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Effect of multiple freeze-thaw cycles on the stability of positive anti-treponemal serum samples

Efeito dos múltiplos ciclos de congelamento e descongelamento na estabilidade de amostras de soro antitreponêmico positivo

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ABSTRACT

Introduction: Biological samples have long been used in multiple laboratory investigations, and this procedure has been an issue of concern, as the samples are submitted to repeated freeze-thaw cycles, which may affect the results of a particular immunodiagnostic assay, due to the occurrence of physical damage to the antibody of interest. **Objective:** This study aimed at investigating the impact of successive freeze-thaw cycles on the stability of serum samples stored at -20ºC regarding the reactivity of anti-treponemal antibodies. **Methods:** At the Immunology Center-Instituto Adolfo Lutz (IAL), the analyzed serum samples analyzed were prepared and established as reference material for anti-treponemal immunodiagnostic assays. Sera stability was evaluated by chemiluminescence assay in samples submitted to 25 successive freeze-thaw cycles, ranging from 6 to 174 cycles. **Results:** Neither statistically significant effect on the reactivity of anti-treponemal antibodies (p-value > 0.05), nor adverse effect were observed in weakly reactive samples, such as the occurrence of false-negative results. **Conclusion:** It was shown that 174 freeze-thaw cycles of anti-treponemal sera did not affect the stability and the quality of samples, when evaluated by chemiluminescence assay.

Key words: serum; antibodies; *Treponema pallidum*; quality control; reactivity-stability.

INTRODUCTION

The effects of repeated freeze-thaw (F/T) cycles directly on the concentrations of several analytes in serum or plasma samples were of minor interest until the expansion of repository banks at the end of the 20th century[1]. Although the need for repeated F/T cycles in serum samples may be minimized by storing fractionated specimens in several small containers, it is often necessary to use that one particular serum which has already undergone one or more F/T procedures. When this occurs, reviewers of papers or manuscripts protocols may challenge the reliability of the data analysis obtained from these samples[1, 2].

In practice, it is possible to reanalyze the stored biological samples to confirm the results previously obtained or to perform further investigations. However, the stability of the analytes must be ensured before issuing the detected results or establishing further investigations[3, 4]. It is imperative to develop the categorical studies to ensure that the applied procedure applied can support the use of stored samples, especially in sensitive immunodiagnostic assays, which detect protein structures (antibodies) susceptible to denaturation. It is a concern that repeated F/T cycles may affect the final result of a given assay, based on the sample showing physical damage and consequent biochemical alteration of the antibodies of interest[2, 5, 6]. Although this procedure is a common practice, the data availability of the data on the effects of multiple F/T cycles on antibody integrity is limiting[7].

Depending on the class of analytes, the universal recommendation for serum treatment is crucial the minimum number of F/T cycles to avoid significant reduction in sensitivity. Antibodies and immunoglobulins have been described as stable components at -20ºC for long periods of time[6]. However, it is necessary to be aware of the F/T cycles in serum samples of each antibody class and in each type of assay used, to ensure that the results achieved in prospective studies are reliable[2, 8].

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Castejon *et al*. (2012)[2] evaluated the effect of 60 consecutive F/T cycles on serum stability for detecting the human immunodeficiency virus (HIV) antibodies. The study showed that there was neither loss of reactivity of specific antibodies in the analyzed samples, nor the occurrence of false positive results in negative samples, when analyzed by different serological methods [enzyme-linked immunosorbent assay (ELISA)/ immunoenzymatic assay (EIA), indirect immunofluorescence and Western blot].

Castro *et al*. (2013)[6] showed that sera submitted to 10 F/T cycles or less of being suitable for performing the serological analysis without significant loss of sensitivity in the immunoenzymatic assay for syphilis.

OBJECTIVE

This study aimed at evaluating the impact of the successive F/T cycles of serum samples, standardized as the reference material with the relative stability of anti-treponemal antibody reactivity, using a chemiluminescence assay.

METHODS

The analyzed serum samples analyzed were prepared and established as reference materials[9-12] to be included as internal quality control (IQC) when performing the treponemal serological tests in the diagnostic laboratories or in conducting researches.

