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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO THE EDTA EXTRACT OF *LEPTOSPIRA INTERROGANS*, SEROVAR *ICTEROHAEMORRHAGIAE*

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Monoclonal antibodies (MABs) were produced against an ethylenediaminetetraacetate (EDTA) extract of Leptospira interrogans *serovar* icterohaemorrhagiae *being characterized by gel precipitation as IgM and IgG (IgG1 and IgG2b). The EDTA extract was detected as several bands by silver staining in SDS-PAGE. In the Western blot the bands around 20 KDa reacted with a monoclonal antibody, 47B4D6, and was oxidized by periodate and was not digested by pronase, suggesting that the determinant is of carbohydrate nature. Immunocytochemistry, using colloidal gold labeling, showed that an EDTA extract determinant recognized by monoclonal antibody 47B4D6, is localized under the outer envelope of serovar* icterohaemorrhagiae. *The MAB raised against the EDTA extract was not able to protect hamsters from lethal challenge with virulent homologous leptospires.*

Key-words: Leptospira. *Monoclonal antibodies. Passive protection.*

Leptospirosis is a zoonosis of world-wide distribution, man being an accidental host.

Several attempts have been made to isolate leptospiral endotoxin. Lipopolysaccharides (LPS) extracted from leptospires showed endotoxic properties[13]. Some authors could not demonstrate any endotoxin activity in LPS extracted from leptospires[8, 7, 28]. The EDTA extract from leptospires showed biological activities characteristic of endotoxin, although these effects were less intense than those of *E. coli* O111:B4 LPS[4].

The development of hybridoma technology facilitated the production of monoclonal antibodies that could be used in identification, localization of antigenic determinants and studies of protective capacity.

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Leptospire genus specific determinant of the protein antigen (GP-Ag) of serovar kremastos recognized by monoclonal antibody was located on the sub-surface of the leptospiral envelope by immunoelectron microscopy and serovar specific TM antigen (LPS) was localized on the cell surface[23]. The same protein antigen was localized on the outer membrane of non pathogenic serovar *andamana*[3]. The LPS of serovar *hardjo* was localized on the outer membrane by means of a monoclonal antibody revealed by immunogold staining in electron microscopy[29]. The determinant to which the MABs against the outer envelope (OE) of serovar *copenhageni* were directed were localized in the leptospiral OE by the immunogold labeling technique[16].

MABs against various antigenic substances of *Leptospira* were prepared and showed protective capacity. The MAB obtained against a glycolipid (OE) passively protected hamsters from leptospiral infection[20]. Hamsters immunized with the MAB against serovar *icterohaemorrhagiae* were protected from challenge with the homologous serovar, but were not protected against the heterologous serovar[31]. The MAB against an OE fraction of serovar *canicola* protected hamsters[30]. Guinea pigs were protected from lethal infection with the daily administration of a MAB against

serovar *copenhageni*[17]. However, the MABs against the outer envelope of serovar *copenhageni* did not protect new-born guinea pigs from challenge with virulent leptospires[16] and MAB LW4 against serovar *lai* Fraction 1 of P-Ag also did not protect hamsters of lethal challenge[20].

This paper reports the production of monoclonal antibodies against the EDTA extract of *Leptospira interrogans*, serovar *icterohaemorrhagiae*, their characterization, localization and passive protection test.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Leptospira interrogans*, serovar *icterohaemorrhagiae*, strain RGA, was grown in synthetic medium[25] at 28°C for 7-10 days and harvested by centrifugation at 6,800g for 20 minutes at 4°C and kept at -20°C until use. The virulent strain of leptospire was isolated from a patient with leptospirosis in Hospital Sarah Kubitschek (Belo Horizonte, MG, Brazil) and was grown in liquid medium (EMJH)[15]. It was identified by the Polymerase Chain Reaction (PCR) as belonging to serovar *icterohaemorrhagiae*[5].

Preparation of the EDTA extract. *Leptospira* cells were extracted by the EDTA method of Leive and Shovlin[19]. The cells were washed three times with 0.12M Tris-HCl, pH 8, ressuspended in the same buffer and brought to 37°C. EDTA (in the same buffer) was added. The final volume contained 0.2g, wet weight, of cells per milliliter and 0.01M EDTA. After 4 minutes of gentle agitation at 37°C, MgCl was added (0.05M final concentration) and the cells were centrifuged at 6,800g for 20 minutes. The supernatant was filtered through millipore filters (0.45mm pore size), dialyzed at 4°C, first against 0.1M sodium phosphate, pH 7.0, for 16-24h and then against several changes of distilled water for 2-4 days. The non-dialyzable material was lyophilized. Hereafter, this material is termed the EDTA extract.

