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SEROLOGICAL STABILITY IN SAMPLES OF *BRUCELLA ABORTUS* SUBJECTED TO FREEZE FOR USE AS A CONTROL IN THE POLARIZED FLUORESCENCE TEST

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Abstract: Brucellosis is one of the most important zoonoses with worldwide distribution, caused by bacteria belonging to the genus Brucella. In Mexico, bovine brucellosis is endemic and is caused by *Brucella abortus*. The objective was to compare the antibody titer in samples before and after freezing without the addition of preservatives and/or molecular stabilizers that could intervene in their biochemical behavior during the polarized fluorescence assay. 200 healthy cows and 200 infected with *Brucella* race Friesian- Holsteins from 38 to 48 months of age, underwent the FPA test and the sera were kept frozen (-20°C). Data analysis was performed through descriptive statistics using the IBM SPSS Statistics 25 Software. Statistical calculations were performed using the Wilcoxon test, comparison between the two means and the average reference values. In all cases, a significance level of 0.01 and a 99% confidence interval were considered. A P value $< \alpha$ (0.01) was obtained in the Kolmogorov-Smirnov normality test. The behavior in the histogram was obtained a mean = 0.53 and standard deviation 3.123. Regarding the individual data of the serological samples of bovines with brucellosis, similar results were obtained before and after freezing. The Wilcoxon signed rank test indicated that 84 samples (42%) decreased their Δ mP values after freezing, while in the remaining 116 samples (58%) this value increased. There was no significant difference. In the serum samples, serological stability was observed for six months analyzed in FPA.
Keywords: Bovine brucellosis, serological stability, freezing.

INTRODUCTION

Brucellosis is one of the most important zoonoses with a global distribution, caused by bacteria belonging to the genus Brucella (Zange et al. 2019). Each of its species

usually has a natural host, however, they can affect most mammals, vertebrates and some amphibians (Godfroid 2018). There are four pathogenic species for humans, which are transmitted due to the proximity that exists with natural hosts: *B. abortion* is the cause of bovine brucellosis (BB) and together with B. *melitensis* reside in bovidae, B. *suis* in suidos and B. canis in canids (Kazemi 2014). The main sectors affected due to this disease are the livestock and socio-sanitary sectors, reflecting in great economic losses (Bano and Lone 2015). In Mexico, BB is endemic, and only the entities of Baja California Sur and Sonora are considered free of the etiological agent (SENASICA, 2019). On the other hand, the States of Coahuila and Durango have the region known as La Comarca Lagunera, which is the main source of bovine milk supply in the country and is classified as the most important dairy basin in Mexico (Loera and Banda 2017).). Contributing with approximately ten million liters of milk per day, it is a geostrategic point due to the economic flow it represents for companies and small producers. According to the SIAP, 2018, in Mexico, the four entities that lead the production of bovine milk are Jalisco, Coahuila, Durango and Chihuahua, which contributed 51.1% of the national total in 2018, which was 12 thousand 8 million 239 thousand liters. In terms of categorization by region, La Comarca Lagunera represented more than 20% of the national total, its contribution being above any individual entity. However, BB continues to be a serious problem in that region and in the main dairy entities since it has not been eradicated (SENASICA, 2019), which is a crucial determinant to facilitate the transmission of this disease to humans. through contaminated food (Dadar, Shahali, and Whatmore 2019), by proximity to infected animals or by exposure to the pathogen in diagnostic laboratories (Traxler et al. 2013). Most of the cases of zoonosis diagnosed bacteriologically in humans correspond to *Brucella* spp, Therefore, it is considered a highly pernicious and invasive disease, qualities for which it has managed to maintain a high prevalence and wide distribution in the country (Lopez-Merino et al. 1992). Currently, there is no specific clinical picture for human brucellosis, finding various signs and symptoms depending on each individual. The most common clinical manifestations in the subacute and acute stages are diaphoresis, wavy fever, hepatomegaly, arthralgia, headache, myalgia, and general malaise, as well as complications from their chronicity can be arthritis, bacteraemia, endocarditis, thrombocytopenia, and meningitis (Singh et al. 2018; Dadar, Shahali, and Whatmore 2019). Although B. abortion is the main species responsible for BB, it has also been isolated from other domestic and wild animals (Godfroid 2017). Regarding cattle, in females it causes clinical signs such as spontaneous late abortions characterized by occurring during the last trimester of gestation, epididymitis, placentitis, metritis, retention of the placenta and obtaining weak offspring susceptible to premature death due to sepsis, in males it produces orchitis and damage to the genitourinary system, likewise, infected cattle can become infertile in addition to spreading the bacteria through milk, semen and body fluids (Corbel et al. 2006).

