Prozone effects in microscopic agglutination tests for leptospirosis in the sera of mice infected with the pathogenic *Leptospira interrogans* serovar Canicola

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Mice experimentally infected with a pathogenic strain of Leptospira interrogans *serovar Canicola produced false negative results (prozone effect) in a microscopic agglutination test (MAT). This prozone effect occurred in several serum samples collected at different post-infection times, but it was more prominent in samples collected from seven-42 days post-infection and for 1:50 and 1:100 sample dilutions. This phenomenon was correlated with increased antibody titres in the early post-infection phase. While prozone effects are often observed in serological agglutination assays for the diagnosis of animal brucellosis and human syphilis, they are not widely reported in leptospirosis MATs.*

Key words: microscopic agglutination test - prozone effect - leptospirosis

Leptospirosis is a globally distributed infectious disease caused by spirochetes of the genus *Leptospira*. It affects humans and a wide variety of domestic and wild animals. This zoonotic disease is endemic in underdeveloped and developing countries and is considered emerging or re-emerging in developed countries (Bharti et al. 2003). Leptospirosis is mainly diagnosed in animals and humans through serological tests (O'Keefe 2002).

The microscopic agglutination test (MAT) is a standard serological assay that detects antibodies against *Leptospira* spp in serum samples, where direct agglutination of serum antibodies with live bacteria is observed via dark-field microscopy. The starting serum dilution for screening using this test is 1:100 and when positive, samples are retested in two-fold serial dilutions to determine the antibody titre (WHO 2003).

The agglutination test has been widely applied for diagnosis because it is simple and easy to perform. However, excess antibodies in a serum sample can inhibit the antigen-antibody interaction and subsequent agglutination reaction, leading to a false negative result, which is known as a prozone phenomenon or effect (Tizard 2004). Prozone effects have often been reported in diagnostic tests for animal and human brucellosis (Plackett & Alton 1975, Guven et al. 2006, Buzgan et al. 2007) and human syphilis (Azevedo et al. 2006), mainly in patients co-infected with human immunodeficiency virus (Lynn & Lightman 2004, Smith & Holman 2004). Apart from the findings of some studies performed in sheep, goats and horses (Malkin 1984, Knudtson & Fetters 1990), it is unknown whether this effect occurs in MATs for leptospirosis.

In mice experimentally infected with a pathogenic strain of *Leptospira interrogans* serovar Canicola, we previously observed false negative reactions (a prozone effect) in MATs of serum samples collected at different times post-infection. In the present study, we evaluated this phenomenon.

We inoculated 60 inbred, albino female Swiss/Uni mice at four-six weeks of age, weighing 30-40 g. The mice were free of pathogens and were acquired from the Multidisciplinary Centre for Biological Research of the State University of Campinas, Campinas, state of São Paulo (SP), Brazil. The animals were allocated to three groups of 20 mice each, 14 of which were inoculated with the pathogenic bacteria, while six were inoculated with sterile Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium as a negative control. The strain used in this study, from *L. interrogans* serovar Canicola, was isolated from a porcine liver in Londrina, state of Paraná, Brazil and is pathogenic in hamsters (Freitas et al. 2004). The animals were kept in 41 x 34 x 16 cm (length x width x height) polypropylene cages with stainless steel perforated floors covered with autoclaved shavings and were supplied with commercial food and drinking water *ad libitum*. The study protocol was approved by the Ethical Committee on Animal Use (CEEA) of the Faculty of Veterinary Medicine and Animal Science of São Paulo State University, Botuca-

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tu, SP, Brazil (64/2006-CEEA). *L. interrogans* serovar Canicola was maintained in the laboratory through alternating passages in hamsters and semi-solid Fletcher medium. To prepare the inoculum, bacteria were cultured in EMJH liquid medium for seven days. Leptospires were counted in a Petroff-Hauser chamber with a dark-field microscope and the final concentration was adjusted to 2.5 x 10^5 microorganisms/mL. The volume of this inoculum administered intraperitoneally to each animal was 0.5 mL.

For serological monitoring, blood samples were initially collected at short, daily intervals until the second week, then weekly until day 77 and, finally, every 14 days until the end of the experiment (on days 4, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 91, 105, 119, 133, 147, 161 and 175). During each sampling event, five inoculated animals (n = 5) and two negative controls were randomly chosen and re-sampling of the chosen animals did not occur until the 35th day post-inoculation. After this date, the animals were again subjected to sampling. This methodology was adopted because at days 7, 14, 21, 28, 35, 91 and 175 post-inoculation, five inoculated animals and two negative controls were sacrificed for tissue collection. For blood collection, the retro-orbital venous plexus was punctured and 0.5 mL of blood was collected in 1.5 mL plastic microtubes via capillary tubes. The blood samples were drained after clot retraction and centrifuged for 10 min at 2,500 rpm. The sera were then stored in new 1.5 mL plastic microtubes at -20ºC until serological testing.