Partial chemical analyses. The LPS was measured by the method of Janda and Work[14], using *E. coli* O111:B4 (Difco Labs.) as standard. Total carbohydrate content was measured by the phenol-sulfuric acid method[5] using glucose as standard. Protein was estimated according to the modified method of Lowry using bovine albumin as standard[16].

Production and screening of monoclonal antibodies. Four weeks old female BALB/c mice were immunized intraperitoneally (i.p.) with the EDTA extract (200µg of LPS) on days zero and 58. Spleen cells were harvested four days later and fusion was performed as described by Galfre and Milstein[9] with P3X63/Ag 8653 myeloma cells. Hybridomas were grown in Dulbecco's minimal essential medium plus hypoxanthine, aminopterine and thymidine (HAT) supplemented with 20% fetal calf serum, 1.0mM pyruvate, 1.0mM oxaloacetic acid, 2.0mM L-glutamine, 0.1 each of a mixture of non-essential aminoacids, 15.0mM HEPES and 0.2 units of bovine insulin. Hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) using the EDTA extract as antigen. They were cloned twice by limiting dilution. Three MABs with high reactivity in ELISA besides good growth characteristics, were selected for further study.

Enzyme-linked immunosorbent assay. An indirect ELISA was carried out according to Adler et al[1]. Microtiter plates were coated either with the EDTA extract (200ng LPS/well) in a 0.06M carbonate-bicarbonate buffer, pH 9.6, for 24h at 4°C. The wells were blocked with a 2% casein-phosphate buffer solution, pH 7.6. Between each stage, the plate was submitted to a series of washings with buffer solution-Tween 20. The wells were probed with the supernatant for 1h at room temperature. A goat anti-mouse horseradish peroxidase conjugate diluted at 1/800 reacted with the antigen-antibody complex for 45 minutes at room temperature. The complex formed was revealed by 0.02M orto phenylen-diamine (Merck) diluted in a 0.01M citrate buffer and added of 0.03% HO for 15 minutes in the dark. The reaction was stopped by 4N $H_2.SO_4$ and read in 492µm (Multiskan, Finlend). The cut-off considered was twice the average optical density (OD) of a leptospire-absorbed mouse serum plus one standard deviation.

Microscopic agglutination test (MAT). The MAT was performed according to the guidelines of the World Health Organization[6].

Isotypes. The monoclonal antibodies were identified by the double immunodifusion method[22] using anti-mouse isotyping reagents (Sigma,USA).

Polyacrylamide gel electrophoresis and Western Blot. The EDTA extract (50µg) was eletrophoresed on 12.5% polyacrylamide gel by the discontinuous method of Laemmli[18] for

four hours at 150V and either silver-stained[12] or transblotted onto nitrocellulose membranes[27]. The membranes were sliced into strips, some of which were treated by periodate, pronase or were left untreated. One strip was then incubated for 1 hour with the anti-EDTA extract MAB. The other strips were incubated with anti-EDTA polyclonal antibody. The immunodetection was completed by incubating with a goat anti-mouse horseradish peroxidase conjugate. Diaminobenzidine and 4-chloronaphtol were used to reveal the bands[11].

EDTA extract treatment with pronase and periodate. Transblotted EDTA extract was treated with pronase (100µg/ml-Promega) in PBS, pH 7.2 at 37°C for 24h. Periodate oxidation was performed as described by Omer et al[21].

Electron microscopy. L. interrogans, serovar *icterohaemorrhagiae* was grown in liquid medium[15] for five days and harvested by centrifugation at 6.800g for 15 minutes. The outer envelope was removed by three exposures to distilled water followed by centrifugation[26]. The pellet was fixed by 0.1% glutaraldehyde for 15 minutes at 4°C and washed three times with PBS pH 8. MAB 47B4D6 was bound to the leptospires after an incubation of one hour at 37°C. Three more washing steps followed. A colloidal gold goat anti-mouse immunoglobulin (Auroprobe EM GAM 10, Ey Labs) was used as second label for one hour at 37°C. After repeated washings, the pellet was deposited on a carbon-coated formvar copper grid. Excess liquid was removed with filter paper. The material was contrasted by 2% ammonium molybdate and observed, after drying, in a transmission electron microscope at 80 KV (Zeiss).

