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Prozone Effect and False Negative Leptospirosis Microscopic Agglutination Test in Experimental Immunized Mice

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Abstract: Leptospirosis is a world-wide bacterial disease affecting many mammals, including man and has been considered to be the zoonosis with widest geographical distribution in the world. MAT and ELISA are the most serological tests used for seroprevalences of susceptible infected mammalian populations world-wide. The detection of positive leptospirosis clinical cases depending on isolation of leptospira, which is affected by the technical difficulties in isolating leptospires from carrier animals or from clinical/pathological material. So, the serological detection of positive cases of leptospira is the most laboratory investigations method for leptospira infection. In this study, Mice experimentally immunized with a three different antigenic preparations (boiled, sonicated, successive frozen and thawed), pathogenic strain of *Leptospira interrogans* serovar Canicola evoked the production of high antibody titer in the immunized mice. All the examined sera collected from 45-tested mice produced positive result with ELISA test. In microscopic agglutination test [MAT], the most of serum samples with high IgM produced false negative results [Prozone Effect], which it was more for 1:50, 1:100 and 1:200 sample dilutions. This phenomenon was correlated with increased antibody titres in the acute and convalescence phases post-infection.

Key words: Leptospira % Prozone effect % Experimental immunization % Mice % ELISA % MAT

INTRODUCTION

Leptospirosis is a zoonotic bacteriological disease, caused by members of the genus *Leptospira*. Although it has a greater incidence in tropical regions than temperate regions, it is considered to be one of the most geographically widespread zoonosis. Leptospirosis is endemic zoonotic disease in underdeveloped and developing countries and is considered emerging or re-emerging in developed countries [1]. Leptospirosis characterized by complex clinical symptoms that vary from a mild flu-like form to lethal pulmonary hemorrhage or hepatorenal failure [2].

Rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification for prompt and proper treatment.

Leptospira serological testing is the most widely and frequently used laboratory procedure to confirm the clinical diagnosis, determine herd prevalence or conduct epidemiological studies in animals and humans [2]. Antibodies are detectable in the blood approximately 5 to 7 days after the onset of illness and persist for weeks or months and, in some cases, years [3].

A wide variety of serological tests that show varying degrees of serogroup and serovar specificity previously described, microscopic agglutination test [MAT] and the enzyme-linked immunosorbent assay [ELISA] play a role in diagnosis of veterinary leptospirosis [2,4].

The standard serologic test, the microscopic agglutination test [MAT], using live antigens is specific and provides useful epidemiologic data in the form of presumptive serogroup [5, 6].

However, excess antibodies in a serum sample can inhibit the antigen-antibody interaction and subsequent agglutination reaction, leading to a false negative result that is known as a prozone phenomenon or effect [7]. Prozone effects prior reported in brucellosis diagnosis [8-10]. Prozone phenomenon reported in MATs for diagnosis of leptospirosis in sheep, goats and horses [11,12], So MAT is inadequate for rapid case

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identification, since it is technically demanding, costly and requires the maintenance of live, hazardous stock serovar cultures; it also requires analyses of paired sera to verify the seroconversion, which delays diagnosis [13].

ELISA could overcome such problem; ELISA designed to detect both IgG and IgM-leptospiral antibodies [14]. IgM, the first immunoglobulin expressed by plasma cells, can help in the rapid leptospirosis diagnosis [15].

This is particularly important in areas where leptospirosis is widespread and residual antibodies of past infection are found in a large part of the population [14].

ELISAs are sensitive [16, 17], but showing a considerable degree of cross-reactivity with *Leptospira* heterologous serovars, as compared with MAT [18].

An example of the potential of ELISAs in the veterinary field is the development of an antibody capture ELISA for Hardjo infection in cattle [19 which detects antibodies to a protective lipopolysaccharide [LPS] outer envelope fraction common to both *Leptospira borgpetersenii* serovar Hardjo and *Leptospira interrogans* serovar Hardjo. It proved to be very sensitive and able to measure the protective antibodies in both milk and sera, giving a better indication of the immune status of the bovine population. Meaningful results were obtained on both bulk and individual animal milk samples, something not possible with the MAT.

