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Homogeneity study of the internal quality control sera for immunodiagnosis of HIV/AIDS

Estudo de homogeneidade dos soros utilizados em controle de qualidade interno de testes imunodiagnósticos de HIV/Aids

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ABSTRACT

Introduction: The present study reports the data from the first homogeneity assessment of samples composing the serum panels produced at the Immunology Center of Instituto Adolfo Lutz, São Paulo. These samples have been distributed to the public laboratories and those partaking in the Brazilian Unified Health System, and to the participants in the Internal Quality Control Program for human immunodeficiency virus (HIV) antibody (Ab) testing. **Objective:** To assess the homogeneity of serum samples in panels from different lots for HIV/acquired immunodeficiency syndrome (AIDS) immunodiagnosis by using the statistical method to ensure quality of the reference material. **Method:** Sera homogeneity was evaluated by means of enzyme-linked immunoassay/enzyme immunoassay (ELISA/EIA) for detection of HIV Ab, and the one-way analysis of variance was employed for analyzing the data. No statistically significant differences were found among the several serum vials. **Conclusion:** The sera dispensed in the vials were homogeneous in the respective lots.

Key words: reference material; HIV; quality control; immunoenzyme techniques.

INTRODUCTION

The laboratory area has been stimulated to implement the Analytical Quality Assurance Programs, which offer to laboratories the possibility of demonstrating the technical competence and the capacity to produce reliable and traceable results[3].

The daily determination of different analytes in several matrices is performed in clinical laboratories. Quality control for these determinations requires the use of reference materials, not only those certified, but samples used in the quality assessment of routine assays[16].

Laboratory test results help the clinicians to make the diagnosis and decisions as to treatment; therefore, the reliability of these tests is extremely important for health care[10].

The establishment of the internal quality control (IQC) for the serological diagnosis of HIV infection, besides being a parameter to validate a serological reaction, it confers advantages as the quality improvement of the obtained results, the identification of lot-to-lot variations of the diagnostic reagent kits, and the detection of random errors during the test running.

Instituto Adolfo Lutz (IAL), the Central Laboratory of Public Health linked to the Coordination for Diseases Control – Secretary of Health of São Paulo State (CDC/SSH-SP) – has been working on these actions to be introduced by the laboratories of State sub-network of the state in their respective units, by means of workshops, training, production of a technical manual and related actions[15].

In 2009, in a pioneering and innovative action, the Immunology Center of IAL began the production and free

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distribution of serum panel constituted by HIV-negative and HIV-positive samples. These have been the reference material for preparing IQC in anti-human immunodeficiency virus (HIV) serological tests (IQC HIV) by laboratories of the sub-network of São Paulo State, enrolled in the Internal Quality Control Program coordinated by IAL[7].

With the production of the reference material, the Central IAL has followed a group of measures to verify and to ensure the quality of the produced lots of serum panels, according to those established by The International Organization for Standardization (ISO) Guide 34 and ISO Guia 35 of Associação Brasileira de Normas Técnicas (ABNT)[6, 11, 12].

The reference material production comprises several activities, such as processing and fractionation of input, packing and choice of measuring methods. After preparation, it is necessary to ensure the sample homogeneity in each produced lot, so that the values obtained from measurement in some units, randomly chosen, are valid for the other units in the same lot[17].

The homogeneity study of a certain material is one of the main factors for assured maintenance of its physical-chemical properties. Even when the material is expected to be homogeneous, it is crucial to analyze the homogeneity variation among vials in a lot, as in the case of solutions. On that account, some tests have to be performed to ensure that no failure in sample processing and packing has occurred; therefore, to guarantee that the samples of a certain lot are sufficiently homogeneous among them[6].

The employment of homogeneity tests in a lot is consisted of defining the sample variations among the adequately packed vials in a certain lot[8]. Assuring homogeneity is a specialized task that requires the use of proper statistical methods[17].

OBJECTIVE

The objective of this work was to assess the homogeneity in serum samples from panels of different lots for HIV/acquired immunodeficiency syndrome (AIDS) immunodiagnosis by means of the statistical method to ensure the quality of the reference material produced at IAL.

