Veterinary Immunology and Immunopathology, 7 (1984) 33—42
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

SERODIAGNOSIS OF CANINE LEPTOSPIROSIS BY SOLID-PHASE ENZYME-LINKED IMMUNOSORBENT ASSAY

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(Accepted 20 December 1983)

ABSTRACT

Hartman, E.G., van Houten, M., van der Donk, J.A. and Frik, J.F., 1984. Serodiagnosis of canine leptospirosis by solid-phase enzyme-linked immunosorbent assay. Vet. Immunol. Immunopathol., 7: 33-42.

An enzyme-linked immunosorbent assay (ELISA) to detect antibodies to Leptospira interrogans serotype canicola in dogs was developed and evaluated. Comparison of the ELISA with the microscopic agglutination test (MAT) showed that, during the first two weeks after an experimental infection with serotype canicola, the ELISA detected antibody at higher dilutions than the MAT. After the second week post-infection both tests detected antibody at almost equal titres (r=0.89). The outer envelope (OE) antigen of serotypes icterohaemorrhagiae, copenhageni and canicola was fairly serotype-specific, whereas the pellet (P) antigen showed more cross-reactivity. Both OE and P antigen of Leptospira biflexa strain Patoc I could be used as cross-reacting antigen in the ELISA. Compared to the MAT, the ELISA has some technical advantages. It is suggested that the ELISA would be useful as a screening test.

INTRODUCTION

The diagnosis of leptospirosis in man and animals has always been a problem (WHO, 1967b). The laboratory diagnosis can be established either by isolating the leptospires from blood, urine and/or organs such as the liver and kidneys or by detecting specific circulating antibodies. The isolation and identification of leptospires is time-consuming and will only permit a retrospective diagnosis. Moreover, isolation often fails because many patients are treated with antibiotics. The microscopic agglutination test (MAT), using live leptospires as antigen, is the standard reference test for the serodiagnosis of leptospirosis (Wolff, 1954; WHO, 1967a; Turner, 1968). Disadvantages of the MAT include the large number of serotypes to be maintained and the risk of infection of technicians. Moreover, the test is complicated and time-consuming, and can only be performed by experienced technicians.

The solid-phase enzyme-linked immunosorbent assay (ELISA) has been used for the serodiagnosis of a wide range of infectious diseases in man and animals,

0165-2427/84/$03.00

including leptospirosis (Bruggmann et al., 1973; Voller et al., 1978; Adler et al., 1980; Terpstra et al., 1980; Adler et al., 1981). Most of the disadvantages of the MAT do not apply to the ELISA. Antigen can be prepared in large quantities and can be stored for several months (Terpstra et al., 1980). The ELISA could be simplified by using a single antigen which cross-reacts with all serotypes detected in an animal species in a particular area. Terpstra et al. (1980) described the use of serotype icterohaemorrhagiae strain Wijnberg antigen in the ELISA as a genus-specific antigen. Leptospira biflexa strain Patoc I was used as a cross-reacting antigen in various genus-specific tests (Turner, 1968; Palit and Gulasekharam, 1973). In the Netherlands only infections with serotypes icterohaemorrhagiae, copenhageni and canicola of Leptospira interrogans are observed in almost every case of canine leptospirosis (Hartman, 1977). A single antigen cross-reacting with the above serotypes could be used for serological screening of dogs suspected of having leptospirosis.

The results of the use of the ELISA in the serodiagnosis of canine leptospirosis are presented in the present paper. The ELISA method is compared with the MAT and, in addition, the degree of cross-reactivity of a number of different antigens is tested.

MATERIALS AND METHODS

Dogs

Five 1- to 2-year-old seronegative (MAT) dogs of either sex (four beagles and one bull-terrier) were experimentally infected by intraperitoneal inoculation of 5 ml of a 10% hamster liver suspension containing 5.4×10^5 to 6.8×10^8 hamster-virulent Leptospira interrogans serotype canicola leptospires. The concentration of the micro-organisms in the liver suspension was determined by counting in a Helber counting chamber (Gelman Hawksley Ltd.).

The dogs were inoculated sequentially with a liver suspension of a hamster which had been inoculated with positive blood of the preceding dog. Blood samples were cultured at each collection date by adding three drops of blood to 5 ml of EMJH medium (Difco). The cultures were incubated at $30^{\circ}C$ and examined by dark-field microscopy weekly for four weeks. Leptospires were recovered from the blood of all dogs during the first five days after infection.