Passive protection assay. Six groups of five golden hamsters (three weeks old, 30 grams average weight) were inoculated intraperitoneally with 1ml of culture supernatant and 24 hours later challenged by the same route with virulent *L. interrogans,* serovar *icterohaemorrhagiae* (107 cells/ml). Deaths were recorded and the animals showed symptoms typical of acute severe leptospirosis (arched back, icterus, hemorrhages).

RESULTS

Screening of monoclonal antibodies. The partial chemical analysis of the EDTA extract revealed the presence of LPS (37.8%), protein (18.3%) and polysaccharides (9.7%).

Fourteen hybridoma cell lines were produced against the EDTA extract. The screening was made by ELISA. The culture supernatants, tested every 48 hours, reacted with the EDTA extract of serovar *icterohaemorrhagiae* and showed an OD of 0.419 to 0.969. All of them were no agglutinating.

The MABs were of the following isotypes: 3 IgG1 (47B4D6, 47B4D11, 47B4F5), 7 IgG2b (47C1C4C8, 47C1C4D4, 47C1C5, 47C1C7, 47A5G11C9, 47A5G11D2, 47A5B4B11) and 1 IgM (47C1C11). Three could not be tested.

Three of them were chosen for the experiments, MABs 47B4D6, 47C1C4C8 and 47C1C11. This selection was based on rapidity of growth, titers in ELISA and isotype.

Gel electrophoresis and immunoblotting antigens antigens. The SDS-PAGE of the EDTA extract demonstrated several bands. Some of them migrated to the same positions as those of *E. coli* O111:B4 LPS showing the same repetitive polysaccharide pattern (Figure 1).

The results of the determination of the chemical nature of the antigenic determinants of the EDTA extract by Western blot are shown in Figure 2. The polyclonal anti-EDTA extract antibody (Strip 1) revealed bands from 17 to 67 kDa. When treated with periodate (Strip 2), the extract bands around 20kDa had their intensities diminished. Strip 3 shows the bands that were not affected by pronase treatment. Strip 4 shows the extract bands revealed by MAB 47B4D6. Also MABs 47C1C11 and 47C1C4C8 were tested and showed the same result. The bands were oxidized by periodate and not digested by pronase, suggesting that the determinant is a carbohydrate.

Electron microscopy. The localization of the EDTA extract determinant in leptospires of serovar *icterohaemorrhagiae* was done by MAB 47B4D6. Figure 3 shows an electronmicrograph of serovar *icterohaemorrhagiae.* Figure 3.1 shows a leptospire with its OE (30,000 X) after reaction with MAB 47B4D6 and a colloidal gold goat anti-mouse conjugate. No binding of the gold particles is seen. Figure 3.2 shows a leptospire without the OE (30,000X) used as a control. Figure 3.3 shows a leptospire without the OE after reaction with MAB 47B4D6, with the goat anti-mouse colloidal gold particles of the conjugate bound to it. We observed that the MAB localized the EDTA extract determinant on the

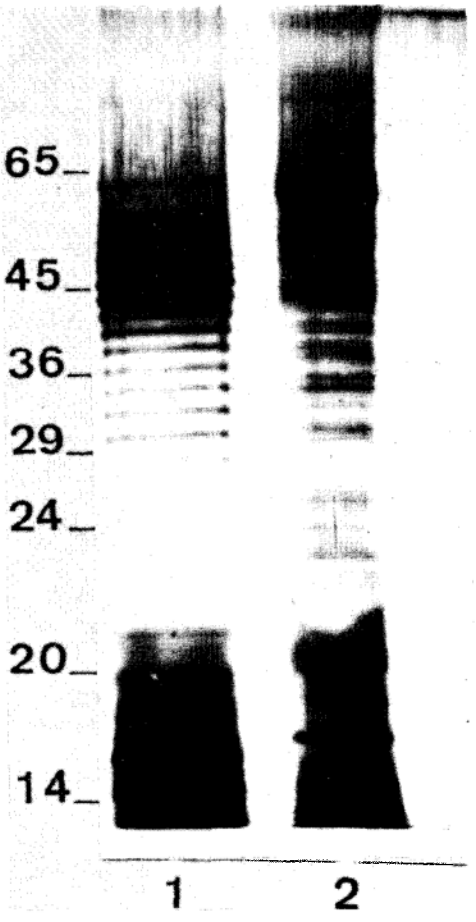


Figure 1 - Polyacrylamide gel electrophoresis of the EDTA extract of Leptospira interrogans, *serovar* icterohaemorrhagie *silver stained. Lane 1 -* E. coli *O111:B4 LPS (50µg). Lane 2 - EDTA extract of* L. interrogans, *serovar* icterohaemorrhagiae *(50µg). Apparent molecular mass markers are indicated on the left of the figure.*