MATERIAL AND METHOD

For the production of serum to prepare IQC HIV panels, a standard operational procedure (SOP) was designed to process the material. The following steps were established: conversion of plasma (plasma bag) into serum; characterization of serum as to the presence of specific anti-HIV antibodies in different serological assays; conduction of sterility and fractionation procedures on sera to compose the different lots.

Plasma processing

Four plasma bags with negative serology result for the markers established in hemotherapic procedures were used, whose data were provided by the blood donors screening[1], besides three HIV-positive plasma bags. Plasma bags were stored at -20°C until being transformed into serum. In order to ensure the sample tracking, all information received on the plasma bags were recorded in a data bank[15]. The process of turning the plasma into serum was carried out by means of the thrombin technique, according to the methodology recommended by the World Health Organization (WHO), with modifications[18].

Serum characterization

Serum characterization was performed by using different diagnostic test kits for detecting anti-HIV antibodies, by means of enzyme-linked immunoassay/enzyme immunoassay (ELISA/EIA), Western blot and indirect immunofluorescence for HIV, routinely used in the HIV/AIDS Immunology Center of IAL.

Identification of serum sample lots

Each serum lot, positive (strongly reactive) and negative, was numbered according to the sequential enumeration established for serum panels produced at the HIV/AIDS Laboratory of the Immunology Center. For the weakly reactive lots (low antibody titers), called IQC, the positive sera numbering was used. Thus, the following lots were set up: (a) two lots of HIV-positive (strongly reactive) serum samples, identified by numbers 067 and 075 (part of serum 067 was used to prepare a weakly reactive sample); (b) two lots of HIV-positive serum samples numbers 061 and 067, which were diluted in negative sera (064 and 062), respectively, to prepare IQC; (c) two lots of negative serum samples, identified by numbers 063 and 065.

Preparation of HIV-positive (weakly reactive) serum samples

The ideal dilution for anti-HIV antibodies in serum samples was established as recommended in the technical manual for preparing the positive IQC to ELISA/EIA[15] assay, routinely employed at the HIV/AIDS Laboratory of IAL.

To establish the ideal dilution of each sample, the positive sera lots 061 and 067 were diluted in series, respectively, with negative sera 064 and 062, respectively. Each sample dilution was tested in ELISA/EIA, Vironostika HIV Uni-Form Plus O – Biomérieux, and the best dilution to be used as IQC was that whose value of optical density (OD) ranged from 1.5 to 4.5 times the cut-off (CO) value or the test cut-off[15].

Serum fractionation

Serum panels were prepared following the standard operational procedures (SOP) of the HIV/AIDS Laboratory of IAL[7, 15] and in accordance with the Good Laboratory Practices for the fractionation of samples in serum aliquots, product packing and labeling procedures[13, 14].

Before fractionation, sera were homogenized in a tilting shaker (rocker type) during 120 minutes. Next, the samples were dispensed in tubes suitable for freezing (cryotubes), which were labeled, numbered and stored in freezer at -20°C in cryogenic storage boxes.

Tube selection for the homogeneity study

In order to select the subset of serum tubes to study the homogeneity among samples of vials from different lots, the method of random sampling was applied, and using the formula established in program Microsoft Office Excel (Microsoft Corp., Redmont, WA, USA), as described in SOP for the homogeneity test for panel serum samples. For conducting the homogeneity study among vials containing sera, the number of serum sample tubes was established according to the lot size (tube numbers obtained after packing), as recommended by ABNT ISO Guia 35[6]. Sixteen vials of each lot of negative serum 063 and 065, and of positive serum 067 and 075, as well as 12 vials of each serum lot IQC 061 (diluted at 1:60,000) and IQC 067 (diluted at 1:20,000) were selected.

Homogeneity test

The development and the implementation of homogeneity evaluation of serum samples were performed according to ABNT ISO Guia 35[6]. In view of the available procedures for this sort of evaluation are often employed in chemical analytical assays, it was necessary to propose an adequate model for biological trials.