Among the diagnostic tests, the conclusive indicator that an animal has brucellosis is bacteriological isolation, however, as it is an expensive, tedious and time-consuming process, it is impractical for eradication campaigns, opting to perform serological tests. Performing a periodic analysis of the animals and having their clinical history available serves as guidance to the Zootechnical Veterinarian and the laboratories to obtain a timely and accurate differentiated diagnosis,

as well as for the issuance and interpretation of correct results. When obtaining as a result of the serological tests an indication that the animal could have had contact with the etiological agent, that it is infected or has been recently vaccinated, it is advisable to integrate various serological tests in the clinical protocols, being very useful for the ability to use some tests as screening and others as confirmatory, likewise, due to the diagnostic performance that some may have, may be of vital importance for the early detection of the disease in asymptomatic animals.

Mexico is among the countries with the highest BB incidence rates in the world (Méndez-Lozano, Rodríguez-Reyes, and Sánchez-Zamorano 2015). Due to the fact that the epidemiological behavior of this disease varies in each region due to various factors such as climate, geography and those of the immunological nature of the animals (de Figueiredo et al. 2015; Ducrotoy et al. 2015), under these circumstances it is recommended, if required, than when selecting a diagnostic method in a laboratory (OIE, 2018).

Under these circumstances it is advisable, if required, that when selecting a diagnostic method in a laboratory, it be examined to perform some additional optimization, consisting of carrying out a series of experiments, followed by a subsequent analysis of the data to its use when monitoring the correct execution of the test, ensuring that it meets the specifications of internal controls, standards and reference materials, all with the aim of establishing national secondary controls with which internal controls can be prepared of work to be used in the daily systematic laboratory diagnosis, making the necessary modifications according to the characteristics of the region in which it operates.

There is no scientifically supported information on the serological stability of the samples that are used as internal controls at the Research Center. Likewise, no evidence was found in the literature that a study on serological stability after freezing had been carried out. Since there are no standardized national controls marketed by the National Veterinary Biologics Producer (PRONAVIBE) for the polarized fluorescence assay (FPA), it is pertinent to carry out a study to find out if the samples of healthy and sick animals selected as internal controls suffer Significant modifications in the conservation process that may affect its performance, therefore, it is essential to know the stability of the anti-Brucella antibody titer in the internal controls before and after being subjected to freezing, to confirm its serodiagnostic validity if used. as indicators of the state of animal health that adjust to the real behavior of brucellosis in the region. Having samples to be used as internal controls from healthy cows and cows sick with brucellosis contributes to the establishment of cut-off points in the region. Therefore, the objective was to compare the antibody titer in samples before and after freezing without the addition of preservatives and/or molecular stabilizers that could intervene in their biochemical behavior during the FPA assay.

MATERIAL AND METHODS

Longitudinal, non-parametric, analytical and comparative study. This study was approved by the Ethics Committee of the Animal Health Research and Diagnostic Center. The stables of the present study belong to La Comarca Lagunera and participated under anonymity. 400 Friesian-Holstein cows from 38 to 48 months of age were sampled, of which approximately 10 ml of blood from each one was collected in vacutainer tubes labeled with the corresponding animal identification number through the coccygeal vein. Subsequently, they were transported in coolers to the laboratory of the Center for

