result (Log)**	**Maximum Limit considering Total Error of table 6 (Log)**	**Variation between LACEN-CE Result and Maximum Value (Log)**
HIV-high	4.00	4.31	0.31
HIV-low	2.66	2.87	0.21
HBV-high	3.32	3.45	0.13
HBV-low	2.52	2.62	0.10
HCV-high	5.73	6.02	0.29
HCV-low	4.39	4.61	0.22

Conclusion

Biological variation describes the observed variation in the concentration or activity of different constituents in an individual, reflecting regulation by homeostatic processes in the body [25]. The use of IQC provides daily elements that allow the operator to identify errors or atypical behaviors in the analytical performance of the system. In the event of IQC errors, the analytical routine should be postponed until the cause of the error is identified, and appropriate actions are taken to correct the analytical performance and initiate the routine [6]. In seeking literature specifications for Intra- and Inter-Individual Biological Variation (CVBi and CVBg) related to the analytes of interest, no information was found in the reference databases, EFLM and Westgard Q.C., regarding HIV, HBV, and HCV or even other analytes measured by RT-PCR techniques. The absence of information, a limitation foreseen since the Stockholm Conference in 1999, leads the clinical laboratory to determine specifications that align with the models presented in the conferences of either Stockholm or Milan [12-16]. The LACEN-CE employs the use of commercial Roche© kits for internal control, which have specifications that should guide the analytical performance of the system at the national and international market levels. From these specifications, presented in Table 2, mathematically determined values of CV and Acceptable Random Error were established to assess the analytical performance of the system. Although the analysis and treatment pattern follows the Model 2, which relates to biological variability, the analytical specification that best suits the procedure adopted in this study is Model 5, which expresses the "state of the art" available for a particular analyte. This term refers to "a methodological procedure that aims to develop a mapping of scientific productions" whose result is the "descriptive inventory of academic and scientific production on the topic investigated" [26]. Given the fact that methodologies involving biological variation have been widely disseminated since the first decade of 2000, after the Stockholm conference, the limitation of content involving the biological variability of analytes such as HIV, HCV and HBV in infected individuals is understandable. The MS, through the Unidade de Assistência e Laboratório da Coordenação Nacional de DST e Aids, Assistance and Laboratory Unit of the National STD and AIDS Coordination, in english, recommended, in a technical note from 1999, as a significant analytical variation for the viral load assay for HIV the value of 0.5 Log10 between measurements of the same patient, that is, a precision parameter [24]. The use of data from the KIT manufacturer, on the other hand, supports the specification of the selected model 5, due

to the fact that it has mastery of the production technique and monitoring of the quality of control KITs and the performance of the equipment itself. Thus, the "optimal" results for HBV and HCV, and "Desirable" for HIV are corroborated by mathematical projections based on the manufacturer's data; and the automation of the equipment, which allows for the minimization of variation in the analytical phase, arising from the operator, instrumentation analytical and related interferences. The EQC can also be evaluated according to the state of the art, just like the IQC, based on the results available in each round by the proficiency test advisory group. However, unlike the IQC, there are a range of factors that disadvantage the model with regard to quantitative assessment, including the number of participants in each round, which directly interfere with the predictive value of random or systematic error.Thus, it was not possible to establish a single model for the critical assessment of systematic error. It was observed, however, that the results obtained during the rounds, for the three analytes evaluated, presented values slightly below the group average, suggesting trends. There is no clear specification regarding the total allowable error for testing HIV, HCV and HBV. This supports the limitation of model 2 parameters, in addition to the scarcity of information that can adapt to the requirements of model 1, making it therefore necessary to resort to model 5, with more restrictions in the evaluation process. To monitor the significance of the total error, reflecting systematic and random error, mathematical simulations were carried out that considered the LACEN result in each round of the proficiency test and the variation in the result, resulting from the calculated total error. This value was compared with the significant clinical variation proposed by the MS. From the data obtained in Table 7, it is possible to verify that the total error measured in the study, when applied to the results obtained, did not demonstrate variations, which in the clinical environment would be considered significant. This corroborates the stability of the analytical process, recommended by current legislation. It is important to note that during the monitored period, there were no rule violation infractions or operational problems. The present study made it possible to determine random, systematic and total error. In order to be able to indicate the analytical performance of the RT-PCR methodology for HIV and viral hepatitis at LACEN-CE. The limitation of biological variability data and clinical studies that provide parameters for clinical laboratories and especially for public health laboratories to use as specifications, demonstrates the relevance of the current initiative. The achievement of "optimal" and "desirable" performance for the monitored analytes indicates the quality with which the processes are evaluated and monitored by LACEN-CE, as well as confirmation that the total error values do not imply clinically significant results. With access to molecular biology technologies by clinical laboratories, it becomes increasingly necessary to pay attention to the data and elements available to manage methodologies and ensure the quality of results released by laboratories.

Disclosure Statement

I, Gabriel Thé Araújo Gomes, the primary author of this manuscript, certify that there are no conflicts of interest or impediments that could influence the results or interpretations presented in this work. I declare that: I have no financial affiliations with organizations or entities that may have a direct or indirect interest in the content of this manuscript. I have not received funding or financial support from any entity that could influence the conduct or presentation of this work. I have no financial interests, patents, or stock holdings in companies related to the topic addressed in this manuscript. This disclosure statement is provided in good faith and reflects complete transparency regarding potential conflicts of interest.

Declaration of Helsinki

The present work did not use information, samples or biological material from patients treated by the Laboratório Central de Saúde Pública do Estado do Ceará. All scientific research was carried out using commercial internal quality control kits.

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