

Do multiple freezing and thawing cycles of serum samples affect antibodies used for leptospirosis diagnosis?

Vários ciclos de congelamento e descongelamento em amostras de soro afetam anticorpos para o diagnóstico de leptospirose?

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ABSTRACT

There are no data on the effect of multiple freezing and thawing (FT) cycles on antibodies in serum samples from patients with leptospirosis. This study aimed to evaluate the impact of repeated FT cycles on antibody stability in banked serum samples used for leptospirosis diagnosis by the Microscopic Agglutination Test (MAT) and the IgM ELISA. A total of 67 serum samples from patients with leptospirosis confirmed by MAT were analyzed. Group 1 included 10 samples subjected to 14 FT cycles, while Group 2 comprised 57 samples stored for up to 23 years at -20°C and thawed only once. No significant differences in antibody levels were observed between groups. Samples with MAT titers of 1:200 became undetectable after repeated cycles, but this did not compromise case interpretation. Regression analysis of IgM ELISA showed a declining trend across FT cycles. Positivity by both MAT and IgM ELISA was maintained for up to 40 FT cycles, except for one sample. These results suggest that antibodies remain stable, supporting the reuse of stored samples in research and laboratory validation studies for leptospirosis.

Keywords. Leptospirosis, Zoonoses, Freezing, Serum, Serology.

RESUMO

Não há dados sobre o efeito de múltiplos ciclos de congelamento e descongelamento (FT) em anticorpos em soros de pacientes com leptospirose. O objetivo deste estudo foi avaliar o impacto de ciclos repetidos de FT na estabilidade de anticorpos em soros armazenados para diagnóstico da leptospirose pelo teste de aglutinação microscópica (MAT) e pelo ELISA IgM. Foram analisadas 67 amostras de soro de pacientes com leptospirose confirmadas pelo MAT. O Grupo 1 foi composto por 10 amostras submetidas a 14 ciclos de FT, enquanto o Grupo 2 incluiu 57 amostras armazenadas por até 23 anos a -20°C e descongeladas apenas uma vez. Não foram observadas diferenças significativas entre os grupos. As amostras que apresentaram títulos de 1:200 no MAT tornaram-se indetectáveis, mas não comprometem a interpretação dos casos. A análise de regressão do ELISA IgM mostrou tendência de queda ao longo dos ciclos de FT. Estima-se que a positividade em ambos os testes seja mantida por até 40 ciclos, exceto em uma amostra. Esses resultados sugerem que os anticorpos permanecem estáveis, permitindo a reutilização de amostras em pesquisas e validações laboratoriais para leptospirose.

Palavras-chave. Leptospire, Zoonoses, Congelamento, Soro, Sorologia.

INTRODUCTION

Leptospirosis is a potentially fatal zoonosis, distributed globally, with a higher incidence in tropical countries. It is caused by *Leptospira* spp., which comprise 66 species and more than 300 serovars¹⁻⁴. Identifying leptospirosis is a diagnostic challenge because its manifestations vary from asymptomatic or mild flu-like cases to a severe fulminant disease presenting with jaundice, renal failure, pneumonia, hemorrhage and shock⁵⁻⁸. Laboratory diagnosis of leptospirosis is based on the detection of antibodies by serological methods, detection of leptospiral DNA by molecular tests, or isolation of the organism through culture methods⁹. Although several techniques have been developed, the microscopic agglutination test (MAT) is still used for leptospirosis diagnosis and for seroprevalence survey. The test is considered the reference serological test and detects both immunoglobulin M (IgM) and immunoglobulin G (IgG) class agglutinating antibodies. However, MAT requires a high level of technical expertise and the maintenance of a large panel of live pathogenic *Leptospira* spp. standard cultures. Agglutination examination by dark-field microscopy is subjective and depends on observer variation, even within laboratories⁹. Detection of immunoglobulin M (IgM) antibodies by enzyme-linked immunosorbent assay (ELISA) has been used widely as a screening test⁹.

Clinical samples are used in several laboratory investigations and generally are submitted to multiple freezing and thawing (FT) cycles, which may affect the stability of antibodies of interest. However, sometimes the use of such samples is unavoidable when it is necessary to reanalyze part or all of the serum to confirm previously obtained results or to perform further investigations. Since there is no data available regarding the effect of repeated FT on stored serum samples to the diagnosis of leptospirosis, the aim of our study was to investigate the stability for detecting the human leptospirosis antibodies detected by MAT and IgM ELISA and the stability of banked sera stored up to 23 years.