Research and Diagnosis in Animal Health for subsequent centrifugation. Serum was separated from the blood by centrifugation at 3000 g for 3 min. Each blood serum sample was deposited in AXYGEN® SCIENTIFIC MCT-200-C LOT NO. 27717064 labeled with the identification numbers of each animal. Subsequently, the FPA test was performed and the sera were frozen (-20°C). After 6 months of frozen preservation, the sera were tempered and the FPA test was performed a second time. The study was carried out on serum samples obtained from a group of 200 healthy cows and another group of 200 infected with Brucella spp. The following serological units were analyzed and compared: delta millipolarization units (Δ mP) and millipolarization units (mP) by means of the BioTek® Synergy™ 2 multimode plate reader. Data analysis was performed through descriptive statistics using the IBM SPSS Statistics 25 Software, comparing the results obtained from the FPA readings in the serological samples before and after freezing, determining the serological stability, average values and the standard deviation. Statistical calculations were made using the Wilcoxon test, comparison between the two means and the average reference values. The assumption of normality was verified by means of the Kolmogórov-Smirnov test. In all cases, a significance level equal to 0.01 and a confidence interval of 99% were considered (Flores-Ruiz, Miranda-Novales, and Villasís-Keever 2017).

RESULTS AND DISCUSSION

A value of $P < \alpha$ (0.01) was obtained in the Kolmogórov-Smirnov normality test between the differences in the values of Δ mP before and after freezing in the serological samples of cows sick with brucellosis, thus it was shown that the data comes from a non-normal distribution (**Table 1**). In the same way, this behavior was graphically observed in the histogram with the respective data, obtaining a mean value = 0.53 and a standard deviation = 3.123. (**Figure 1**), as well as in the Q-Q graph, the trajectory of points obtained deviations with respect to the line of the identity function (**Figure 2**).

Regarding the individual data of the serological samples of bovines with brucellosis, similar results were obtained before and after freezing(**Table 2**). The Wilcoxon signed rank test indicated that 84 samples (42%) decreased their Δ mP values after freezing, while in the remaining 116 samples (58%) this value increased(**Table 3**). However, there was no significant difference (**Table 4**).

Because the value of the asymptotic significance (0.016) of the Wilcoxon signed rank test was greater than the value of α (0.01) with a confidence interval of 99%, it was shown that the median of the differences between the values of the Δ mP Post and Δ mP Pre in the serological samples of cows sick with brucellosis is equal to zero, being sufficient evidence to retain the null hypothesis of the present investigation and demonstrate that the blood sera of cows sick with brucellosis do not undergo any modification. in its biochemical behavior at the level of anti-Brucella antibodies during frozen storage (-20°C) in a period of 6 months without the addition of molecular stabilizers.

The diagnostic performance of FPA also depends on the precision and accuracy of the laboratory worker, which influences the results obtained that will later be analyzed, interpreted and issued by the clinical laboratory, this type of variable belonging to other related factors that are not objects of study. in the present investigation since a training and validation of the analyst was carried out by the laboratory.

Likewise, the implementation of samples from animals sick with brucellosis as positive internal serological controls can be considered

an alternative in order to have references that are more closely related to the real epidemiological behavior of the disease in the La Comarca Lagunera region, as well as to avoid a total dependence on the supply of external controls, which could affect the number of samples that the laboratory can process daily. An important point in using internal controls is to demonstrate that they perform correctly and do not affect the delivery of reliable results for your customers. On the other hand, working on obtaining, collecting and testing controls that serve as a reference in the region for subsequent commercialization to other laboratories would increase the efficiency of these to advance with the eradication of bovine brucellosis in Mexico.

When comparing the titer of antibodies in the serum samples, serological stability was observed after six months that they were kept frozen when analyzed in FPA, without the need to use any preservative, an aspect to take into account for its serodiagnostic application. For this reason, they could be used as internal positive controls in the laboratory of the Center for Diagnostic Research in Animal Health.

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	Kolmogórov-Smirnov [a]		
Δ mP	Statistical	Degrees of freedom	P-value
Post[b] – Pre[c]	0.081	200	0.003

[a] Lilliefors significance correction
[b] Value of Δ mP of serological samples after freezing
[c] Value of Δ mP of serological samples before freezing

Table 1. Values in the normality test in the serological samples of cows sick with brucellosis before and after freezing.