In the present study, the chemiluminescence assay –Advia Centaur-Syphilis (Siemens Healthcare Diagnostics, Inc, NY, USA) was used in the Laboratory of Syphilis-Centro de Imunologia do Instituto Adolfo Lutz (CIM-IAL).

For conducting this study were produced four batches of anti-treponemal positive IQC sera [P01N176 (dilution 1:1000), P03N177 (dilution 1:100), P04N178 (dilution 1:100) and P06N180 (dilution 1:300)]; and one batch of negative serum (batch 165) was produced.

The mentioned batches of sera mentioned were prepared and then stored in a freezer at -20ºC for a short time, around three weeks. Immediately after storage, 25 vials of serum were selected from each batch to be submitted to the F/T process (F/T serum) and one vial as the reference sample (RS), which remained stored in a freezer at -20ºC. The F/T serum samples were taken from the freezer and they were placed at room temperature until completely thawed, and then frozen again. F/T procedure was repeated until the following numbers of cycles were completed: 6-13-20-27-34-40-48-55-62-69-73-80-89-97-104-111-117-125-132-140-146-153-160-167-174. Serum samples were grouped and identified according to the number of cycles and stored in the freezer at -20ºC until the assay be performed.

In order to avoid the effects of any variations that might occur in the laboratory daily routine, the stability analyses of the samples of different F/T and of the RS cycles were performed under the same conditions used for the anti-treponemal IQC sera.

Stability was assessed by analyzing the presence or absence of analyte reactivity variations (antibody positivity or negativity) by chemiluminescence assay – Advia Centaur-Syphilis – for detecting anti-*T. pallidum* antibodies, by following the procedures recommended by the manufacturer of the respective set of immunodiagnostic reagents. The samples of each cycle of F/T and RS were calculated by using the means from three determinations results.

The results of the chemiluminescent assay were expressed by the index value, and the sample was considered reagent when value was ≥ 1.0 and the value of < 1.0 as negative. These results were evaluated by simple linear regression analysis, using STATISTICA version 11, together with analysis of single-factor of variance (One-Way Anova)[13-15], using the Microsoft Office Excel Program.

This study was approved by the IAL – Human Research Ethics Committee (CAAE: 57496516.7.0000.0059).

RESULTS

According to the procedures established for preparing the reference material in the CIM-IAL, the batches of sera produced for this study were previously characterized for analyzing their reactivity, by using different sets of diagnostic tests for the detection of treponemal and non-treponemal antibodies.

In chemiluminescent testing from the Advia Centaur diagnostic reagent set, the positive anti-treponemal antibodies serum sample was diluted in accordance with the technical guide[9]; thus, the ideal dilution of positive IQC corresponded to the index value between 1.5-4.5 times the cut-off value of the test (equal to 1.0). The process for evaluating the homogeneity of serum samples was carried out in accordance with the ABNT ISO Guia 35[15].

The results detected in RS were used as reference parameter for evaluating the adverse effects on the antibody reactivity in sera submitted to multiple F/T processes.

Aiming at applying the linear regression analysis to the results found in chemiluminescence assay, a table was drawn up including the following data: the mean values of results detected in three determinations of F/T samples in different cycles of each batch of anti-treponemal IQC and in the negative serum (batch 165). Also the values of their respective RS stored at -20ºC were included (**Table 1**).

The results obtained from anti-treponemal IQC samples (P01N176, P03N177, P04N178, P06N180) showed index values between 1.5-4.5 times the cut-off value. The coefficient of variation (CV) mean calculated in each batch was, respectively 1.1%, 1.4%, 1.1% and 0.9%, which did not represent significant variations in sera results even after multiple F/T cycles (174 cycles).

The negative control serum (batch 165) showed no change in reactivity after being subjected to multiple F/T cycles, which was evidenced by the non-occurrence of false positive reactions in the specific antibody detection assays.

Table 2 shows the results of the regression analysis of mean values of three determinations of samples submitted to 174 F/T cycles and of the RS, according to the anti-treponemal IQC batch (P01N176, P03N177, P04N178 and P06N180).