Serum samples

Serial serum samples were collected from each dog before and after experimental infection and stored in a frozen state ($-70^{\circ}C$) until use. Control sera were obtained from seronegative (MAT) beagles.

The bull-terrier was not available for the experiment after day 23.

Microscopic agglutination test (MAT)

The MAT was carried out in Cooke Microtiter plates. Serial two-fold dilutions of serum starting with 1 in 20 were made in Chang (1947) buffer. The titre is expressed as the reciprocal of the highest dilution of serum at which at least 50% of the leptospires were agglutinated.

Cultures of Leptospira interrogans serotype canicola strain Hond Utrecht IV, serotype icterohaemorrhagiae strain Kantorowics and serotype copenhageni strain Wijnberg were used as antigen. The density of each antigen was approximately 10^8 leptospires per ml. Titres equal to or higher than 160 were considered to be positive (WHO, 1967a).

ELISA

Preparation of outer envelope (OE) antigen. Leptospira interrogans serotype canicola strain Hond Utrecht IV, serotype icterohaemorrhagiae strain Kantorowics and serotype copenhageni strain Wijnberg, and Leptospira biflexa strain Patoc I were grown for seven days in EMJH medium (Difco) at 30°C to a density of approximately 10^9 leptospires per ml. OE antigen was prepared using the method described by Bey et al. (1974) with slight modifications. Leptospires (1l) were harvested by centrifugation at 15000 x g for 50 min at 4°C in a Sorvall RC 2 B centrifuge. The pellet was washed three times with PBS 7.4 to remove bovine albumin and resuspended in 50 ml of a 1M NaCl solution. After approximately 45 min the then spherical leptospires were sedimented at 15000 x g and resuspended in 2.5 ml of distilled water. This cell suspension was treated with 50 ml of 0.04% sodium dodecyl sulfate (SDS) solution (Sigma Chemical Co.) for 2 min at 23°C to solubilize the OE. The SDS-treated cells were sedimented at 15000 x g and the supernatant was passed through a 0.45 µm membrane filter (Millipore Corp.). The supernatant was dialysed against PBS 7.4 containing 0.02% sodium azide and 1 mM EDTA to prevent reaggregation of the solubilized OE on removal of SDS (Bruggmann et al., 1977). The protein content of the antigen preparation was determined by the method of Lowry et al. (1951).

Preparation of pellet (P) antigen. The pellet of the 0.04% SDS-treated leptospires was resuspended in 25 ml of 1% SDS. After incubation for 30 min at 37°C, the suspension was centrifuged at 15000 x g. The supernatant was passed through a 0.45 µm Millipore filter. SDS was removed as described above with the exception that 10 mM EDTA was used to prevent reaggregation. The protein content was determined by the method of Lowry et al. (1951).

Determination of the optimum coating procedure. Antigen was diluted (5 µg/ml) in either Chang buffer, PBS 7.4, 0.1M carbonate buffer pH 9.6 or distilled water. Polystyrene microtitre plates (Cooke Dynatech, Microelisa) were coated overnight at room temperature (wet antigen plates). The most satisfactory results were obtained with Chang buffer. Incubation overnight at room temperature

resulted in a more uniform coating than did 2 h at 37°C or 2 h at room temperature. Overnight incubation at 37°C (dried antigen plates) resulted in non-specific reactions with negative control sera. Antigen-coated plates could be stored at -70°C for at least two months.

Determination of the optimum antigen concentration. Microtitre plates were incubated overnight at room temperature with various concentrations of SDS-treated antigen (OE and P) in Chang buffer and tested in the ELISA with serial two-fold dilutions of MAT-positive serum as well as MAT-negative serum. Optimum antigen concentrations (µg/ml of protein) were: Hond Utrecht IV (OE and P), Wijnberg (OE and P) and Kantorowics (OE and P) 2 µg/ml, Patoc I OE 4 µg/ml and P 2 µg/ml. Antigen concentrations exceeding 5 µg/ml resulted in a reduced ELISA titre.

Determination of the optimum serum diluent. Various serum diluents were tested: PBS 7.4, PBS + 0.1% (v/v) Tween 20, PBS + 1% (w/v) egg albumin (Difco), PBS + 0.1% Tween 20 + 1% egg albumin, PBS + 0.1% Tween 20 + 1% bovine albumin fraction V (BDH Chemicals Ltd.) and PBS + 0.1% Tween 20 + 1% horse serum. Optimum results were obtained with PBS + 0.1% Tween 20 + 1% egg albumin.