Sample homogeneity was assessed by checking variations in the analyte contents (presence or absence of anti-HIV antibodies) by ELISA/EIA, where the variability may be estimated by the optical density values of the reaction. The ELISA Vironostika HIV Uni-Form Plus O – Biomérieux (lot A41EY) diagnostic kits and the following equipment Organon Teknika Biomérieux were used: Incubator 5000, Microwell System Washer 400, and microplate Reader 230.

Homogeneity assays were carried out after bottling. Serum samples of the selected tubes of each lot were analyzed in triplicates and in a single assay, for testing in repeatability conditions. The sample order during the assay was chosen at random.

Aiming at determining whether the variability of the metrological process of serum sample homogeneity would be significant before the variability of the employed immunoenzymatic methodology[4, 5], 20 consecutive assays were carried out by ELISA/EIA, what represented 100 determinations in each of the sera of IQC HIV lots 067 and 063.

In the evaluation of the homogeneity test results, the one-way analysis of variance was used (One-Way ANOVA)[2, 6, 9], seeing that this method is statistically robust and reliable[17] and to verify whether the variation in serum sample composition would be non-significant for the proposed objective. Data from the analysis of variance provided the standard uncertainty due to the serum homogeneity, which was considered uncertainty between bottles (Ubb).

This study was approved by the Ethics Committee for Research in Human Beings of IAL (nº. 046/2010).

RESULTS

In order to apply the statistical method of analysis of variance in the results obtained in ELISA/EIA, a spreadsheet was created containing the data from ratio OD/CO, that is, the ratio between the optical density (OD) and the cut off (CO) values, besides the mean and the relative standard deviation (RSD) of triplicates in each serum lot, as presented in **Table 1**.

Samples of HIV-positive serum (strongly reactive) lots 067 and 075 showed results above the maximum value of detection (OD > 3,000) of ELISA plate reader; thereby, for these samples the analysis of variance was not feasible. However, results found in weakly reactive HIV-positive serum samples (IQC 061 and 067) were the most suitable to assess the homogeneity in different tubes, for they demonstrated OD/CO ratio values from 1.5 to 4.5 times the cut-off value.

The homogeneity tests in anti-HIV antibodies negative sera (lots 063 and 065) were carried out to assess whether the material kept the conditions of uniformity of structure or composition regarding the studied property.

Homogeneity assessment

Data analysis of variance was performed to assess the occurrence of significant differences in analyte contents, that is,

TABLE 1 – Results expressed by ratio OD/OC at immunoenzymatic assay (ELISA/EIA) in samples from vials selected at different serum lots

HIV-negative sera									HIV-positive sera								
	065				063					IQC 061				IQC 067			
Vials	1	2	3	Mean	1	2	3	Mean	Vials	1	2	3	Mean	1	2	3	Mean
1	0.36	0.55	0.46	0.46	0.38	0.43	0.38	0.39	1	2.51	2.38	2.26	2.38	2.41	2.39	2.34	2.38
2	0.39	0.38	0.48	0.42	0.32	0.38	0.36	0.35	2	2.6	2.28	2.07	2.32	2.33	2.25	2.28	2.28
3	0.39	0.49	0.5	0.46	0.34	0.42	0.43	0.4	3	2.55	2.41	2.07	2.35	2.26	2.44	2.22	2.3
4	0.54	0.4	0.45	0.46	0.33	0.35	0.4	0.36	4	2.48	2.26	1.97	2.24	2.42	2.5	2.27	2.4
5	0.39	0.42	0.42	0.41	0.3	0.38	0.42	0.37	5	2.36	2.14	2.24	2.25	2.55	2.25	2.33	2.38
6	0.37	0.41	0.41	0.4	0.34	0.34	0.4	0.36	6	2.27	2.32	2.45	2.34	2.33	2.42	2.26	2.33
7	0.35	0.33	0.4	0.36	0.41	0.35	0.36	0.37	7	2.23	2.29	2.15	2.22	2.43	2.44	2.49	2.45
8	0.62	0.38	0.46	0.49	0.34	0.45	0.35	0.38	8	2.5	2.26	2.15	2.31	2.44	2.48	2.2	2.37
9	0.35	0.44	0.4	0.4	0.39	0.35	0.39	0.38	9	2.3	2.09	2.05	2.15	2.38	2.42	2.22	2.34
10	0.36	0.37	0.36	0.36	0.34	0.31	0.39	0.35	10	2.37	2.29	2.18	2.28	2.48	2.4	2.3	2.39
11	0.4	0.48	0.45	0.44	0.31	0.33	0.38	0.34	11	2.25	2.43	2.08	2.25	2.41	2.27	2.15	2.28
12	0.35	0.31	0.45	0.37	0.32	0.36	0.43	0.37	12	2.13	2.24	2.15	2.17	2.6	2.27	2.35	2.4
13	0.32	0.36	0.4	0.36	0.33	0.34	0.41	0.36	–	–	–	–	–	–	–	–	–
14	0.36	0.37	0.43	0.39	0.28	0.36	0.42	0.36	–	–	–	–	–	–	–	–	–
15	0.36	0.39	0.46	0.41	0.34	0.32	0.41	0.35	–	–	–	–	–	–	–	–	–
16	0.38	0.45	0.44	0.42	0.3	0.34	0.44	0.36	–								
Overall mean				0.41				0.37					2.27				2.36
Relative standard deviation (RSD)				9.63%				4.32%					3.15%				2.23%