MATERIAL AND METHODS

Serum samples

A total of 67 serum samples from patients with leptospirosis confirmed by MAT were analyzed after undergoing the FT process. Of these, 10 serum samples were also diagnosed by IgM ELISA. The inclusion criteria were convalescent samples from laboratory confirmed cases of leptospirosis and titers above 1:200 by MAT. Serum samples were stored at -20 °C and divided into two groups: one group consisted of ten serum samples positive for MAT and IgM ELISA that underwent 14 cycles of freezing and thawing (Group 1). The other group comprised 57 banked sera stored from 1999 to 2017 frozen at -20 °C and thawed only once (Group 2). All serum samples were checked visually for hemolysis and lipemia to avoid possible interferences.

Ethics approval

This study was approved by the Ethics Committee of Instituto Adolfo Lutz (approval number 6.327.950) in accordance with the standards recommended by the National Research Ethics Commission (CONEP), and the standards required by Resolution 466/2012 of the Ministry of Health – National Health Council.

Microscopic agglutination test

The MAT was performed using the standard procedure as described by Faine et al¹⁰ with the following serovars as live antigens: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Copenhageni,

Cynopteri, Djasiman, Grippytyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Sejroe, Tarassovi and Wolfii. These serovars represent the serogroups known to be prevalent in São Paulo, Brazil^{11,12}. Briefly, suspensions of live leptospire were mixed with serially diluted serum samples in 96-well microtiter plates and incubated at 30 °C for 2 hours. Agglutination was examined using dark-field microscopy at 100x magnification. Titers were calculated as the highest serum dilutions showing at least 50% agglutination of the cells for each serovar tested. A titer of 1:200 or above was considered as a significant titer. The serogroup with the highest titer was considered to be the presumptive infecting serogroup. Internal controls were included in each assay run to ensure the reliability of results. These included a positive control serum with known antibody titers against *Leptospira* spp. and phosphate-buffered saline (PBS) as a negative control. Both were tested in parallel with the samples and confirmed the assay's proper performance and the validity of the agglutination reactions observed.

IgM ELISA

IgM ELISA for the detection of IgM antibodies against *Leptospira* spp. was performed using a commercial *Leptospira* IgM ELISA (Abbott diagnostics, Inc., Korea). The assay was performed according to the manufacturer's instructions. Serum was diluted 1:100 in the diluents provided with the ELISA kit before transfer to *Leptospira* antigen-coated microwell strips. One hundred µL of diluted patient samples, calibrator, positive and negative controls were added into their respective microwell. The plate was covered and incubated for 30 minutes at 37 °C ± 1 °C and then washed six times with diluted wash buffer. One hundred µL of HRP conjugated anti-human IgM were added into each well. One hundred µL of TMB substrate was added into each well and the plate was incubated for 10 minutes at room temperature (20 – 25 °C). One hundred µL of stop solution was added into all wells. Within 30 minutes the absorbance of each well was read at a wavelength of 450 nm. The cut-off value was calculated as the average absorbance of the calibrator triplicates, multiplied by the calibration factor. The corresponding calibration factor was properly used as indicated by the manufacturer. The results were expressed as index value calculated by the ratio of sample absorbance to the mean cut-off absorbance. A value of > 1.1 index was considered as positive. A value of 0.9-1.1 index and < 0.9 index was considered equivocal and negative, respectively.

Freezing and thawing (FT) process

Previously unthawed serum samples from each patient were divided into two aliquots: one was stored at 4 °C as a baseline control, and the other was subjected to freeze-thaw (FT) cycles. One set of aliquots, designated as the baseline, was immediately stored at 4 °C for the remainder of the study. The remaining aliquots were repeatedly taken from the freezer (–20 °C), left at room temperature until completely thawed, and then refrozen. For Group 1 the titration process was repeated after each FT cycle, for a total of 14 cycles, each separated by a 15-day interval. For Group 2, a single FT cycle was performed. All tests were performed and evaluated by the same individual, who was blinded to the results.

Data analysis

Simple linear regression was used to assess the trend (slope) of IgM ELISA index values (Group 1) over FT cycles. All calculations were performed using STATISTICA 64, version 11 (StatSoft Inc., USA).