Since the anti-treponemal IQC batches presented *p*-value higher than 0.05, it could be concluded that there was no significant difference among the values; therefore, the material was considered stable even after 174 F/T cycles. For the negative sample 165, included in the stability analysis, the *p*-value was lower than 0.05, which demonstrated the occurrence of a statistically significant difference among these values. However, the interpretation of the laboratory result was not changed, remaining as a "non-reagent sample", and being in accordance with the acceptance criteria established by the manufacturer of the used diagnostic set. Therefore, the sera were considered non-reactive because they showed a relation value index of < 1.00. In addition, this serum was also analyzed by the fluorescent treponemal antibody absorption (FTA-Abs) technique, which confirmed the negativity of sample.

TABLE 1 – Results expressed by the index value, in the chemiluminescence assay – Advia Centaur-Syphilis –, calculated by mean of the values of three determinations in RS and F/T serum samples

Serum	RS	F/T																								
		6	13	20	27	34	40	48	55	62	69	73	80	89	97	104	111	117	125	132	140	146	153	160	167	174
Anti-treponemal																										
P01N176	2.66	2.7	2.61	2.69	2.67	2.65	2.67	2.69	2.63	2.69	2.66	2.67	2.68	2.66	2.64	2.64	2.63	2.66	2.67	2.66	2.64	2.64	2.79	2.66	2.64	2.64
P03N177	2.99	2.99	3.00	3.04	3.02	3.04	3.07	3.04	3.00	3.00	3.00	3.03	3.07	2.96	2.97	2.99	2.98	2.95	2.95	3.14	3.01	3.05	3.1	3.04	2.95	3.06
P04N178	3.59	3.65	3.63	3.6	3.64	3.58	3.66	3.74	3.74	3.72	3.83	3.84	3.91	3.91	3.57	3.58	3.59	3.62	3.67	3.73	3.66	3.58	3.81	3.73	3.61	3.67
P06N180	2.73	2.81	2.84	2.69	2.73	2.75	2.64	2.73	2.72	2.71	2.67	2.69	2.74	2.67	2.64	2.65	2.66	2.68	2.66	2.72	2.69	2.67	2.86	2.71	2.68	2.71
Negative																										
165	0.1	0.11	0.06	0.06	0.07	0.06	0.07	0.08	0.08	0.09	0.09	0.12	0.11	0.1	0.12	0.11	0.12	0.12	0.12	0.12	0.11	0.12	0.12	0.11	0.11	0.11

RS: reference sample; F/T: freeze/thaw.

TABLE 2 – Results of the simple linear regression analysis applied to the mean values of the three determinations of serum from different batches of anti-treponemal IQC submitted to the 174 F/T cycles, with 95% of confidence interval

Regression analysis					
	P01N176	P03N177	P04N178	P06N180	165
Multiple R	0.0375	0.0559	0.0292	0.2487	0.7536
R-squared	0.0014	0.0031	0.0009	0.0618	0.568
Adjusted R-squared	-	-	-	0.021	0.5492
Standard error	0.0346	0.0495	0.1054	0.0561	0.0142

Anova																					
Degrees of freedom	Sum of squares					Mean square					F					*p*-value				
	P01N176	P03N177	P04N178	P06N180	165	P01N176	P03N177	P04N178	P06N180	165	P01N176	P03N177	P04N178	P06N180	165	P01N176	P03N177	P04N178	P06N180	165
Regression (1)	0.00004	0.0002	0.0002	0.0048	0.0061	0.00004	0.0002	0.0002	0.0048	0.0061	0.0324	0.0722	0.0196	1.5158	30.2382	0.8587	0.7905	0.8898	0.2307	0.000014
Residual (23)	0.0275	0.0563	0.2557	0.0723	0.0046	0.0012	0.0024	0.0111	0.0031	0.0002										
Total (24)	0.0275	0.0564	0.256	0.0771	0.0107															