MATERIALS AND METHODS

Laboratory Mice: Three groups of total 45 healthy Balb/c mice [6-7 weeks old], were delivered from Animal Production Department, Theodor Bilharz Research Institute, Giza, Egypt. The mice allocated into three groups, coded [I], [II] and [III], each group composed of 15 mice. Group [I] of mice was injected by boiled antigen, the other group [II] was infected by sonicated antigen and the last group [III] was subjected to successive freeze and thawed antigen.

Leptospira Reference Live Strains for MAT: According to Ellis and Little 1986 [20] the strains used for serological testing by the MAT were cultures of live leptospires serovar Canicola. Leptospires were grown in EMJH medium [Difco] and incubated for 5-7 days at 30°C. The strains are maintained by weekly subculture into fresh medium. Each flask is subcultured to two new flasks of fresh medium, one of them to be used in the MAT in the next week and the other to be kept for the next week's subculture. The subculture of strains is done with asepsis conditions and using a laminar flow chamber. Each subculture of each antigen is always checked for possible unwanted contamination with other bacteria by inoculating of a few drops of the subcultured leptospires into Nutrient Broth. The cultures are incubated at 39° C and the purity checks at 37°C. Each week the purity checks are examined before the leptospira cultures are subcultured and if contamination has occurred, the respective flasks are discarded and substitute ones, free from contamination, are used for subculturing. At the same time, a loopful of the culture was examined by dark field microscopy to confirm the presence of viable leptospires and the absence of contamination.

Preparation of Antigens for Mice Injection: The leptospira antigen was prepared by different three methods

Boiled Antigen Preparation: The antigenic preparation was produced according to the procedure of Terpstra *et al.* [21], with special modification. The organisms were grown as above and harvested after 6 d by centrifugation [10,000 X g for 45 min]. The organisms were washed twice by centrifugation [10,000 X g for 45 min] in phosphate buffered saline [PBS; pH 7.3]. The pellet from 100 mL of culture was resuspended in 100 mL of PBS. The culture was killed heating in a bath with boiling water for 30 min and then centrifuged at 10 000 X g for 30 min to remove insoluble material. The supernatant was used as antigen. Antigens were stored at -70°C and were found to be stable for at least 4 month.

Preparation of Sonicated Antigen: Sonicated antigen was prepared as follows. The organism was cultivated in EMJH medium [Difco]. One-liter flasks containing 500 ml of the medium were inoculated with 50 ml of fresh culture containing 10^7-10^8 cells/ml and incubated at 30°C with shaking for 7 days to yield a cell density of about 10^8 cells/ml. The organisms were killed with 0.5 mg/l sodium azide for 30 minutes. After being frozen at -20°C for 7 days, they were centrifuged at 10,000g for 30 minutes at 4°C. The pellet was washed twice with 0.01 M PBS [pH 7.2] and resuspended to 25% of the original volume in PBS. After disruption by sonication at 20 kHz for 3 periods of 5minutes each, Pool sonicated antigen was stored at -20°C in small aliquots until used. [22]

Preparation of Successive Frozen and Thawed Antigen: Leptospiral antigen was prepared using extraction technique, as described by Molloy *et al.* [23], with some modifications. Seven-day leptospira culture was

harvested and washed three times with phosphate-buffered saline [PBS, pH 7.2]. The pellet was resuspended with 40mM Tris [pH 7.8] at the ratio of 1:2 and vortexed vigorously for 5 min. The bacterial suspension was then lysed by three cycles of freeze-thawing using liquid nitrogen and 37°C water bath. After centrifugation, the supernatant was stored at -20°C in small aliquots until Antigens used.