RESULTS

Sixty-seven positive serum samples were analyzed, and results were compared with those obtained from the initial analysis. For Group 1, no notable alterations were observed in antibody titers between unfrozen and frozen sera even after 14 FT cycles as assessed by both methods. Antibody titers at baseline and after 14 cycles measured by MAT are presented in the [Table](#). While slight fluctuations were detected, they did not appear to follow a consistent pattern indicative of titer degradation. Similarly, for Group 2, which included sera stored for up to 23 years, no clear trend of variation was observed.

Table. Antibody titers determined by Microscopic Agglutination Test (MAT) at baseline and after 14 freeze-thaw cycles in ten leptospirosis serum samples (Group 1)

serovar* sera samples	Australis	Autumnalis	Canicola	Castellonis	Copenhageni	Cynopteri
1	200/400	400/800	na	na	6400/12800	800/800
2	na	na	na	200/200	na	na
3	3200/3200	na	6400/12800	1600/1600	6400/12800	400/200
4	100/200	na	6400/3200	1600/1600	1600/3200	100/100
5	400/400	200/200	400/800	na	1600/3200	1600/1600
6	1600/800	400/400	800/800	12800/6400	6400/6400	na
7	na	na	100/100	na	200/100	na
8	400/400	800/400	1600/800	na	6400/6400	na
9	800/800	na	200/100	na	3200/1600	na
10	200/neg	400/400	na	na	3200/3200	na

serovar* sera samples	Djasiman	Hebdomadis	Icterohaemorrhagiae	Pomona	Sejroe	Wolfii
1	na	na	6400/6400	100/100	na	na
2	200/200	na	na	na	na	na
3	200/neg	200/neg	6400/12800	na	400/200	na
4	200/100	200/200	1600/1600	na	na	na
5	200/neg	200/100	1600/1600	na	na	na
6	na	800/400	12800/25600	na	na	200/400
7	na	na	200/100	na	na	200/200
8	na	800/800	1600/1600	na	na	200/200
9	na	100/100	1600/800	na	na	na
10	na	na	1600/1600	na	na	na

Baseline/after 14 FT cycles

na – no agglutination (negative result)

* Only serovars with at least one positive reaction are shown. Serovars Bataviae, Grippityphosa, Hardjo, Javanica, Panama, Pyrogenes and Tarassovi were non-reactive in all samples

By MAT, results consistent with the baseline were obtained after 14 cycles, except for the anti-Hebdomadis titers in one serum sample, anti-Djasiman in two samples and anti-Australis in one sample, all of which had initial titers of 1:200 that became undetectable. No changes in IgM ELISA positivity were observed when antibody levels were analyzed by index. The index values for each sample were plotted against the number of FT cycles (Figure). Regression analysis indicated a significant downward trend ($p < 0.01$; 99% confidence interval), suggesting a decline in seropositivity with increasing FT cycles. It was estimated that samples with an initial index > 3.0 would not decrease to the cutoff index 1.1 before 40 FT cycles. In contrast, a sample with an index of 2.7 was estimated to reach 1.1 after 24 cycles.