IQC: internal quality control.

of specific antibodies in sera after vial bottling; also to estimate the standard uncertainty associated with homogeneity. The results of this evaluation for assessing the lot-to-lot and within-lot homogeneity are presented in **Table 2**.

From the results obtained by the analysis of variance, it was observed that in each lot the value of $F_{calculated}$ was lower than that of $F_{critical}$, being p-value > 0.05. These data indicate that there were no statistically significant differences were found among the results obtained for the diverse vials. Therefore, it might consider that vial samples were homogeneous among them in their respective lots.

Assessment of uncertainty associated with homogeneity

From the results of analysis of variance (Table 2), repeatability standard deviation (SR) was estimated, as well as the SD of homogeneity between vials (Sbb), standard uncertainty associated to homogeneity between vials (Ubb) and the relative standard uncertainty associated with the mean (Ubb %), expressed in **Table 3**.

TABLE 2 – Analysis of variance (One-Way ANOVA) for the homogeneity study between serum vials in the different lots

Lot number	Variation source	Degrees of freedom (df)	Quadratic mean (QM)	$F_{calculated}$	p-value	$F_{critical}$
065	Between vials	15	0.0048	1.381	0.215	1.992
	Within vials	32	0.0034	–	–	–
063	Between vials	15	0.00075	0.326	0.988	1.992
	Within vials	32	0.0023	–	–	–
IQC 061	Between vials	11	0.015	0.556	0.845	2.216
	Within vials	24	0.028	–	–	–
IQC 067	Between vials	11	0.0083	0.662	0.759	2.216
	Within vials	24	0.013	–	–	–

IQC: internal quality control.

TABLE 3 – Estimate of standard uncertainty associated with serum homogeneity between vials

Lot number	Overall mean	SR	Sbb	Ubb	Ubb (%)
065	0.41	0.059	0.021	0.021	5.1
063	0.37	0.048	0	0.014	3.8
IQC 061	2.27	0.166	0	0.052	2.3
IQC 067	2.36	0.112	0	0.035	1.5

SR: repeatability standard deviation; Sbb: standard deviation of homogeneity between vials; Ubb: standard uncertainty associated to homogeneity between vials.

The quadratic mean between groups (QM between) for lot 065 was higher than the quadratic mean within the groups (QM within). In this case, Sbb might be used as an estimate for Ubb, and be calculated by equation 1.

Equation 1: Ubb = Sbb =
(QM between – QM within/n) ^ 0,5

For sera of lots 063, IQC 061 and IQC 067, the Sbb values were interpreted as null, because they presented values of the quadratic mean among groups (QM between) lower than those of the quadratic mean within the groups (QM within). Thus, The Sbb values were lower than SR values. As the method repeatability was more significant than variation among samples, the contribution of variance within vials was considered; as a result, the standard uncertainty associated with homogeneity was estimated according to equation 2.