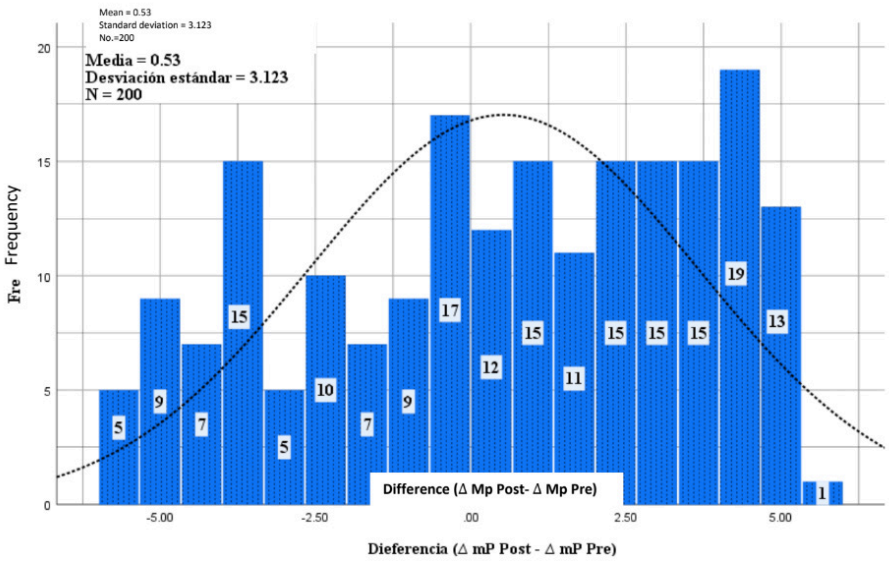

Figure 1. Histogram of the differences in the values of Δ mP (Post – Pre) in the serological samples of cows sick with brucellosis.

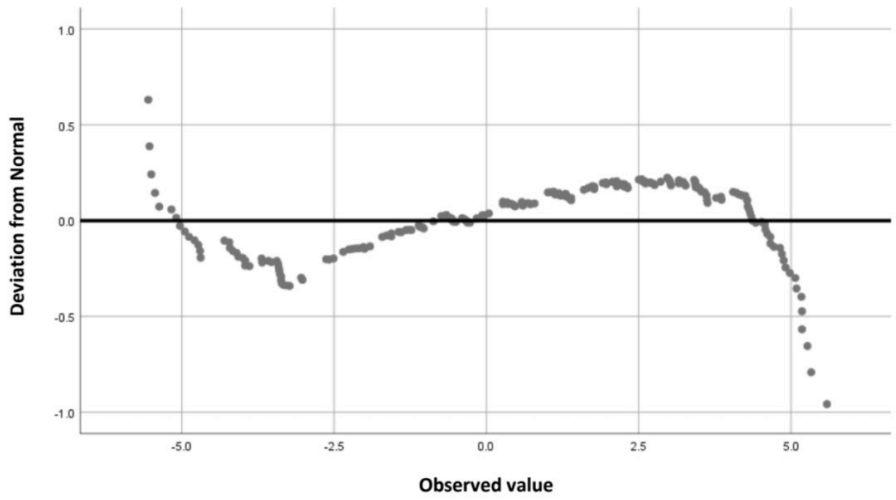

Figure 2. Normal Q-Q graph without trend between the values of the differences of Δ mP (Post – Pre) in the serological samples of cows sick with brucellosis.

Δ mP	N	Half	Standard deviation	Minimum value	Maximum value
Pre	200	150.0535	64.79665	20.10[a]	229.80[b]
Post	200	150.5834	64.92717	20.84[a]	228.53[b]

Table 2. Comparisons between the mean, standard deviation, maximum and minimum values of Δ mP in serological samples from cows infected with brucellosis before and after freezing.

[a] Corresponds to the same sample

[b] Corresponds to the same sample

	N	Average range	Sum of ranks
Negative ranks	84[a]	96.20	8081.00
Positive ranks	116[b]	103.61	12019.00
Draws	0[c]		
Total	200		

[a] Δ mP Post < Δ mP Pre

[b] Δ mP Post > Δ mP Pre

[c] Δ mP Post = Δ mP Pre

Table 3. Wilcoxon signed rank test on serological samples from cows infected with brucellosis after freezing.

	Δ mP Post – Δ mP Pre
Value of Z	-2.403[a]
Asymptotic significance (two-sided)	0.016

[a] It is based on negative ranks.

Asymptotic significances are shown. Significance level 0.01; confidence interval 99%.

Table 4. Wilcoxon signed rank test statistics