Coefficients	Results					Standard error					Estatistics *t*					*p*-value				
	P01N176	P03N177	P04N178	P06N180	165	P01N176	P03N177	P04N178	P06N180	165	P01N176	P03N177	P04N178	P06N180	165	P01N176	P03N177	P04N178	P06N180	165
Intersection	2.6661	3.0139	3.6859	2.7335	0.071	0.0141	0.0202	0.0431	0.0229	0.0058	188.7762	149.2178	85.5993	119.3935	12.2446	0.0000	0.0000	0.0000	0.0000	0
Inclination	-0.00002	0.0001	0.0001	-0.0003	0.0003	0.0001	0.0002	0.0004	0.0002	0.0001	-0.18	0.2687	0.14	-1.2312	5.4989	0.8587	0.7905	0.8898	0.2307	0.000014

IQC: internal quality control; F/T: freeze/thaw.

Figures 1 to **5** show the relation of 95% confidence interval among the index values mean based on the F/T cycles respectively, for the anti-treponemal IQC batches – P01N176, P03N177, P04N178 and P06N180 – and negative sample (165), respectively.

In Figures 1 to 5, the regression lines from each batch were constructed to analyze the significant trend in the stability of positive or negative sera to anti-treponemal antibodies. The obtained slope was tested for significance by the *p*-value, and for the anti-treponemal IQC batch the regression was not significant and it did not present a trend (*p*-value > 0.05). Therefore, the mean values of the sera indexes did not change according to the cycles and they were

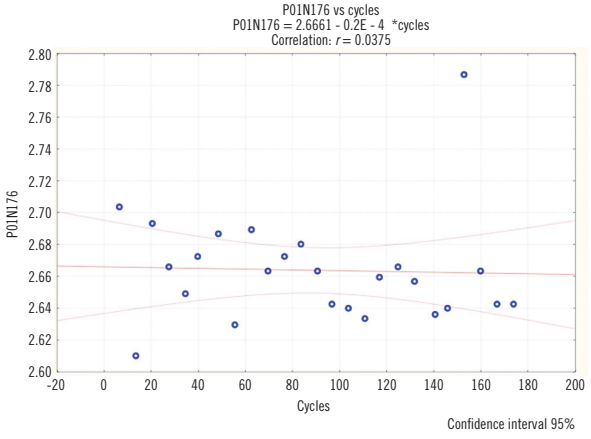

FIGURE 1 – *Linear regression of the IQC anti-treponemal P01N176 batch based on 174 cycles of F/T*

IQC: internal quality control; F/T: freeze/thaw.

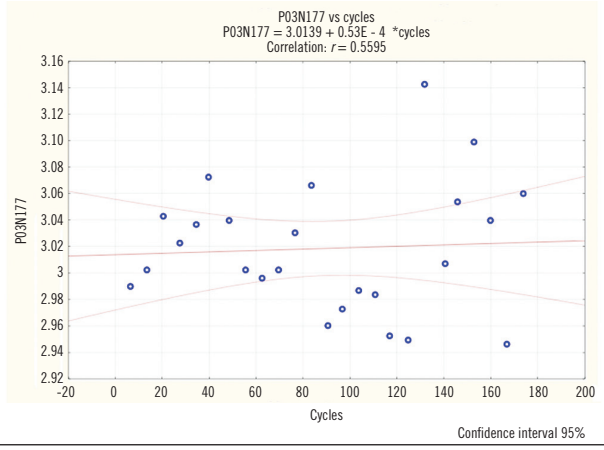

FIGURE 2 – *Linear regression of the IQC anti-treponemal P03N177 batch based on 174 cycles of F/T*

IQC: internal quality control; F/T: freeze/thaw.