Mice Immunization Scheme: Before immunization, the mice were bled individually via the retro-orbital plexus and the sera were collected, pooled and used as a pool of negative control serum. After bleeding, each mouse was injected intraperitoneally with 0.2 ml of a mixture of equal volume of 50 µg of the *L. interrogans* serovar Canicola antigen [500 µg/ml in normal saline solution] and Freund's complete adjuvant. The mice were reimmunized four more times at 2-week intervals using the same immunogen and same route but with Freund's incomplete adjuvant. The second, third, fourth and fifth booster doses were 50, 50, 100 and 120 µg, respectively [24]. Seven days after the fifth immunization, the mice were bled and their sera were assessed for titers of antibody against the homologous antigens by an indirect ELISA, described below. While five mice were inoculated with 1 ml sterile Ellinghausen-McCullough-Johnson-Harris [EMJH] liquid medium as a negative control. The study protocol was approved by the Ethical Committee on Animal Use of the Faculty of Veterinary Medicine, Cairo University.

Microscopic Agglutination Test [MAT]: MAT was performed as described previously [23], with the following antigen of *L. interrogans* serovar Canicola. Serial two fold dilutions of the sera were made in 0.01 M phosphate buffered saline [pH 7.2] starting from 1:50 (then 1:100, 1:200 to 1:800), in microtitre plates. MAT was performed by incubating the sera at 37°C for 90 min with suspensions of live leptospires [1 x 10^8 to 2 x10^8 per ml] of each strain. The titre of a sample was considered as the highest dilution in which an agglutination of 50% or more of leptospires was observed. The degree of agglutination was assessed in terms of the proportion of viable, motile free leptospires present, when compared with the negative control.

Protein Concentration: The total protein content of the different three types of antigenic preparation were estimated with the BCA protein assay kit, according to the manufacturer's instructions Qubit® Protein Assay Kits [Invitrogen, UK].

Determination of Optimal Antigen Concentration for MAT: The plates were incubated with the following sonicated antigen concentrations: 64, 32, 16, 8, 4, 2, 1 µg 500 ng and 250 ng of protein/ml. They were tested against different dilutions begins by 1:40 to 1/81080 dilution of positive sera against serovars Canicola. The 1:400 constant dilution of conjugate goat anti-mouse IgM conjugated to horseradish peroxidase [1:4000 dilution] [SERVA, Germany] was applied.

Preparation of the ELISA Test: The antibody responses against various antigen preparations were evaluated by the Enzyme- Linked Immunosorbent Assay as had been described previously by Chalayon *et al*. [25].

Determination of Cut-Off Points and ELISA Controls: Three positive and five negative controls were always included in each plate. To determine the cut off points, MAT negative control sera diluted at 1:50 were tested by ELISA with each antigen and conjugate and mean optical densities [OD] were calculated. The mean OD450 value was calculated to be 0.405 with a standard deviation (SD) of 0.088. The values corresponding to the negative OD means plus three standard deviations were chosen as cut-off points, calculated to be 0.671.

RESULTS

Determination of the Optimal Antigen Concentration: The optimal concentration of the antigenic preparations obtained was determined to be 16 µg protein/ml. In this ELISA, the different three antigenic preparations reacted with mice antisera against *L. interrogansserovars* Canicola. The three antigenic preparations from *L. interrogans* serovars Canicola showed reactivity against all tested mice 45 antisera listed in Table [1]. The 3 antigenic preparations gave considerably higher A450 values with mice antiserum.

Results of the ELISA Tested Sera: The cut-off value was determined 0.681. None of the negative control samples reacted to MAT at different dilutions. All tested mice sera showed IgM detectable level by ELISA. From the results, the use of the three different antigenic preparations at concentration 16 µg/ml distinguish most clearly the negative control and positive tested group sera. All of the positive-group sera gave higher titers [IgM titers; 1.924 vs. 0.894], while the titers in the negative-group sera OD titers were; 0.302 vs. 0.498.