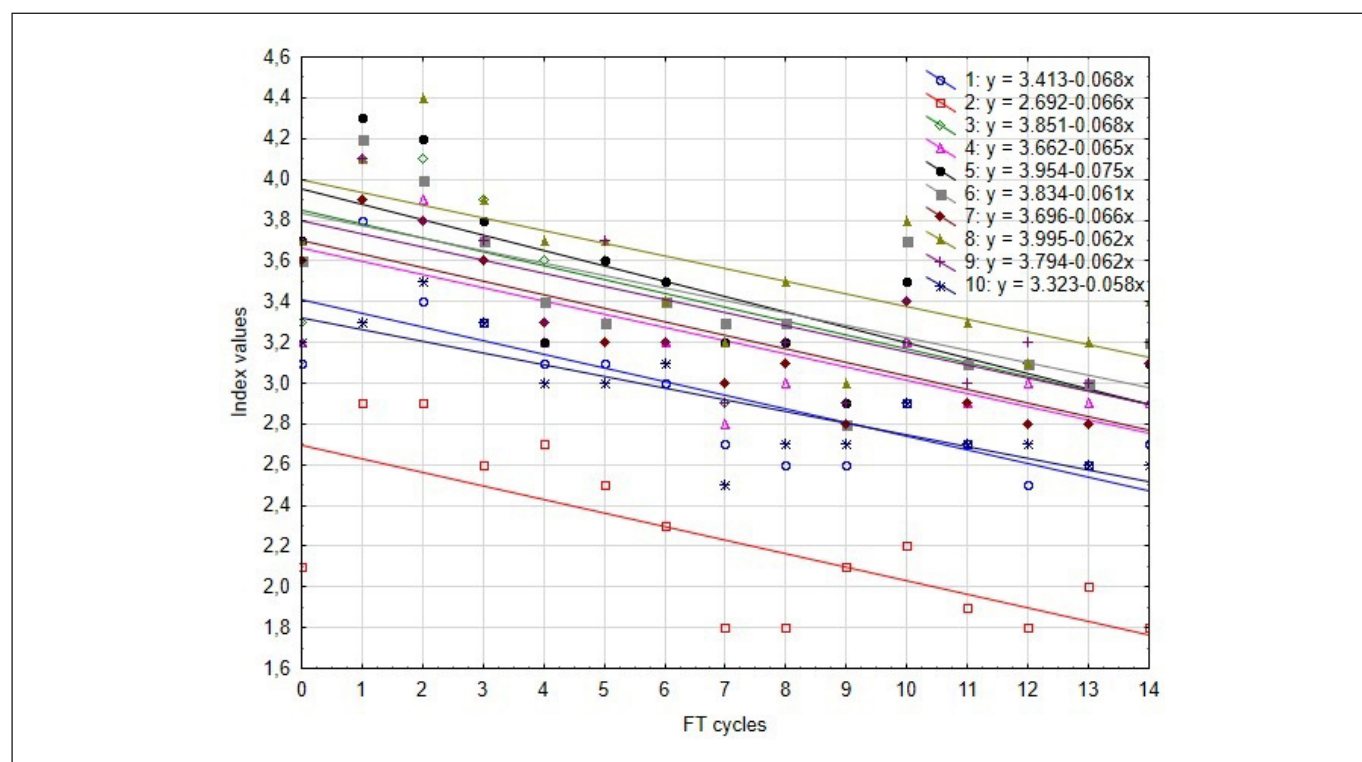


Figure. IgM ELISA results for each serum sample against the number of freeze-thaw cycle (Group 1). The horizontal line indicates the linear fit equation for each sample result

DISCUSSION

The pre-analytical phase occurs from the medical request until the moment of analysis. Proper sample collection, storage, and transport are essential to ensure reliable laboratory results, making this phase a critical component of the analytical process with direct implications for patient outcomes in clinical research. For stored serum samples to be reanalyzed, whether to confirm previous findings or to conduct new analyses, it is essential to ensure their stability. Despite the widespread use of serum samples subjected to multiple freeze-thaw (FT) cycles, limited data are available regarding their impact on antibody integrity in the context of human leptospirosis diagnosis.

Generally, serum samples for clinical analyses are stored at -20°C for short-term storage and at -70°C for long-term preservation. However, repeated thawing and refreezing during reuse raise concerns about potential degradation of antibody targets and the reliability of serological results. Therefore, it is crucial

to assess whether repeated FT cycles influence antibody titers and to consider the number of cycles tolerated by each diagnostic method. Depending on the antibody class, it is crucial to minimize the number of FT cycles to avoid potential loss of sensitivity. Although antibodies are generally stable at -20°C for extended periods, some assays, such as ELISA, may be more sensitive to protein denaturation. Immunoglobulin M is a pentameric macromolecule that is more susceptible to structural denaturation compared to other immunoglobulin classes such as IgG¹³. Numerous investigations have shown that repeated freeze-thaw cycles can compromise IgM stability by inducing conformational changes that impair its antigen-binding capacity, thereby reducing the sensitivity of immunoassays such as ELISA. Degradation of IgM can result in an underestimation of seroreactivity, especially in samples with borderline antibody levels. Furthermore, the functional integrity of IgM can be affected not only by the number of freeze-thaw cycles but also by the duration of exposure to different temperatures during thawing, the type of storage conditions, and sample handling protocols¹³⁻¹⁵.