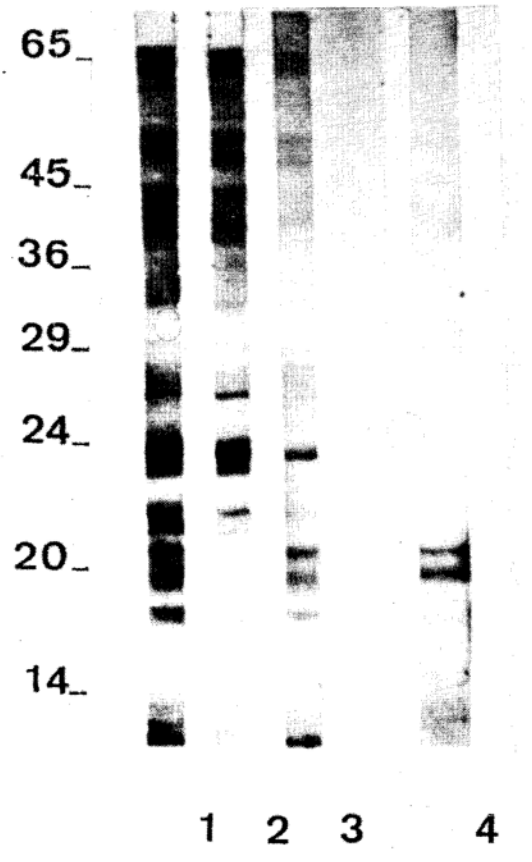


Figure 2 Determination of the chemical nature of the EDTA extract determinants by Western blot. Antigen:EDTA extract of Leptospira interrogans, *serovar* icterohaemorrhagiae. *Strip 1-EDTA extract revealed by polyclonal antibodies. Strip 2-EDTA extract revealed after treatment with periodate. Strip 3-EDTA extract revealed after treatment with pronase. Strip 4-EDTA extract revealed by Monoclonal antibody 47B4D6. Apparent molecular mass markers are indicated on the left of the figure.*

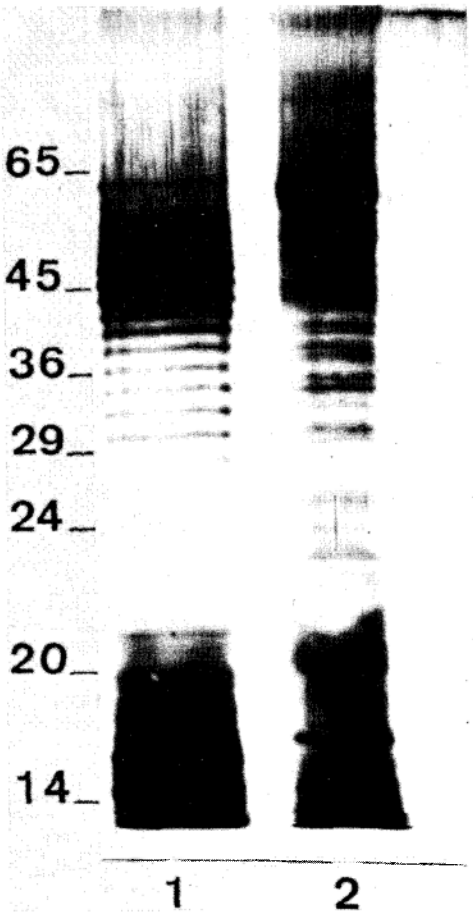
surface layer of the leptospire, under the OE (56,000 X).

Passive protection assay. The results of the passive protection assay with MAB 47B4D6 is shown in Table 1. The control animals died after an average survival time of 3.3 days. Those groups that received the undiluted monoclonal antibody had an average survival of 5.2 days and those that received the 1/10 dilutions of 4.0 days. The kidneys of the

Table 1 - Passive protection of hamsters by monoclonal antibody 47B4D6 against challenge with Leptospira interrogans, serovar icterohaemorrhagiae.