Table 1: The ELISA OD values of the 45 tested sera of three groups of experimental injected mice with different antigen preparation methods

	Boiled Antigen	Sonicated Antigen	Successive frozen and thawed antigen
S-1	1.454	1.012	1.761
S-2	1.245	1.431	1.524
S-3	1.381	1.011	1.511
S-4	1.033	1.508	1.921
S-5	1.021	1.121	1.812
S-6	1.555	1.357	1.714
S-7	1.002	1.682	1.914
S-8	0.994	1.222	1.741
S-9	0.894	1.072	1.988
S-10	1.247	1.535	1.924
S-11	1.100	1.566	1.879
S-12	1.757	1.324	1.787
S-13	1.155	1.601	1.652
S-14	1.121	1.555	1.921
S-15	1.645	1.420	1.824

Standard deviation: 0.089, Cut-off value 0.681.

Table 2: MAT result of mice sera group [I] injected by boiled leptospira antigen

	1ST GROUP [I]: MICE EXPERIMENTALY INJECTED WITH BOILED LEPTOSPIRA ANTIGEN														
	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10	S-11	S-12	S-13	S-14	S-15
1st Dilution 1:50	N.	N.	N.	P.	P.	N.	P.	P.	P.	P.	N.	N.	P.	N.	P.
2nd Dilution 1:100	N.	N.	N.	P.	P.	N.	P.	P.	P.	P.	N.	N.	P.	N.	P.
3rd Dilution 1:200	N.	N.	N.	P.	P.	N.	P.	P.	N.	P.	N.	N.	N.	N.	P.
4th Dilution 1:400	P.	P.	P.	P.	P.	P.	P.	N.	N.	P.	P.	P.	N.	P.	N.
5th Dilution 1:800	P.	N.	N.	N.	N.	P.	N.	N.	N.	N.	P.	P.	N.	N.	N.
6th Dilution 1:1600	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.

P.: Positive, N.: Negative

Table 3: MAT result of mice sera group [II] injected by sonicated leptospira antigen

	2nd GROUP [II]: MICE EXPERIMENTALY INJECTED WITH SONICATED LEPTOSPIRA ANTIGEN														
	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10	S-11	S-12	S-13	S-14	S-15
1st Dilution 1:50	P.	N.	P.	N.	N.	N.	N.	N.	P.	N.	N.	N.	N.	N.	N.
2nd Dilution 1:100	P.	N.	P.	N.	N.	N.	N.	N.	P.	N.	N.	N.	N.	N.	N.
3rd Dilution 1:200	P.	N.	P.	N.	N.	N.	N.	P	P.	N.	N.	N.	N.	N.	N.
4th Dilution 1:400	P	P.	P	P.	P.	P.	P.	P.	P	P.	P.	P.	P.	P.	P.
5th Dilution 1:800	N.	N.	N.	P.	N.	P.	P.	N.	N.	P.	P.	P.	P.	P.	N.
6th Dilution 1:1600	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.

Table 4: MAT result of mice sera group [III] injected by successive frozen and thawed leptospira antigen

	3nd GROUP [II]: MICE EXPERIMENTALY INJECTED WITH FREEZED and THAWED LEPTOSPIRA ANTIGEN														
	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10	S-11	S-12	S-13	S-14	S-15
1st Dilution 1:50	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
2nd Dilution 1:100	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
3rd Dilution 1:200	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
4th Dilution 1:400	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.
5th Dilution 1:800	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	N.	P.	P.
6th Dilution 1:1600	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.

Considering the cut-off values, several sera with high antibody titer in ELISA are negative by MAT.-Such in group [I] of injected mice as in the case of sera No. 1, 2, 3, 6, 11, 12 and 14 were negative by MAT titre <1:200 [Table 2].

From all the tested sera within group [II] negative MAT titres sera, sample NO. 2, 4, 5, 6, 7, 8 and 10-15 at dilution [< 1:200] although, all previous samples are positive with ELISA test [Table 3].