The MAT was performed according to the methodology described by the World Health Organization (WHO 2003) using 24 *Leptospira* spp serovars (Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcombi, Cynopteri, Djasiman, Sentot, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Hardjo, Wolffi, Shermani, Tarassovi and Patoc). Live cultures were grown for seven days in EMJH liquid medium and diluted 1:3 in 0.01 M phosphate buffered saline, pH 7.2, which corresponded to a concentration of approximately 2.0 x 10^8 leptospires/mL. Each serum sample was initially diluted 1:50 and tested for all serovars; samples showing 50% or more agglutination than the negative control were considered positive. The positive samples were tested again in two-fold serial dilutions for the serovar(s) that reacted previously. The endpoint titre corresponded to the reciprocal of the highest serum dilution that showed 50% agglutination, as measured by comparison with the negative controls. The obtained data were tabulated in an Excel spreadsheet. For statistical analysis, the data were compared using a chi-square test at a significance level of $\alpha = 0.05$ (Triola 2005).

The animals inoculated with the pathogenic strain of *L. interrogans* serovar Canicola seroconverted, but showed no susceptibility to the disease symptoms. Serological screening produced no reactions to any *Leptospira* spp serovars other than that used for inoculation, indicating a specific immune response against the Canicola serovar.

False negative reactions were observed in several serum samples at dilutions of 1:50, 1:100 and 1:200. Ten of the 95 serum samples were discarded due to inconsistent results. Of the 85 remaining samples, two tested negative in the initial 1:50 dilution. Among the 83 reactive samples, false negative reactions occurred in 47 (56.6%) of the 1:50 dilutions, 26 (31.3%) of the 1:100 dilutions and one (1.2%) of the 1:200 dilutions.

Based on the distribution of false negative results for the 1:50 and 1:100 dilutions and the final titres obtained from each serum sample, we found that the prozone effect increased when the titre of antibodies was high. This trend was statistically significant ($p < 0.05$) (Tables I, II).

The false negative results were also compared between samples collected at different times (Tables III, IV). There was a significant difference ($p < 0.05$) between the number of false negative results obtained during the initial post-infection period, especially between seven-42 days post-infection and the negative results obtained later in the infection.

Such false negative results correspond to an excessive increase in the concentration of antibodies against a specific antigen. An antibody concentration much higher than the antigen density may inhibit agglutination (Plackett & Alton 1975, Azevedo et al. 2006, Guven et al. 2006, Buzgan et al. 2007).

The complement system may also interfere with agglutination, as shown by Malkin (1984). To reduce this phenomenon, complement can be inactivated by heat-treating serum in a 56ºC water bath or by adding ethylenediamine tetraacetic acid. Serum inactivation is performed in MATs to diagnose human leptospirosis (WHO 2003), but it is rarely applied when testing animal samples. To eliminate excess antibodies and decrease the prozone effect, serum samples can be diluted further.

The cut-off value that is commonly used for sample dilutions assayed by MATs is 1:100, which is the standard value for screening (WHO 2003). Because live antigens are used, antigen standardisation, which depends on the growth time and leptospire density of cultures, is difficult (O'Keefe 2002). Considering that false negative results were produced by several samples in our study, we must emphasise the importance of reducing the prozone effect in serum samples tested via MATs.

In both diagnostic and research protocols, serum inactivation (Knudtson & Fetters 1990), higher serum dilutions and higher antigen concentrations can help minimise the prozone effect. However, these procedures should be carefully evaluated because they directly influence the results and standardisation of MATs. Although the antigens were prepared consistently for all of the MATs performed in this study, unknown factors led to differences in their density. These differences in the ratios of antigens to antibodies may have produced prozone effects. Although prozone effects are rarely reported in MATs, they should be further studied so that the results obtained during screening and titration are correct and reliable, especially in animal serum samples that are not inactivated prior to testing.

TABLE I

False negative results for 1:50 dilution in microscopic agglutination test of sera from mice experimentally infected with *Leptospira interrogans* serovar Canicola, according to the final titres obtained in each sample

	False negative result for 1:50 dilution		
Titre[a]	Yes n (%)	No n (%)	Total n (%)
≤ 800	17 (37.8)	28 (62.2)	45 (100)
≥ 1.600	30 (78.9)	8 (21.1)	38 (100)
Total	47	36	83 (100)

a: chi-square test ($p < 0.05$).

TABLE II

False negative results for 1:100 dilution in microscopic agglutination test of sera from mice experimentally infected with *Leptospira interrogans* serovar Canicola, according to the final titres obtained in each sample

	False negative result for 1:100 dilution		
Titre[a]	Yes n (%)	No n (%)	Total n (%)
≤ 800	8 (17.8)	37 (82.2)	45 (100)
≥ 1.600	18 (47.4)	20 (52.6)	38 (100)
Total	26	57	83 (100)

a: chi-square test ($p < 0.05$).

TABLE III

False negative results for 1:50 dilution in microscopic agglutination test of sera from mice experimentally infected with *Leptospira interrogans* serovar Canicola, according to the sampling period

	False negative result for 1:50 dilution		
Period (day)[a]	Yes n (%)	No n (%)	Total n (%)
≤ 42	22 (75.9)	7 (24.1)	29 (100)
> 42	25 (46.3)	29 (53.7)	54 (100)
Total	47	36	83 (100)

a: chi-square test ($p < 0.05$).

TABLE IV

False negative results for 1:100 dilution in microscopic agglutination test of sera from mice experimentally infected with *Leptospira interrogans* serovar Canicola, according to the sampling period

	False negative result for 1:100 dilution		
Period (day)[a]	Yes n (%)	No n (%)	Total n (%)
≤ 42	16 (55.2)	13 (44.8)	29 (100)
> 42	10 (18.5)	44 (81.5)	54 (100)
Total	26	57	83 (100)

a: chi-square test ($p < 0.05$).

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