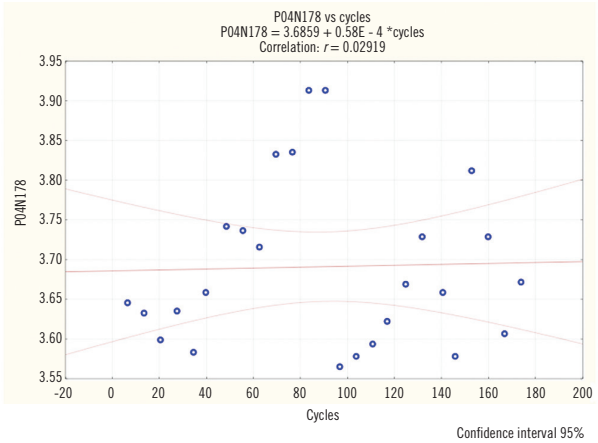

FIGURE 3 – *Linear regression of the IQC anti-treponemal P04N178 batch based on 174 cycles of F/T*

IQC: internal quality control; F/T: freeze/thaw.

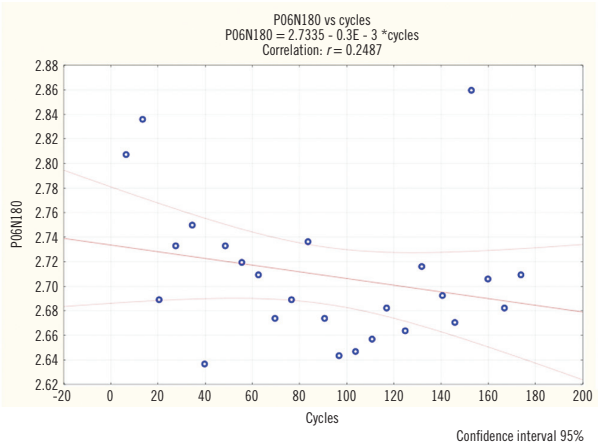

FIGURE 4 – *Linear regression of the IQC anti-treponemal P06N180 batch based on 174 cycles of F/T*

IQC: internal quality control; F/T: freeze/thaw.

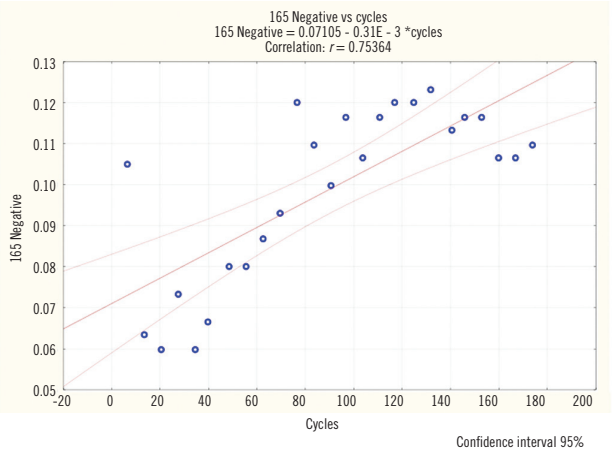

FIGURE 5 – *Linear regression of negative serum (165) batch based on 174 cycles of F/T*

considered stable with 95% confidence level. However, in the negative serum (batch 165), the regression line was significant and presented a trend (p-value < 0.05). But according to the results shown in Table 1 no change in reactivity was detected after multiple F/T cycles, which was evidenced by the absence of false positive reactions in chemiluminescence assay.

DISCUSSION

The study demonstrates that serum samples submitted to 174 F/T cycles do not show changes in the reactivity of anti-treponemal antibodies that could cause adverse effects in the results of the used chemiluminescence assay used. These data are important for researchers seeking information on the use of biological material in serological evaluations.

Despite the recommendation on the serum treatment in a minimum number of F/T cycles to avoid significant reduction in sensitivity, few studies are available on this matter[1, 5, 16]. Thus, the present study was designed to investigate the invariability of the reactivity characteristic of anti-treponemal antibodies in samples prepared for IQC. Since the IQC samples present low positive reactivity, any changes in their components are easily detected in the final test result. Hence, these samples are the tools to evaluate the integrity of the sera after having been submitted to multiple F/T cycles.

Controlling of the pre-analytical process is fundamental for the stored sera, as they can be used in the retrospective and prospective studies, or even to maximize the laboratory results information. One of the quality indicators to detect errors at this stage is the improper storage of biological samples[16]. It is worth pointing that appropriate care should be taken during handling and storing sera, as many other factors might induce protein degradation, such as storage time, microbial contamination, among others, which affect the final results of the studies. Therefore, it is recommended to store the biological material in small aliquots; however, this is not achievable for large studies[2, 3, 7].