Monoclonal antibody 47B4D6	Number of survivors/ animals tested	Survival average (days)
Undiluted culture supernatant	0/5	5.2
Culture supernatant (1/10 dilution)	0/5	4.0
Control	0/5	3.3

Monoclonal antibody 47B4D6-intraperitoneal inoculation
Leptospires-1 x 10^7 cells/ml, intraperitoneal inoculation



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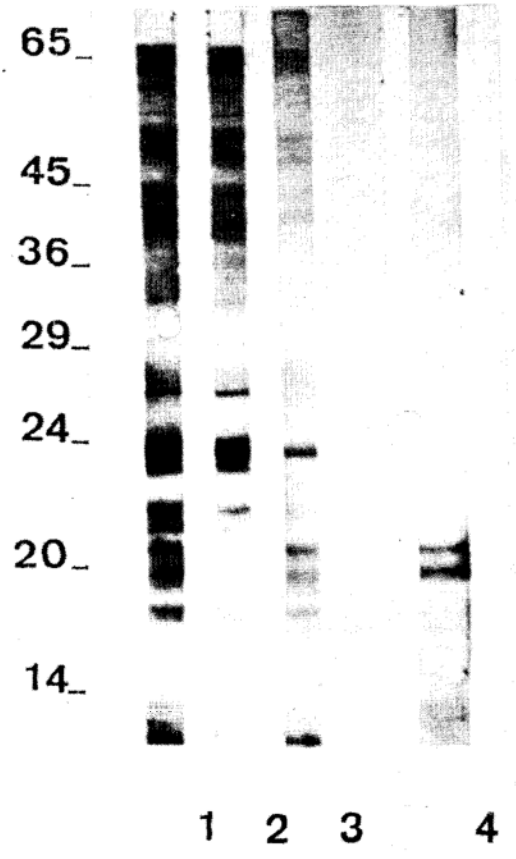

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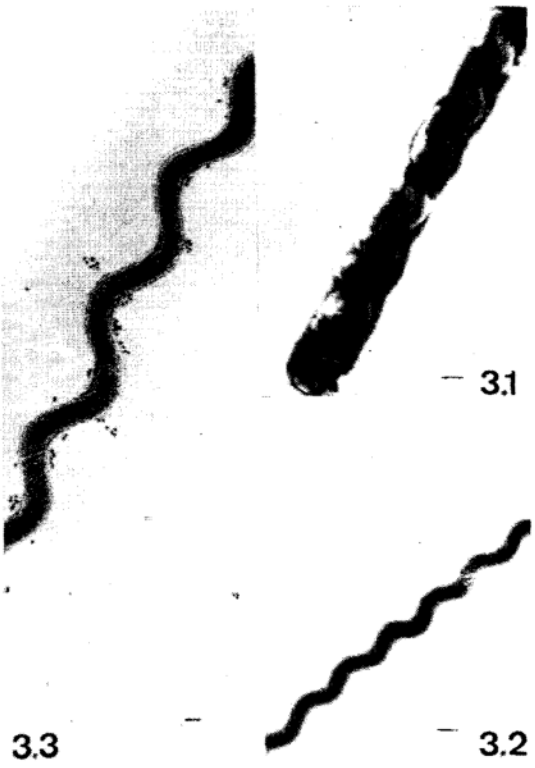

Figure 3 - Electronmicrograph of Leptospira interrogans, *serovar* icterohaemorrhagiae. *3.1 With the outer envelope after reaction with MAB 47B4D6 and a colloidal gold goat anti-mouse conjugate. 30,000x Bar = 0.1µm. 3.2 Without the outer envelope, 30,000x. 3.3 Without the outer envelope bound to the monoclonal antibody 47B4D6 and to a goat-anti-mouse colloidal gold conjugate, 56,000x, Bar=0.2µm.*

animals were cultured for 30 days and no evidence of leptospira growth was observed.

DISCUSSION

Fourteen MABs against the EDTA extract were produced and characterized as IgG1 (3), IgG2b (7), IgM (1) and three were not tested. All of them were non agglutinating. The MABs produced against the OE of serovar *copenhageni*, were also non agglutinating[16].