In group [III] of mice injected by successive frozen and thawed antigen produced the highest OD levels in IgM. Moreover, all tested sera in this group all negative with MAT titer [= 1:200], that phenomena evoke is clearer in this group as, the highest antibody titer response appear in this group [Table 4].

DISCUSSION

Based on our observations in this study, successive injections of mice leptospirosis resistant animal model-with different leptospira antigens evoked variable IgM antibody titers.

Screening for leptospira antibodies in immunized mice after 60 days were applied by two tests, ELISA and MAT. The IgM ELISA detected all cases of immunized mice by the used three (boiled, sonicated and repeated frozen and thawed) antigens of *L. interrogans* serovars Canicola.

It is also interesting to observe that several sera with high ELISA antibody titers were negative by MAT [= 1:200]. It is known that ELISA detects more reactors than MAT, as ELISA detects IgM appears earlier before agglutinins detected by MAT [26-29].

In addition, sera contain non-agglutinating leptospiral ELISA can detect both agglutinating and non-agglutinating antibodies, that explains its higher sensitivity than the MAT. Another, explanation of the false negative result for first dilutions of MAT [= 1:200] observed during the latter post-infection period, especially between 45-60 days post-infection correspond to an excessive increase in the concentration of antibodies against a specific antigen [Prozone effect]. An antibody concentration much higher than the antigen density may inhibit agglutination [30-33].

Malkin [11] correlated the problem of false negative result with MAT to complement system affect, which may also interfere with agglutination, He reduced this phenomenon, by complement inactivation via heat-treating serum in a 56°C water bath or by adding ethylenediamine tetraacetic acid.

Obviously, in our study prozone effect, decreased with dilution of serum till reach MAT =1:200. On differ, of WHO recommendations, the cut-off value that is commonly used for sample dilutions assayed by MATs is 1:100, which is the standard value for screening [34]. MAT depends on the use of live antigen and the standardization of the used antigen is difficult, which depends on the growth time and leptospires density of cultures [35].

Add a paragraph about the difference in percentages of positive and negative with various antigens used in the study.

CONCLUSION

Our study and observation recommended that, the MAT cannot be regarded as providing a reliable estimate of the prevalence of leptospiral prevalence, especially if low titres are given equal consideration to higher ones. This may have happened in some surveys and could be particularly important in the true estimation and evaluation of experimental infection. Regarding to prozone effect, it has a direct influence on both diagnostic and research protocols so, more attention to take precautionary measures for reduction of prozone effect to minimize false negative MAT result by serum dilution but, more standardization parameters and studies on this phenomenon should be approved to avoid any deviation from MAT standard method.

REFERENCES

1. Bharti, A.R., J.E. Nally, J.N. Ricaldi, M.A. Matthias, M.M. Diaz, M.A. Lovett, P.N. Levett, R.H. Gilman, M.R. Willig, E. Gotuzzo and J.M. Vinetz, 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis., 12: 757-771.
2. Levett, P.N., 2001. Leptospirosis. Clin. Microbiol. Rev., 14: 296-326.
3. Silva, M.V., E.D. Camargo, L. Batista, A.J. Vaz, A.P. Brandõa, P.M. Nakamura and J.M. Negrão, 1995 Behaviour of specific IgM, IgG and IgA class antibodies in human leptospirosis during the acute phase of the disease and during convalescence. J. Trop. Med. Hyg., 98: 268-272.
4. O.I.E., 2000. Manual of Standards for Diagnostic Tests and List A and B diseases Vaccines, pp: 165-175.