The present study has some limitations. Firstly, the results of F/T cycles were based on samples stored for a short time (103 days). It would have been ideal to perform the study also in samples stored for longer periods in order to more thoroughly analyze the stability of sera concerning F/T cycles. Although the antibodies have been reported to remain stable at -20ºC for prolonged periods of storage[6, 17], comparing samples with different storage times and subjected to F/T cycles are an important data on the sera integrity for performing prospective investigations.

Another limiting factor was the failure in performing the immunoglobulin measure in samples before and after F/T and the antigen-antibody binding variance tests. This investigation was based on results detected in the used methodology, in which the applied F/T cycles applied did not interfere in the performance of the samples regarding the reactivity of specific antibodies. No false negative reactions and false positive results occurred, which demonstrates the stability of studied sera studied after performing successive F/T cycles.

In both biobanks design and use of stored sera, this study might be valuable in evaluating the integrity of samples, especially for those that have already undergone different laboratory tests. It is important to establish a quality assurance program to monitor the behavior of the stored samples[3]. Thus, knowledge regarding the stability of serum antibodies during repeated F/T cycles will enable the use to its maximum potential.

CONCLUSION

Based on the results found in the anti-treponemal antibody detection tests observed in the chemiluminescence assay and considering the conditions established in the present analysis, it can be concluded that the sera remained stable after successive F/T cycles.

Although further research is required to assess the stability of sera submitted to the F/T procedure, the present study showed that neither a change in the reactivity characteristic of anti-treponemal antibodies in weakly reactive sera, nor absence of expressive effect on the results of the analyzed samples were occurred.

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RESUMO

Introdução: *As amostras biológicas têm sido utilizadas em múltiplas investigações por um longo período de tempo, e existe a preocupação a respeito dos seus repetidos ciclos de congelamento e descongelamento que podem afetar os resultados de determinado ensaio imunodiagnóstico pela ocorrência de dano físico do anticorpo de interesse.* ***Objetivo:*** *O objetivo deste estudo foi investigar o impacto dos sucessivos ciclos de congelamento e descongelamento na estabilidade das amostras de soro armazenadas a -20ºC quanto à reatividade de anticorpos antitreponêmicos.* ***Métodos:*** *No Centro de Imunologia do Instituto Adolfo Lutz (IAL), as amostras de soro analisadas foram preparadas e estabelecidas como material de referência de teste imunodiagnóstico antitreponêmico. A estabilidade dos soros foi avaliada por meio de ensaio de quimioluminescência, em amostras submetidas a 25 sucessivos ciclos de congelamento e descongelamento, que variaram de 6 a 174 ciclos.* ***Resultados:*** *Não houve efeito estatisticamente significante na reatividade dos anticorpos antitreponêmicos (valor de* $p > 0,05$*), e nenhum efeito adverso foi observado nas amostras fracamente reagentes, como a ocorrência de resultados falso negativos.* ***Conclusão:*** *Foi demonstrado que os 174 ciclos de congelamento e descongelamento dos soros antitreponêmicos não afetaram a estabilidade e a qualidade das amostras, quando avaliados por meio do ensaio de quimioluminescência.*

Unitermos: *soro; anticorpos;* Treponema pallidum*; controle de qualidade; reatividade-estabilidade.*

REFERENCES

1. Comstock GW, Burke AE, Norkus EP, Gordon GB, Hoffman SC, Helzlsouer KJ. Effects of repeated freeze-thaw cycles on concentrations of cholesterol, micronutrients, and hormones in human plasma and serum. Clin Chem. 2001; 47(1): 139-42. PubMed PMID: 11148194.

2. Castejon MJ, Yamashiro R, Oliveira CC, Olivieri JC, Oliveira CAF, Ueda M. Avaliação dos múltiplos ciclos de congelamento e descongelamento na estabilidade dos soros para a detecção de anticorpos anti-HIV. Rev Inst Adolfo Lutz. 2012; 71(3): 573-81.