Prior incubation with 1% egg albumin in PBS to prevent possible non-specific binding of antibodies to the solid-phase was not required.

Determination of the conjugate dilution. Dilutions of 1 in 500, 1 in 750 and 1 in 1000 of horse-radish peroxidase-conjugated rabbit anti-dog Ig (Miles Laboratories) were tested in a chequer-board titration with positive and negative sera. The conjugate dilution which produced the highest titre with MAT-positive serum and no non-specific reaction with MAT-negative serum, was 1 in 750. The optimum conjugate dilution varies, however, from batch to batch.

Test procedure. The antigen-coated plates were washed thoroughly three times in a total quantity of 2 l of tapwater containing 0.05% (v/v) Tween 20. Then 100 µl of PBS 7.4 with 0.1% Tween 20 and 1% egg albumin were added to each well and serial two-fold dilutions of serum were made, starting with 1 in 20. The plates were incubated for 2 h at 37°C. After washing three times with 2 l of tapwater-Tween 20, 100 µl of horse-radish peroxidase-conjugated rabbit anti-dog Ig, diluted 1 in 750 with PBS 7.4 containing 0.1% Tween 20 and 1% egg albumin, was added and incubated for 1 h at 37°C. Finally, after the plates had been washed three times as previously described, 100 µl of substrate was added.

The substrate was prepared just before use by dissolving 80 mg of 5-amino-2-hydroxybenzoic acid (Merck) in 100 ml of hot distilled water (70°C). The solution was then cooled to room temperature and the pH adjusted to 6.0 with 1N NaOH. To 90 ml of the 5-amino-2-hydroxybenzoic acid solution 10 ml of freshly prepared 0.05% H_2O_2 was added. As enzymic degradation of 5-amino-2-hydroxybenzoic acid results in a partially insoluble product, this substrate is only acceptable for visually read ELISA tests (Voller et al., 1978). Positive wells can

easily be distinguished from negative wells. The titre is expressed as the reciprocal of the highest serum dilution presenting a clearly visible brown colour. Controls of antigen, conjugate and substrate were included in the test.

RESULTS

Table I shows the results of the titration of the sera of one out of five experimentally infected dogs (dog no. 4). As the titration of the sera of the other four dogs gave comparable results, these are omitted. The titration of the sera was performed by both the MAT and ELISA techniques using various antigens.

TABLE I

MAT and ELISA titres in serum of one out of five experimentally infected dogs (dog no.4).

Antigen:	Hond Utrecht IV			Wijnberg			Kantorowics			Patoc I	
Day	MAT	OE	P	MAT	OE	P	MAT	OE	P	OE	P
0	θ	θ	θ	θ	θ	θ	θ	θ	θ	θ	θ
2	θ	θ	θ	θ	θ	θ	θ	θ	θ	θ	θ
4	θ	θ	θ	θ	θ	20	θ	θ	θ	θ	θ
7	θ	80	160	θ	20	160	θ	20	160	80	80
9	θ	160	160	θ	80	160	20	80	320	160	160
14	320	320	320	θ	160	320	40	160	640	320	320
24	320	160	160	θ	40	160	20	80	320	160	160
31	320	160	160	θ	40	160	20	80	320	80	80
45	160	160	160	θ	80	160	θ	80	320	40	40
52	80	80	160	θ	80	80	θ	80	160	40	80
87	20	40	40	θ	θ	20	θ	θ	40	20	20
122	20	40	40	θ	θ	20	θ	θ	40	20	20

θ = <20

Cross-reactivity of various antigens

ELISA. When the homologous antigen (Hond Utrecht IV) was used in the ELISA, the OE and P titres were approximately equal. When heterologous P antigens (Wijnberg and Kantorowics) were used, the titres were almost as high as those attained with the homologous OE and P antigens.

The use of heterologous OE antigens resulted in titres which were decidedly lower than those with the homologous OE antigen.

