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DOT ELISA AS A RAPID METHOD FOR SEROLOGICAL DIAGNOSIS OF CANINE BRUCELLOSIS

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In this work, we describe a dot-enzyme linked immunosorbent assay with two different antigens of Brucella canis. The antigens, soluble sonic and outer membrane proteins were adsorbed on to a solid nitrocellulose surface. The test was applied to 40 field serum samples previously analysed by the rapid slide agglutination test and the 2-mercaptoethanol tube agglutination test. Sera were obtained from clinically suspected dogs of different age, sex and breed. Sera were also obtained from four clinically healthy animals and used as a negative control. One sample originated from a female with a high antibody titer and positive isolation of Brucella canis. Out of the 44 examined sera, 19 had agglutination titers > 1/50. A positive reaction to proteins of the soluble sonic extract, was obtained with 10 sera and with outer membrane proteins with 7 sera. All positive sera in the dot-ELISA had agglutination titers > 1/100.

This study shows that proteins of soluble sonic extract of Brucella canis in dot-ELISA are reliable and highly specific for rapid diagnosis of brucellosis in dogs.

Key words: brucellosis, dog, Brucella canis, dot-ELISA.

INTRODUCTION

Brucellosis of dogs caused by *Brucella canis* is present in most countries in the world, especially those with many stray dogs (Flores-Castro et al., 1977). With infected animals, clinical signs occur in the genital tract, causing abortion in pregnant females, while orchitis is present in males. Usually, one testicle is affected (Carmichael and Kenny 1968). *Brucella canis* is highly adapted to dogs, although human infections have been reported (Alton et al., 1988; Corbel 1997). Therefore, this species of *Brucella* is also significant for public health. Isolation of the microorganism is the most reliable diagnostic method. Unfortunately, in most cases the isolation is unsuccessful, because of antibiotic therapy applied to the animals prior to sampling and objective diagnosis (Alton et al., 1988). For serological diagnosis several tests are applied, but problems of cross reactivity are present. There are no standardized antigens and diagnostic protocols, so the results obtained in different laboratories do not agree (Mateu de Antonio et al.

1994). On the other hand, tests used for serological diagnosis of classical brucellosis are not useful, because *Brucella canis* is a rough (R) species which differs from smooth (S) species. To diagnose brucellosis of dogs a rapid slide agglutination test with *Brucella ovis* (also an R species with similar antigens) can be used, as well as a tube agglutination test with 2-mercaphoethanol which has to eliminate the reactivity of IgM antibody responsible for non-specific reactions. Both tests are highly sensitive, but with a great number of incorrect results. On the other hand, the use of cytoplasmic antigens of *Brucella canis* or other *Brucella* overcomes the problem of non-specific reactions, but these antigens do not differentiate between antibodies to R and S *Brucella* species.

Enzyme immunoassays are widely used for diagnosis of classical brucellosis, mainly detecting antibodies to outer antigens of these species. Unfortunately, there is not enough information about their application for diagnosis of brucellosis in dogs. Use of a less mucoid variant (M-) of *Brucella canis* to obtain antigen showed favourable results. This type of antigen (mainly R-lipopolysaccharide (R-LPS), gave reliable results, while the reference strain RM 6/66 was unsuitable due to high mucosity (Mateu de Antonio et al., 1993). On the other hand, attempts to standardize an ELISA using cytoplasmic antigens (CAg) have been unsuccessful because of their poor ability to bind to polystyrene plates and the difficulty in completely removing R-LPS.

The dot-ELISA which we made proved to be reliable because cytoplasmic proteins in the appropriate concentration are easily adsorbed on nitrocellulose membranes, and the reading itself is much easier than with classical ELISA.

MATERIAL AND METHOD

Bacterial strain and antigen production.