Equation 2: Ubb =
(QM within/n) ^ 0.5 * (2/df QM within) ^ 0.25

Where n represents the number of replicates of the vial replicates (sample); df, the degree of freedom; and QM within, the quadratic mean within the groups.

Variability in the metrological process originated from the non-homogeneity of serum samples in relation to the variability of the employed immunoenzymatic methodology was assessed by means of the analysis of quality control graphs, which indicated that the variability of the employed metrological process is of the order of 25%. Thus, the values of relative standard uncertainty associated with the mean were low for serum lots, according to data presented in **Table 4**.

These data demonstrated that uncertainty of homogeneity among serum vials was adequate for the desired aims. Errors associated with measurement result did not alter the result of anti-HIV antibodies detection for the used assay.

TABLE 4 – EValues of relative standard uncertainty associated with the mean for serum lots

Lot number	0.65	063	IQC 061	IQC 067
Ubb (%)	5.1	3.8	2.3	1.5

IQC: internal quality control; Ubb: standard uncertainty associated to homogeneity between vials.

DISCUSSION

Homogeneity study is essential to confirm and to detect possible flaws in the preparation of the reference material, which helps the producer to assess the quality of the product to be at hand. Besides, it is one of the main requirements for certification award. It has been a difficult process which demands a significant quantity of testing and relatively large volumes of sera, but it has been a fundamental step to assure quality and reliability of the produced material.

As the analytical laboratories need to demonstrate the reliability in their result analysis, the producers of reference material ought to assure that their products meet a series of quality requirements by implementing adequate quality systems. In this context, the quality assessment of the products offered to laboratories should not be limited to homogeneity, but to all of the activities that may affect the product quality.

Scientific articles reported by other investigators[8, 17] have directed to the public who works on chemistry area, where the different measurements used in chemical analytical assays are published. The methods used by those authors were adequate and in conformity to help in standardizing and developing the conduction of homogeneity tests in the biological samples assessed in this study.

In practice, the confirmation of sera homogeneity is an extremely relevant property for the clinical area regarding the production of reference material of excellent quality in the Immunology Center of IAL. The use of such reference material implies the obtainment of reproducible results. Consequently, the IQC HIV used by laboratories participating in the Program of Internal Quality Control will improve the conduction and the release of anti-HIV exams with reliable results.

In search for improving and to assure the highest stability of serum samples during transportation and storing, the next challenge for the HIV/AIDS Laboratory will be the use of serum samples preserved by freezing-drying process.

CONCLUSION

This paper reports the correct and indispensable conduct of methodologies for monitoring the metrological quality of

measuring analyte in serum samples composing panels for HIV/AIDS immunodiagnosis. These data on the sample homogeneity indicate the appropriate quality of the reference material produced in the Immunology Center of IAL and distributed to the laboratories network that participate in the Program of Internal Quality Control, aiming at the improvement of HIV/AIDS serological diagnosis in the state of São Paulo.

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RESUMO

Introdução: *No presente estudo estão descritos os resultados das primeiras análises feitas sobre a avaliação da homogeneidade das amostras componentes de painéis de soros produzidos no Centro de Imunologia do Instituto Adolfo Lutz e distribuídos aos laboratórios públicos e conveniados ao Sistema Único de Saúde e participantes do Programa de Controle de Qualidade Interno para imunodiagnóstico de vírus da imunodeficiência humana/síndrome da imunodeficiência adquirida (HIV/AIDS).* ***Objetivo:*** *Avaliar a homogeneidade das amostras de soro componentes de painéis de diferentes lotes para imunodiagnóstico de HIV/Aids por meio de método estatístico para garantir a qualidade do material de referência.* ***Material e método:*** *A homogeneidade das amostras de soro foi avaliada por meio de enzyme-linked immunoassay/enzyme immunoassay (ELISA/EIA) para detecção de anticorpos anti-HIV, e os resultados foram submetidos à análise de variância fator único. Não foram encontradas diferenças significativas entre os resultados obtidos para os diversos frascos de soro.* ***Conclusão:*** *As amostras distribuídas nos frascos foram homogêneas entre si nos respectivos lotes.*

Unitermos: *material de referência; HIV; controle de qualidade; técnicas imunoenzimáticas.*

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