With the exception of dog no. 1 the use of OE and P antigen of Patoc I usually resulted in lower titres. However, the ELISA using Patoc I antigen became positive at the same time as the ELISA with Hond Utrecht IV antigen. MAT-negative control sera were also negative in the ELISA when the OE antigen of Hond Utrecht IV, Wijnberg or Kantorowics was used. However, in dog no. 2 titres up to 20 were recorded in preinoculation sera when the P antigens of these strains were used.

Again, with the exception of dog no. 1 (from which a titre up to 20 was observed with OE antigen of Patoc I prior to the experimental infection), when Patoc I OE and P antigen were used, the controls were negative. From the above it follows that only ELISA titres of 40 or higher may be regarded as specific.

MAT. Few if any cross-reactions were observed in the MAT with serotype copenhageni strain Wijnberg. Cross-reactions with serotype icterohaemorrhagiae strain Kantorowics occurred at titres as high as 80, with the exception of dog no. 1 in which sera titres up to 640 with respect to strain Kantorowics were detected.

Comparison of ELISA and MAT

A comparison between MAT titres and ELISA (OE antigen) titres using Hond Utrecht IV antigen of sera drawn from day(D)0 to D14 after infection is shown in Fig. 1. ELISA titres were significantly higher than MAT titres during this period (chi^2=8.47; df=1; $P < 0.01$). The correlation coëfficient obtained from a linear regression analysis was r=0.57 (df=12; $P < 0.05$). Samples with a titre of < 20 in one or both of the serological tests were not included in the statistical analysis.

Three serum samples taken during the first two weeks after the infection did not show any reaction in the MAT, whereas the ELISA titre was already positive ($\geq$40).

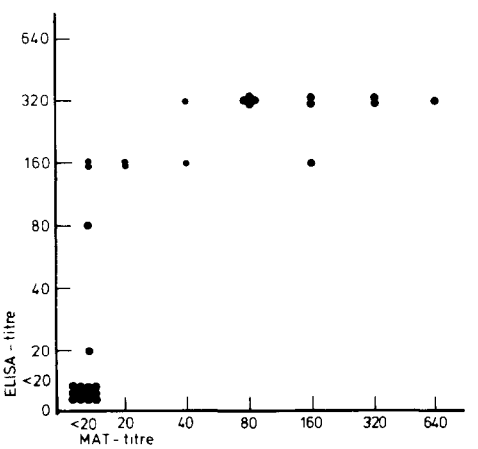

Fig. 1. Comparison between MAT and ELISA titres of sera drawn from D0 to D14 after infection of five dogs with serotype canicola.

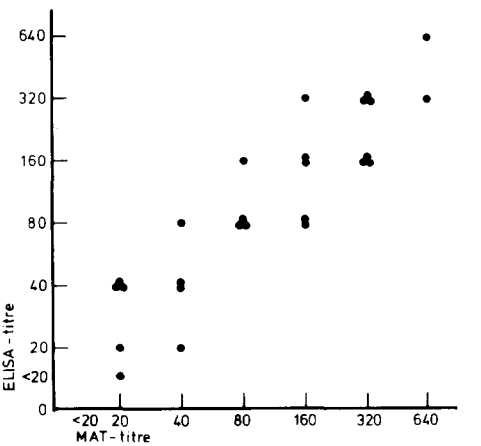

Fig. 2. Comparison between MAT and ELISA titres of sera drawn from D14 after infection of five dogs with serotype canicola.

A comparison of MAT and ELISA (OE antigen) titres of sera drawn after D14, as presented in Fig. 2, shows that the two tests detected antibody at almost equally high titres ($r=0.89$; $df=23$; $P<0.001$). The ELISA also detected antibody at higher titres than the MAT from D0 to D14 when homologous or heterologous P antigens were used. As a rule, this was less obvious using the heterologous OE antigens and the OE and P antigen of Patoc I (Table I).

Table II shows the days on which the MAT and ELISA (OE antigen) titres were ≥ 160 for the first time, using Hond Utrecht IV antigen. With the ELISA technique this titre was attained on average 5.0 days earlier in the course of the infection than it was with the MAT. Using Student's t-test for related observations, this difference proved to be significant ($t=5.27$; $df=4$; $P<0.01$).

Dog no.	MAT	ELISA
1	6	4
2	15	8
3	11	7
4	14	9
5	13	6

TABLE II

Number of days after infection when MAT titres and ELISA titres were ≥ 160 for the first time.