5. Cumberland, P., C.O. Everard and P.N. Levett, 1999. Assessment of the efficacy of an IgM-ELISA and microscopic agglutination test [MAT] in the diagnosis of acute leptospirosis. Am. J. Trop. Med. Hyg., 61: 731-734.
6. Brandão, A.P., E.D. Camargo, E.D. da Silva, M.V. Silva and R.V. Arbão, 1998. Macroscopic agglutination test for rapid diagnosis of human leptospirosis. J. Clin. Microbiol., 36: 3138-3142.
7. Tizard, I.R., 2004. Veterinary immunology: an introduction, 7th ed., Saunders Company, Philadelphia, pp: 494.
8. Plackett, P. and G.G. Alton, 1975. A mechanism for prozone formation in the complement fixation test for bovine brucellosis. Aust. Vet. J., 51: 374-377.
9. Guven, G.S., B. Cakir, G. Oz, M.D. Tanriover, E. Turkmen and T. Sozen, 2006. Could remembering the prozone phenomenon shorten our diagnostic journey in brucellosis? A case of *Brucella* spondylodiscitis. Rheumatol Int., 26: 933-935.
10. Buzğan, T., H. Karsen, M.K. Karahocagil, H. Akdeniz and M. Sunnetçioğlu, 2007. A case of brucellosis presenting as high titer negative result by standard tube agglutination test. Mikrobiyol. Bul., 41: 151-154.
11. Malkin, K., 1984. Enhancement of *Leptospira hardjo* agglutination titers in sheep and goat serum by heat inactivation. Can. J. Comp. Med., 48: 208-210.
12. Knudtson, W.U. and M. Fetters, 1990. The effect of heat-inactivation on agglutinating antibody titers to *Leptospirainterrogans*. J. Vet. Diagn. Invest., 2: 149-150.
13. Faine, S., B. Adler, C. Bolin and P. Perolat, 1999. *Leptospira* and leptospirosis. 2nd ed. Melbourne, Australia: MediSci.
14. Adler, B., A.M. Murphy, S.A. Locarnini and S. Faine, 1980. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J. Clin. Microbiol., 11: 452-457.
15. Cousins, D.V., G.M. Robertson and L. Hustas, 1985. The use of the enzyme-linked immunosorbent assay [ELISA] to detect the IgM and IgG antibody response to *Leptospirainterrogans* serovars hardjo, pomona and tarassovi in cattle. Vet Microbiol., 10: 439-50.
16. Thiermann, A.B. and L.A. Garrett, 1983. Enzyme-linked immunosorbent assay for the detection of antibodies to *Leptospirainterrogans serovars hardjo* and *pomona* in cattle. Am. J. Vet. Res., 44: 884-887.
17. Bercovich, Z., R. Taaijke and B.A. Bokhout, 1990. Evaluation of an ELISA for the diagnosis of experimentally induced and naturally occurring *Leptospira hardjo* infections in cattle. Vet Microbiol., 21: 255-262.
18. Cho, H.J., S.P. Gale, S.A. Masri and K.L. Malkin, 1989. Diagnostic specificity, sensitivity and cross-reactivity of an enzyme-linked immunosorbent assay for the detection of antibody against Leptospira interrogans serovars pomona, sejroe and hardjo in cattle. Can. J. Vet. Res., 53: 285-289.
19. Yan, K.T., W.A. Ellis, D.P. Mackie, M.J. Taylor, S.W. McDowell and J.M. Montgomery, 1999. Development of an ELISA to detect antibodies to a protective lipopolysaccharide fraction of *Leptospira borgpetersenii* serovar hardjo in cattle. Vet Microbiol., 69: 173-187.
20. Ellis, W.A. and T.W.A. Little, 1986. The present state of leptospirosis diagnosis and control Martinus Nijhoff Publishers, AD Dordrecht, the Netherlands, pp: 85.
21. Terpstra, W.J., G.S. Ligthart and G.J. Schoone, 1980. Serodiagnosis of human leptospirosis by enzyme-linked-immunosorrbent-assay [ELISA]. Zentralblatt Für Bakteriologie-1-Abt-Originale-A: Medizinische Mikrobiologie, Infektionskrankheiten Und Parasitiologie, 247: 400-405.
22. Tansuphasiri, U., S. Deepradit, D. Phulsuksombati and W. Tangkanakul, 2005b. Two simple immunoassays using endemic leptospiral antigens for serodiagnosis of human leptospirosis southeast Asian j trop med Public Health, 36(2): 302-3011.
23. Molloy, M.P., B.R. Herbert, B.J. Walsh, M.I. Tyler, M. Traini, J.C. Sanchez, D.F. Hochstrasser, K.L. Williams and A.A. Gooley, 1998. Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. Electrophoresis, 19: 837–844.
24. Monte, L.G., F.R. Conceição, M.L. Coutinho, F.K. Seixas, E.F. da Silva, F.A. Vasconcellos, L.A. deCastro, C.P. Hartleben, O.A. Dellagostin and J.A. Aleixo, 2011. Monoclonal antibodies against the leptospiral immunoglobulin-like proteins A and B conserved regions. Comp Immunol Microbiol Infect Dis., 34(5): 441-6.