3. Paltiel L, Rønningen KS, Meltzer HM, Baker SV, Hoppin JA. Evaluation of freeze thaw cycles on stored plasma in the biobank of the Norwegian mother and child cohort study. Cell Preserv Technol. 2008; 6(3): 223-30. PubMed PMID: 20428472.

4. Cuhadar S, Koseoglu M, Atay A, Dirican A. The effect of storage time and freeze-thaw cycles on the stability of serum samples. Biochem Med. 2013; 23(1): 70-7. PubMed PMID: 23457767.

5. Pinsky NA, Huddleston JM, Jacobson RM, Wollan PC, Poland GA. Effect of multiple freezer-thaw cycles on detection of measles, mumps, and rubella virus antibodies. Clin Diagn Lab Immunol. 2003; 10(1): 19-21. PubMed PMID: 12522034.

6. Castro AR, Jost HA. Effect of multiple freeze and thaw cycles on the sensitivity of IgG and IgM immunoglobulins in the sera of patients with syphilis. Sex Transm Dis. 2013; 40(11): 870-1. PubMed PMID: 24113410.

7. Rivers JL, Jones J. Antibody titer variance due to freezing of patient specimens. TXSTUR. 2013; 1(2): 6-14.

8. Kronenberg F, Lobentanz EM, König P, Utermann G, Dieplinger H. Effect of sample storage on the measurement of lipoprotein[a], apolipoproteins B and A-IV, total and high density lipoprotein cholesterol and triglycerides. J Lipid Res. 1994; 35: 1318-28. PubMed PMID: 7964193.

9. São Paulo. Secretaria de Estado da Saúde. Coordenadoria de Controle de Doenças. Manual técnico para implementação do controle de qualidade interno nos procedimentos laboratoriais para diagnóstico sorológico da infecção pelo HIV no estado de São Paulo. São Paulo: IAL; 2007. [Accessed on: January 24, 2017]. Available at: bvsalud.org.

10. Castejon MJ, Yamashiro R, Oliveira CAF, et al. Implementação de controle de qualidade interno (CQI) nos ensaios sorológicos anti-HIV. Produção e distribuição de painéis de soro pelo Instituto Adolfo Lutz Central. Bepa. 2009; 6(65): 30-2.

11. Castejon MJ, Yamashiro R, Oliveira EL, Silveira EPR, Oliveira CAF. Manual do participante – controle de qualidade interno (CQI) em ensaios de imunoblot rápido HIV e de quimioluminescência antitreponêmico. 2016. [Accessed on: January 24, 2017]. Available at: http://ses.sp.bvs.br/lildbi/docsonline/get.php?id=6126.

12. Castejon MJ, Yamashiro R, Oliveira EL, Silveira EPR, Oliveira CAF. Instituto Adolfo Lutz inicia produção do controle de qualidade interno para diagnóstico sorológico de sífilis. Bepa. 2016; 13(150): 11-6.

13. Altman DG. Pratical statistics for medical research. Comparing groups – continuous data. Londres: Chapman & Hall; 1991. p. 179-320.

14. Ellison SLR, Barwick VJ, Farrant TJD. Pratical statistics for the analytical scientist – a bench guide. Analysis of variance. Cambridge: RSC Publishing; 2009. p. 48-113.

15. ABNT. Associação Brasileira de Normas Técnicas. ISO Guia 35: materiais de referência – princípios gerais e estatísticos para certificação. Brasil; 2012.

16. Demir M, Cevahir N, Kaleli İ, Bulus N, Ozansoy FA, Yiğit ÖD. Does multiple freezing and thawing cycles of serum affect the detection of anti-nuclear antibodies and anti-neutrophil cytoplasmic antibodies by indirect immunofluorescent method? Biomed Res. 2014; 25(4): 522-7.

17. Castejon MJ, Yamashiro R, Oliveira CC, Oliveira CAF, Ueda M. Stability of anti-HIV antibodies in serum samples stored for two to eighteen years periods. J Bras Patol Med Lab. 2014; 50(4): 272-7.

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