The study of the chemical nature of the antigenic determinant of the EDTA extract by PAGE and Western Blot (WB) techniques showed that this extract was not affected by pronase, but was oxidized by periodate, suggesting a carbohydrate nature. Immunodiffusion and WB experiments showed that MAB MUM/F1-1 of serovar *copenhageni* reacted with a carbohydrate determinant in the *leptospiral* LPS[17]. The MAB against serovar *copenhageni* also reacted with an epitope of carbohydrate nature when sonicated antigens were digested with pronase[24]. The MAB against the OE of serovar *hardjo* reacted with a protein determinant in WB experiments[16].

When localizing the antigenic determinant, we observed that MAB 47B4D6 bound to the *leptospira* cell only after the removal of the OE, indicating that it is under this structure. The MAB against the genus specific protein antigen (GP-Ag) of serovar *kremastos*, labeled with peroxidase or iodinated, also bound to the sub-surface of the OE[23]. However, the MAB against GP-Ag of non-pathogenic serovar *andamana* bound to the OE[2]. The MAB against LPS of serovar *hardjo* or the OE of serovar *copenhageni* bound to the outer membrane of leptospire as evidenced by immunogold labeling techniques[16,29].

The MAB against the EDTA extract of serovar *icterohaemorrhagiae*, when inoculated in hamsters could not protect them from lethal challenge with virulent leptospire. MAB against serovar *copenhageni* and MAB LW4 against serovar lai did not protect new-born guinea pigs[16] or hamsters[20] against lethal infection. However, MAB against serovar *copenhageni* could passively protect guinea-pigs against lethal leptospirosis when daily administered in the dose of 175mg to ensure a protective titer[17]. The MABs against serovar *copenhageni* also protected hamsters, dogs and monkeys[24] and the MAB obtained against a glycolipid of the OE (PAg) from serovar *lai* protected hamsters from *leptospiral* infection[20]. Immunized hamsters with V1 MAB against serovar *icterohaemorrhagiae* were protected from lethal challenge with the homologous serovar[31].

In conclusion, we have a non agglutinating anti-EDTA extract MAB, that did not protect hamsters from challenge with virulent leptospires of the homologous serovar. The determinant is of carbohydrate nature and is located under the *leptospiral* OE.

RESUMO

Anticorpos monoclonais (AcM) foram produzidos contra o extrato EDTA obtido de Leptospira interrogans, *sorovar* icterohaemorrhagiae.

Pelo teste de precipitação foram caracterizados como IgM e IgG (IgG1 e IgG2). A eletroforese em gel de poliacrilamida do extrato EDTA revelou diversas bandas quando corada pela prata. No "Western blot", as bandas em torno de 20 kDa reagiram com o AcM 47B4D6, foram oxidadas pelo periodato e não digeridas pela pronase, sugerindo que o determinante é de natureza carboidrato. O determinante reconhecido pelo AcM 47B4D6 está localizado sob o envelope externo como revelado pela imunocitoquímica usando marcação com ouro coloidal. O AcM contra extrato EDTA do sorovar icterohaemorrahagiae *não protegeu hamsters quando inoculados com lepstopira homóloga virulenta.*