In this work we used *Brucella canis* strain RM 6/66. The bacteria were grown aerobically in 1 l Roux flasks on tryptose agar, for 24 hours at 37°C. Bacteria were harvested with sterile distilled water and centrifuged at 3000 revolutions per minute (rpm) for 30 minutes. After 3 cycles of centrifugation, the bacterial pellet was resuspended in sterile distilled water (1g wet cells in 3 ml water). The suspension was then inactivated for 1 hour at 60°C and after this, sonicated in an MSE ultrasonic apparatus on ice for 30 minutes. The bacterial suspension was centrifuged at 7000 rpm for 30 minutes. The supernatant was collected, filtered through a 0.45 μm filter (Flowpore, ICN Pharmaceuticals, CA) and dialysed in distilled water for 48 hours at 4°C with constant stirring. During this process the water was changed frequently. Then, the dialysate was lyophilised and stored at 4°C until used in this test. The concentration of proteins was determined by the biuret method before they were adsorbed on nitrocellulose.

Extraction of proteins with potassium thiocyanate

The bacterial suspension for this method was obtained in the same way. Briefly, harvested and washed bacteria were suspended without inactivation in 10 volumes extraction solution (0.5 M potassium thiocyanate, 0.08 M NaCl, pH 6.8) and stirred at 4°C overnight. After this, cell debris was removed by centrifugation at 7000 rpm for 30 minutes. Then, the supernatant was passed through a 0,45 μm filter and dialysed against distilled water for 48 hours. The dialysate was lyophilised and stored at 4°C before the application of the test.

Dot-ELISA

The dot-enzyme linked immunosorbent assay was performed in polystyrene microtiter plates with flat bottom wells. Small circular pieces of nitrocellulose (nitrocellulose membrane, pore size 0.45 μm, in roll, Serva) of 5 mm diametar were used for adsorption of both antigens. The antigens were adsorbed on to the surface of the nitrocellulose as follows: soluble sonic extract (1 μl) in rows A, C, E and G and outer membrane proteins (3 μl) in rows B, D, F and H. After this, the wells were filled with bovine serum albumin solution diluted in Tris-HCl buffered saline (0.02M Tris-HCl, 0.15 M NaCl, BSA, 1%, pH 7.5), and incubated for 30 minutes at 37°C. After blocking the nitrocellulose, the plates were drained and washed 3 times with 200 μl of TBS containing 0.05% of Tween 20 (TTBS-0.02 M Tris-HCl, 0.15 NaCl, 0.05% Tween 20, pH 7.5). Test sera were diluted in TTBS in the proportion of 1/100. Diluted sera (100 μl) were dispens in the wells according to the plate layout. The plates were incubated for 1 hour at 37°C and then washed 3 times with 200 μl of TTBS. After this, 100 μl of horseradish peroxidase conjugate (IgG, *H+L* anti-dog, ICN Pharmaceuticals, CA) was added at its working dilution (1/1000). After 1 hour of incubation at 37°C, the plates were washed 3 times with TTBS and once with TBS. 4-Chloro-1-naphthol (Serva) was added as substrate in the quantity of 100 μl to each well. After 15 minutes of incubation at room temperature the reaction was stopped by washing with distilled water. The solution of chromogen was made immediately before use (30 mg 4-chloro-1-naphtol in 10 ml methanol added to 50 ml TBS and 30 μl 30% hydrogen peroxide) (Henderson and Wolf 1992). All samples were assayed in duplicate.

RESULTS

With dot-ELISA 44 sera were examined. Forty sera originated from animals with reproductive disorders; 25 sera, including negative controls did not have any antibody titre to *Brucella canis*. The other 19 sera had antibody titres equal to or more than 1/50 in 2-ME-TAT, while one serum originated from a female in which *Brucella canis* had been isolated from blood after abortion. Seven of the 19 sera, had a titer of 1/50, four 1/100 and eight 1/200. The arrangement of the tested sera as well as the titre in 2-ME-TAT is shown in Table 1.

Figure 1 shows the obtained results. The test was standardised by employing different concentrations of both antigens by checkerboard titration with varying dilutions of highly positive and negative sera. Checkerboard analysis indicated that approximately 500 ng antigen per dot was optimal.

A positive reaction was visible as clear brown circular dots on the nitrocellulose. Distinct positive reactions with both antigens was seen in wells A1B1, A3B3, A10, C7D7 and C9. Weak positive reactions occurred in wells A4B4, B10, A5, C2, C5 and C6. Figure 1 shows that the intensity of colour is not only related to agglutination titre, but is probably related to avidity and/or affinity of the antibody as well.

