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Effect of refrigeration, room temperature, and processing time on serum immunofluorescent antibody titers for *Sarcocystis neurona*

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Abstract

Background: Evaluating antibody titers for *Sarcocystis neurona* for the diagnosis of equine protozoal myeloencephalitis from serum samples is a common practice. However, ensuring timely and proper refrigeration is not always possible.

Objectives: To evaluate immunofluorescent antibody (IFA) titers for *S. neurona* from serum samples stored at room temperature and 4°C.

Samples: Twenty-two serum samples.

Methods: Prospective longitudinal study. Two serum aliquots of 1 mL each were stored at room temperature (20-23.3°C) and 4°C. The unrefrigerated aliquot was immediately tested for IFA titers. Both aliquots were retested on Days 5 and 10 after collection. A paired *t* test was used to compare IFA titers at different time points.

Results: There was no significant difference between IFA titers from baseline with those stored at room temperature at Days 5 ($P = .741$, 95% CI [−56.83, 78.65]), 10 ($P = .677$, 95% CI [−50.01, 75.46]), and between 5 and 10 days ($P = 0.949$, 95% CI [−57.50, 61.14]). There was no significant difference from baseline with those stored at 4°C for Days 5 ($P = .964$, 95% CI [−81.81, 85.45]), 10 ($P = 0.573$, 95% CI [−109.4, 62.15]), and between 5 and 10 days ($P = .5$, 95% CI [−102.6, 51.67]). There was no statistical difference between samples stored at room temperature and 4°C ($P = .688$, CI [−55.51, 37.33]) on Days 5 and 10 ($P = .104$, CI [−80.8, 8.07]).

Conclusions and Clinical Importance: Immunofluorescent antibody test titers for *S. neurona* are stable for up to 10 days at room temperature and 4°C.

KEYWORDS
ambient temperature, antibodies, cold, protein, protozoa, stability

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; EPM, equine protozoal myeloencephalitis; IFA, immunofluorescent antibody; IFAT, immunofluorescent antibody test.

1 | INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is a progressively debilitating neurological disease reported in horses in the Americas.[1-9] It is mainly associated with the protozoan *Sarcocystis neurona* although *Neospora hughesi* and *Toxoplasma gondii* have also been proposed as etiologic agents associated with disease because of evidence of intrathecal antibody production for both pathogens.[10-14] Clinical signs depend on the neuroanatomic location within the central nervous system (CNS), and can vary from acute to subacute to chronic, focal or multifocal neurological signs involving the brain, spinal cord, or both.[10] The definitive diagnosis of EPM requires confirmation of protozoal infection in the CNS through postmortem examination.[1] However, antemortem diagnosis can be suspected by performing a complete neurological examination to confirm signs compatible with the disease, ruling out other possible neurological diseases, and determining the production of intrathecal antibodies against the parasite by immunological diagnostic techniques such as immunofluorescent antibody test (IFAT), and *S. neurona* surface antigen (SnSAG) immunosorbent assays (ELISA).[10,11,15,16]

Considering the risks and costs associated with performing a cerebrospinal fluid (CSF) collection, evaluating serum antibody titers for *S. neurona* in many cases is the first step in the investigation of EPM as a possible clinical diagnosis. Although the presence of antibodies in serum does not confirm the clinical disease,[10,17,18] their determination is used to estimate the probability of having EPM,[11,16] rule out infection, or conduct epidemiological studies to determine seroprevalence.[4,18-20] A common practice among veterinarians when transporting serum samples for diagnosis is to refrigerate them; however, in some cases, because of distance or challenges related to the shipment of samples, ensuring timely and proper refrigeration might not be possible in every case. Additionally, veterinarians from countries without access to specialized laboratories with validated diagnostic tests send serum samples to the United States of America for processing. This might result in delay of processing and analyzing samples soon after collection, often taking several days. Preanalytical considerations such as temperature and time of storage have been described in humans to optimize immunological test results.[21] To our knowledge, there are no studies evaluating the effect of refrigeration and room temperature on serum antibody titers for *S. neurona* in horses. Therefore, the objective of the study was to evaluate the effect of refrigeration and room temperature as well as time of storage on immunofluorescent antibody (IFA) titers for *S. neurona* in serum. We hypothesized that IFA from serum samples stored at room temperature, provided temperate weather, would be stable for at least 5 days.

2 | MATERIALS AND METHODS

This is a prospective longitudinal study comparing IFA titers from serum samples stored at room temperature (20-23.3°C [68-74°F]) and under refrigeration at 4°C (39.2°F) at 3 different time points: Day 1 = day of collection (baseline), Day 5, and Day 10 after collection.

2.1 | Serum samples

Serum samples from 22 horses from our research herd, previously identified as having IFA titers for *S. neurona* were used for the study. Ten milliliters of whole blood was collected in red top tubes with no anticoagulant by venipuncture of the jugular vein. Once clotted, the samples were centrifuged at 1000*g* for 10 minutes and serum was collected. Two aliquots of 1 mL each were separated and stored, 1 at room temperature and the other under refrigeration at 4°C. The unrefrigerated aliquot was immediately tested for antibodies to *S. neurona* using IFAT as previously described.[15] Both aliquots were processed again for the detection of IFA on Days 5 and 10 after collection. Serum dilutions started at 1 : 40 and the end-point titer was the last serum dilution showing distinct, whole parasite fluorescence. Although it has been reported that a difference in IFA titers of 1 to 2 dilutions (increased or decreased) is not considered significantly different based on validation studies,[11,22,23] comparison of baseline samples to Days 5 and 10 in this study was based on IFA end-point titers. This approach is consistent with clinicians being able to calculate serum to CSF IFAT ratios.[16,24] This is important because the antemortem clinical diagnosis of EPM is supported by a low IFAT ratio ≤64.[24] The study was approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC, protocol #23833).