Several studies using ELISA have shown how multiple freeze-thaw cycles impact the stability and detection of antibodies in serum samples. Previous research has examined the influence of FT cycles on antibody stability across various infectious diseases¹⁶⁻¹⁸. Castro and Jost¹⁶ reported that multiple freeze-thaw cycles produced only a minimal detrimental effect on IgG and IgM assay sensitivity in syphilis sera. Similarly, Castejon et al¹⁷ found no statistically significant loss of anti-treponemal antibody reactivity ($p > 0.05$) even after up to 174 freeze-thaw cycles. Although these investigations mainly focus on pathogens *Treponema pallidum*, their findings are highly relevant to *Leptospira* research, as the stability of *Leptospira*-specific antibodies may be similarly affected. Additionally, studies involving viral infections such as SARS-CoV-2¹⁸, measles, mumps, rubella¹⁹ and influenza²⁰, have demonstrated that antibody integrity remains stable after multiple freeze-thaw cycles, with no clinically or statistically significant loss of reactivity. Rastawicki et al²¹ further demonstrated that repeated FT cycles did not significantly affect the detection of IgA, IgM and IgG antibodies against some bacterial pathogens, reinforcing the robustness of antibody-based assays. These insights underscore the importance of proper sample handling in serological diagnostics for leptospirosis.

In the present study, we observed no significant effect of up to 14 FT cycles on the stability of antibodies against *Leptospira* spp. as measured by MAT and IgM ELISA. All tested samples remained seropositive throughout the 14 cycles. Additionally, samples stored for up to 23 years at -20°C , including those subjected to repeated thawing, maintained satisfactory stability. To our knowledge, this is the first study to evaluate the combined impact of long-term storage and multiple FT cycles on antibody integrity in serum samples used for leptospirosis diagnostics. These findings support best practices for long-term serum storage in clinical and research settings. Nevertheless, it remains advisable to minimize FT cycles to preserve antibody integrity whenever possible. Further, we evaluated the stability of sera collected between 1999 and 2017 that were tested exclusively by MAT. IgM ELISA was not performed at the time, and therefore those results are unavailable. No clinically significant variations were observed, even in samples stored for over two decades. Although overall MAT results were consistent across the 14 FT cycles, a few minor changes were observed: anti-Hebdomadis titers in sample 3, anti-Australis in sample 10, and anti-Djasiman in samples 3 and 5 decreased from 1:200 to undetectable. However, these serovars were not part of the infecting serogroup, and cross-reactions between serovars of different serogroups are common⁹. MAT uses live antigens, which can lead to slight variations in titers due to day-to-day differences in antigen preparation. Thus, titer fluctuations of one dilution above or below the baseline were not considered significant. Moreover, the subjectivity of MAT may result in inter-operator variability in interpretation.

Overall, serum samples demonstrated strong antibody stability even after 23 years of storage at -20°C and up to 14 FT cycles. One limitation of this study concerns the inclusion criteria of the Group 1

since no serum samples with an index value below 2.7 (by IgM ELISA) were available in this group, despite being positive by MAT. Regression analysis of IgM ELISA results showed a downward trend in index values over multiple FT cycles. Nevertheless, seropositivity is estimated to persist through at least 40 FT cycles in most samples, except for one sample with an index below 3.0, for which seropositivity is estimated to persist up to 24 FT cycles. Another limitation is the absence of IgM ELISA results in Group 2 as this assay was not performed at the time of sample collection.

CONCLUSION

These findings are valuable for researchers interested in the use of biological samples for serological diagnosis of leptospirosis. Despite the limited number of samples analyzed, the results demonstrated no significant effect of repeated FT cycles on antibody stability. Antibody levels measured for *Leptospira* spp. by MAT and IgM ELISA remained stable and detectable in serum samples subjected to up to 14 FT cycles.

Furthermore, the serum samples demonstrated satisfactory stability even after 23 years of storage at -20°C , including those that underwent 14 freeze-thaw cycles. To our knowledge, this is the first study to evaluate the impact of storage duration and repeated freeze-thaw cycles on antibodies integrity in serum samples used for leptospirosis diagnosis. Our findings provide valuable insights to guide routine laboratory procedures involving serum samples, and importantly, emphasize the need to minimize freeze-thaw cycles to preserve antibody stability.

CONFLICT OF INTEREST

The authors declare that there are no conflicting interests.

FUNDING

The authors did not declare.

AUTHOR'S CONTRIBUTIONS

All authors have contributed significantly to this study. Eliete Caló Romero: idealized the project, elaborated the study protocol, analyzed data and wrote the manuscript. Roberta Morozetti Blanco and Elaine dos Santos Lima: performed the lab experiments, analyzed the data and revised the manuscript. Camila Cardoso de Oliveira: analyzed the data, performed the statistical analyses and reviewed the manuscript.

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