Palavras-chaves: Leptospira. *Anticorpos monoclonais. Proteção passiva.*

REFERENCES

1. Adler B, Murphy AM, Locarnini SA, Faine S. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 11:452-457, 1980.
2. Cacciapuoti B, Ciceroni L, Saito T, Ono E, Yanagava R. Broad reacting surface antigens in *Leptospira biflexa* serovar *andamana*. Zentralbatt fur Bakteriologie Mikrobiologie und Hygiene Series A 269:86-99, 1988.
3. Caballero OLSD, Neto ED, Koury MC, Romanha AJ, Simpson AJG. Low-stringency PCR with diagnostically useful primers for identification of Leptospira serovars. Journal of Clinical Microbiology 32: 1369-1372, 1994.
4. De Souza L, Koury MC Isolation and biological activities of endotoxin from *Leptospira interrogans*. Canadian Journal of Microbiology 38:284-289, 1992.
5. Dubois M, Gilles KA, Hamilton JK, Rebers A, Smith F. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-355, 1956.
6. Faine S (Ed). Guidelines for the control of leptospirosis. WHO Offset Publication 37, Geneva, 1982.
7. Faine S, Adler B, Palit A. Chemical, serological and biological properties of a serotype-specific polysaccharide antigen in Leptospira. Australian Journal of Experimental Biology and Medical Science 52: 311-319, 1974.
8. Finco DR, Low DG. Endotoxin properties of *Leptospira canicola*. American Journal of Veterinary Research 28:1863-1872, 1967.
9. Galfré G, Milstein C. Preparation of monoclonal antibodies; strategies and procedures. Methods in Enzymology 73:3-46, 1981.
10. Hartree EF Determination of protein: a modification of Lowry method that gives a linear photometric response. Analitical Biochemistry 48:422-427, 1972.
11. Hawkes R, Niday E, Gordon J. A dot immunobinding assay for monoclonal and other antibodies. Analitical Biochemistry 119:142-147, 1982.
12. Hitchcock P, Brown T. Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. Journal of Bacteriology 154:269-277, 1983.
13. Isogai E, Isogai H, Kurebayashi Y, Yto N. Biological activities of leptospiral lipopolysaccharide Zentralbatt fuer Bakteriologie Mikrobiologie und Hygiene Series A 261:53-64, 1986.
14. Janda J. Work E. A colorimetric estimation of lipopolysaccharides. Febs Letters 16:343-345, 1971.
15. Johnson RC, Harris VJ. Differentiation of pathogenic and saprophytic leptospires. 1. Growth at low temperatures. Journal of Bacteriology 94:27-31, 1967.
16. Jost BH, Adler B, Vinh T, Faine, S. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. Journal of Medical Microbiology. 22:269-275, 1986.
17. Jost BH, Adler B, Faine S. Reaction of monoclonal antibodies with species-specific determinants in *Leptospira interrogans* outer envelope. Journal of Medical Microbiology 27:51-57, 1986.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.
19. Leive L, Shovlin VK, Mergenhagen E. Physical, chemical, and immunological properties of lipopolysaccharide released from *Escherichia coli* by ethylenediamine tetraacetate. Journal of Biological Chemistry 243:6384-6391, 1968.
20. Masuzawa T, Nakamura R, Hashiguchi Y, Shimizu T, Iwamoto Y, Morita T, Yanagihara Y Immunological reactivity and passive protective activity of monoclonal antibodies against protective antigen (Pag) of *Leptospira interrogans* serovar lai.

Zentralbatt fuer Bakteriologie Mikrobiologie und Hygiene Series A 272: 328-336, 1990.

21. Omer PA, Manson M, Woody JN. Antibody to carbohydrate and polypeptide epitopes on the surface of schistosomula of *S. mansoni* in Egyptian patients with acute and chromic shistosomiasis. Parasitology 98:417-424, 1989.
22. Ouchterlony O. Diffusion in gel methods for immunological analysis. Progress in Allergy 5:178, 1968.
23. Sakamoto N, Ono E, Kida H. Production of monoclonal antibodies against leptospiral genus-specific protein antigen and localization of the antigen by immunoelectron microscopy. Zentralbatt fuer Bakteriologie Mikrobiologie und Hygiene Series A 259:557-563, 1985.
24. Schoone GJ, Everard COR, Korver H. An immunoprotective monoclonal antibody direct against *Leptospira interrogans* serovar copenhageni. Journal of General Microbiology 135:73-78, 1989.
25. Shenberg E. Growth of pathogenic Leptospira in chemically defined media. Journal of Bacteriology 93:1598-1606, 1967.
26. Takeya K, Mori R, Toda T. Studies on the structure of Leptospira as revealed by the electron microscope. Japanese Journal of Microbiology 1:99-101, 1957.
27. Towbin H, Staehelin T, Gordon J. Eletrophoretic transfer of protein from polyacrylamide gel to nitro cellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America 76:4350-4354, 1979.
28. Vinh T, Adler B, Faine S. Glycolipoprotein cytotoxin from *Leptospira interrogans* serovar copenhageni. Journal of General Microbiology 132:111-123, 1986.
29. Vinh T, Shi M, Adler B, Faine S. Characterization and taxonomic significance of LPS of *Leptospira interrogans*, serovar hardjo. Journal of General Microbiology 135:2663-2673, 1989.
30. Yanagihara Y, Masuzawa T, Matsumoto T, Shimizu T, Takayanagi K. Monoclonal antibody against surface antigen of *Leptospira interrogans* serovar *canicola*. Israel Journal of Veterinary Medicine 44:56-58, 1988.
31. Zhang WH, Xiu-Fen B, Qing-Dong D, Di-Kai N. Investigation on monoclonal antibodies against two serovars of the icterohaemorrhagiae serogroup of Leptospira. Zentralbatt fuer Bakteriologie Mikroskopie und Hygiene Series A 270:329-336, 1989.