2.1.1 | Sample size calculation

To determine the required sample size for our study, we conducted a sample size analysis based on the expected effect size and the desired 80% statistical power. Assuming an effect size (Cohen's *d*) of 0.8, the analysis indicated that a minimum of 14 samples was necessary to achieve this power at a significance level of $P = .05$. This number is smaller than the current sample size of 22 samples.

2.2 | Statistical analysis

For statistical analysis, SAS software version 9.4 (SAS Institute, Cary, NC) was used. The normality of serum titers was assessed with the Shapiro-Wilk test. Given the normality of the sample, a paired *t* test was employed for comparing antibody titers across different time points, assuming equal variances between groups. The dependent or outcome variable was the IFAT titer. The independent variables were time point (Day 0, Day 5, Day 10) and storage condition (room temperature or 4°C). The outcome comparison was the difference in IFAT titers over time and under different storage conditions.

3 | RESULTS

The dataset comprised serum samples from 22 horses (14 geldings, 8 mares) of mostly Thoroughbred breed (N = 11), with a mean age of 12.5 years old. Upon analyzing the data, no statistically significant

differences ($P > .05$) were observed in IFA titers from baseline (Day 1) to the subsequent titers on Days 5 and 10 at room temperature and under refrigeration at 4°C. Furthermore, there were no differences in IFA titers at each time point between samples stored at room temperature and under refrigeration at 4°C.

3.1 | Serum IFA titers at room temperature over time

Comparing serum IFA titers for *S. neurona* at baseline with those from samples stored at room temperature for 5 days showed no statistically significant difference ($P = .741$, 95% CI [−56.83, 78.65]) as determined by the paired *t* test. Comparing serum IFA titers for *S. neurona* at baseline with those from samples stored at room temperature for 10 days showed no statistically significant difference ($P = .677$, 95% CI [−50.01, 75.46]). Comparing serum IFA titers for *S. neurona* at room temperature between Days 5 and 10, there was no statistical difference ($P = 0.949$, 95% CI [−57.50, 61.14]).

3.2 | Serum IFA titers under refrigeration at 4°C

Comparing serum IFA titers for *S. neurona* at baseline with those from samples stored at 4°C for 5 days showed no statistically significant difference ($P = .964$, 95% CI [−81.81, 85.45]). Comparing serum IFA titers for *S. neurona* at baseline with those from samples stored at 4°C for 10 days showed no statistically significant difference ($P = 0.573$, 95% CI [−109.4, 62.15]). Comparing serum IFA titers for *S. neurona* at 4°C between Days 5 and 10, there was no statistical difference ($P = .5$, 95% CI [−102.6, 51.67]).

3.3 | Serum IFA titers at room temperature and under refrigeration at 4°C

There was no statistical difference in IFA titers for *S. neurona* between samples stored at room temperature and under refrigeration ($P = .688$, CI [−55.51, 37.33]) on Day 5. There was no statistical difference in IFA titers for *S. neurona* between samples stored at room temperature and under refrigeration ($P = .104$, CI [−80.8, 8.07]) on Day 10.

4 | DISCUSSION

The present study demonstrated that immunofluorescent antibodies for *S. neurona* in serum remain stable for at least 10 days regardless of whether the serum is refrigerated at 4°C or kept at room temperature.

Preanalytical factors include all the steps before the performance of the diagnostic test in the laboratory and have been classified into 2 general categories: (1) technical effects because of sampling technique and management before analysis, including stability of the specimen during storage and shipping to the laboratory, and (2) biological factors inherent to the animal sampled.[25,26] Despite the importance of the preanalytical phase, there are a few studies evaluating preanalytical conditions in human and veterinary medicine,[27] including those that might affect serum antibody measurements in horses.

Previous studies conducted in horses and humans evaluating the effect of room temperature and refrigeration over time on different antibodies have reported stability of immunoglobulins up to 72 hours and 6 days at room temperature and 4°C, respectively.[21,28] Another study evaluated the effects of delayed centrifugation, temperature, and storage stability of specific IgE antibodies in serum and found that serum IgE is stable at room temperature for 48 hours before centrifugation and 10 days at 5°C after centrifugation.[29]

Limitations of the study included that IFA for other etiologic pathogens associated with EPM such *N. hughesi* and *T. gondii*, as well as CSF antibodies, which are indispensable to confirm intrathecal production of antibodies, were not evaluated because of availability. Another limitation is that the samples were centrifuged immediately after coagulation, which is not commonly done by field veterinarians when taking samples and needs investigation. Also, this study was conducted with room environmental temperatures within the range of 20 to 23.3°C (68-74°F), and the effects on the stability of antibodies from serum samples under other environmental temperatures were not evaluated here. Therefore, it is uncertain if similar results might have been observed.

In conclusion, our study indicates that serum IFA for *S. neurona* is stable for up to 10 days at both, room temperature or stored at 4°C. This suggests that results obtained from unrefrigerated samples on which the cold chain cannot be guaranteed or those that are not tested soon after collection are reliable. However, refrigeration might still be the best practice if major fluctuations in environmental temperature from transportation to analysis cannot be predicted.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of California Davis IACUC, protocol #23833.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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