DISCUSSION

The present study shows that the P antigens of the strains Kantorowics (serotype icterohaemorrhagiae) and Wijnberg (serotype copenhageni) cross-react in the ELISA test to almost the same titre as the homologous antigen with sera positive for serotype canicola. The OE or P antigen of Patoc I also cross-reacted in the ELISA with these sera. Studies on dogs most probably infected with serotype icterohaemorrhagiae or copenhageni showed that the P antigens of Wijnberg and Kantorowics could also be used for screening. Because cross-reactions with serotype canicola strain Hond Utrecht IV (OE and P) antigen occurred to a lesser extent in these cases, these serotype canicola antigens are not suitable for screening dogs by ELISA under the conditions prevailing in the Netherlands. The results of the present study are in accordance with the results obtained by Terpstra et al. (1980), who reported that both the Wijnberg strain and the L. biflexa strain Patoc I antigen prepared from a heated culture were reactive over a wide range in the ELISA in serological testing for human leptospirosis.

Bruggmann et al. (1977) successfully employed SDS-prepared antigen in serological testing by the ELISA technique of pigs infected with Mycoplasma suipneumoniae. Moreover, the fact that the outer envelope of leptospires was shown to be a primary target for the antibody-complement bactericidal reaction (Anderson and Johnson, 1968) and to be a potent immunogen in cattle (Bey and Johnson, 1978b), dogs (Bey and Johnson, 1978a), foxes (Glosser et al., 1974) and hamsters (Auran et al., 1972; Bey et al., 1974; Glosser et al., 1974) argues in

favour of the choice of OE antigen. Cross-reactions, particularly with pellet (P) antigen, and the more serotype-specific character of the OE antigen are in accordance with the theory advanced by Rothstein and High (1957) that at least two different antigens are present in leptospires, the peripheral or P antigen (not to be mistaken for the present author's P = pellet antigen) which is believed to be serotype-specific, and the somatic or S antigen which is genus-specific. The OE antigen is comparable with the P (peripheral) antigen of Rothstein and High, and the P (pellet) antigen used by the present authors with the S (somatic) antigen. Fathalla and Coghlan (1980) demonstrated that sucrose-density-gradient fractions of SDS-treated leptospires could be divided into genus-specific, serogroup-specific, and serotype-specific pools. This supports our conclusions.

Although cross-agglutination reactions are frequently observed in leptospirosis serology (Turner, 1968), marked cross-agglutination reactions did not occur in the present experiment with the exception of one dog. The higher agglutination titres detected in the serum of that dog in response to serotype copenhageni strain Kantorowics are probably not due to an intercurrent infection with this serotype because ELISA titres, using OE antigen of this serotype were lower than those recorded using P antigen, as they were in the four other dogs. In this experiment ELISA titres, using OE or P antigen homologous to the infecting strain, were almost identical in all five dogs. In view of this, it could be expected that the OE and P titres with respect to serotype copenhageni would have been equal in the case of an intercurrent infection with this serotype.

In the present experimental design the ELISA using homologous OE antigen became positive on average 5.0 days earlier in the course of the infection than did the MAT, on the basis of a titre equal to or exceeding 160 for the two tests. If the ELISA were based on titres equal to or exceeding 40, this difference would probably increase. When homologous or heterologous P antigens were used, the ELISA was also more sensitive than the MAT during the initial stage of infection (Table I). The antigen-antibody system measured by the ELISA apparently differs from that measured by the MAT in which only agglutinating antibodies are involved. The test may be regarded as specific as only titres < 40 were observed in control sera.

CONCLUSIONS

Introduction of the ELISA technique into the serodiagnosis of canine leptospirosis may be regarded as a welcome addition. The test is fairly simple to perform and could be easily adopted in laboratories in which the ELISA technique is already being used in the serodiagnosis of infectious diseases. The antigen can be prepared in large quantities and stored over a prolonged period. The

ELISA technique in this form will not distinguish between titres which are due to acute leptospirosis or result from a previous infection or (recent) vaccination. This problem requires closer study of specific anti-leptospiral IgM and IgG based on the fact that the production of IgM can be detected during the initial stage of an infectious disease, whereas the production of IgG starts some time later.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. G.M.S. van Dam-Knubben for excellent technical assistance, Mr. P. Bradley for linguistic advice and Prof.Dr. W.A. de Voogd van der Straaten for statistical assistance. Duphar Nederland B.V. is gratefully acknowledged for the supply of beagles.

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