25. Chalayon, P., P. Chanket, T. Boonchawalit, S. Chattanadee, P. Srimanote and T. Kalambaheti, 2011. Leptospirosis serodiagnosis by ELISA based on recombinant outer membrane protein Trans. R. Soc. Trop. Med. Hyg., 105: 289-297.
26. Cumberland, P., C.O. Everard and P.N. Levett, 1999. Assessment of the efficacy of an IgM-ELISA and microscopic agglutination test [MAT] in the diagnosis of acute leptospirosis. Am. J. Trop. Med. Hyg., 61: 731-734.
27. Terpstra, W.J., G.S. Ligthart and G.J. Schoone, 1985. ELISA for the detection of specific IgM and IgG in human leptospirosis. J. Gen. Microbiol., 131: 377-385.
28. Hartman, E.G., M. van Houten, J.A. van der Donk and J.F. Frik, 1984-b. Determination of specific anti-leptospiral immunoglobulins M and G in sera of experimentally infected dogs by solid-phase enzyme-linked immunosorbent assay. Vet Immunol Immunopathol., 7(1): 43-51.
29. Surujballi, O., D. Henning, R. Marenger and C. Howlett, 1997. Development of a monoclonal antibody-based competitive enzyme-linked immunosorbent assay for the detection of *Leptospira borgpetersenii* serovar hardjo type hardjobovis antibodies in bovine sera. Can. J. Vet. Res., 61: 267-274.
30. Plackett, P. and G.G. Alton, 1975. A mechanism for prozone formation in the complement fixation test for bovine brucellosis. Aust. Vet. J., 51: 374-377.
31. Guven, G.S., B. Cakir, G. Oz, M.D. Tanriover, E. Turkmen and T. Sozen, 2006. Could remembering the prozone phenomenon shorten our diagnostic journey in brucellosis? A case of *Brucella* spondylodiscitis. Rheumatol Int., 26: 933-935.
32. Buzğan, T., H. Karsen, M.K. Karahocagil, H. Akdeniz and M. Sunnetçioğlu, 2007. A case of brucellosis presenting as high titer negative result by standard tube agglutination test. Mikrobiyol. Bul., 41: 151-154.
33. Azevedo, L.K.A., P.S.G. Fernandes, D.G.K. Cavalcantim, M.J. e Silva, M.G.L. Batista Neto, V.C.R. Queiroz, V.C.R. Dantas, V.S. de, F. Sales and G.B. Cavalcanti junior, 2006. Caracterização e correlação do fenômeno prózona com títulos de soro-reatividade do VDRL e reação de imunofluorescência indireta em soros de pacientes com sífilis. Rev. Bras. Anal. Clin., 38: 183-187.
34. WHO, 2003. Human leptospirosis: guidance for diagnosis, surveillance and control, WHO Library Cataloguing-in-Publication Data, Geneva, pp: 109.
35. O'Keefe, J.S., 2002. A brief review on the laboratory diagnosis of leptospirosis. N. Z. Vet. J., 50: 9-13.