Table 1. Arrangement of tested sera in dot ELISA

A1B1 (1/800)	A2B2	A3B3 (1/3200)	A4B4 (1/400)	A5B5 (1/100)	A6B6 (1/50)	A7B7	A8B8	A9B9	A10B10 (1/400)	A11B1 nc1	A12B2 nc1
C1D1	C2D2 (1/100)	C3D3 (1/100)	C4D4 (1/50)	C5D5 1/200	C6D6 1/400	C7D7 (1/400)	C8D8 (1/100)	C9D9 (1/400)	C10D0	C11D1 nc2	C12D2 nc2
E1F1	E2F2 (1/50)	E3F3 (1/50)	E4F4	E5F5 (1/50)	E6F6	E7F7	E8F8	E9F9	E10F10	E11F1 nc3	E12F2 nc3
G1H1 (1/50)	G2H2	G3H3 (1/50)	G4H4	G5H5	G6H6	G7H7	G8H8	G9H9	G10H10	G11H1 nc4	G12H2 nc4

Legend: nc1,2,3,4, negative controls

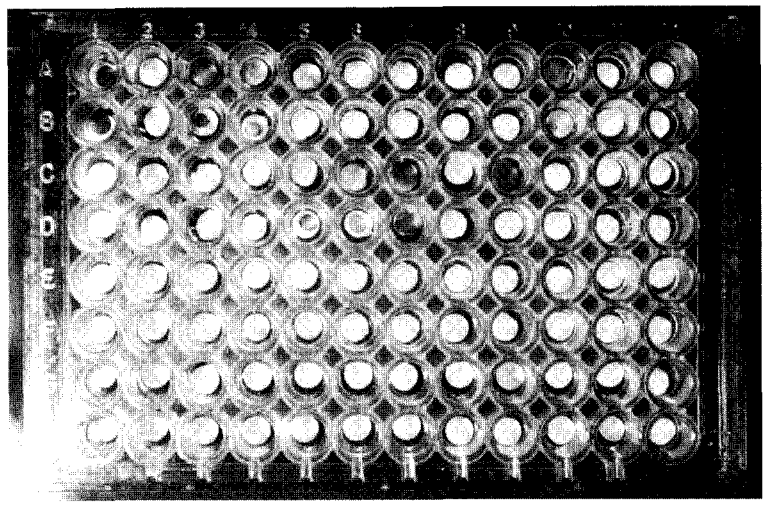

Figure 1. Results of dot-ELISA. The plate shows the development of a brown dot in positive cases using dot-ELISA (e.g. A1B1-clear positive and A4B4-weak positive reaction). See Table 1 for key.

DISCUSSION

Serological diagnosis of infections caused by *Brucella canis* is very difficult, especially in cases without distinct clinical signs and an adequate immunological response. Some, serological negative cases have been described with positive bacterial isolation. The cause of false positive reactions may be infection of the organism with *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *Bordetella bronchiseptica*, etc. The use of cytoplasmic proteins of *B. canis* in the AGID test or immunoblot is very precise, but these tests cannot differentiate between R and S *Brucella* species (Mateu de Antonio et al., 1993, 1994).

An objective diagnosis is directly influenced by the choice of the appropriate antigens. During the process of protein extraction with different methods, a common problem is protein denaturation, which can lead to inappropriate diagnosis (Chin and Turner 1990). On the other hand, LPS of *Brucella* is an immunodominant antigen, but its use causes cross reactions thus decreasing the specificity of the test. A great number of cross reactions between different bacteria and S and R *Brucella* species has been reported (Corbel 1997, Alton et al. 1988).

The choice of method for extracting antigens directly influences the results obtained by different researchers. Selective extraction of outer membrane proteins is connected with complicated procedures and the use of an ultracentrifuge. The antigens obtained in this way are a mixture of proteins and polypeptides of the outer and inner membrane, or they originate from the bacterial cytosol which is detected electrophoretically. Differences in the efficiency of the chemical components applied for this purpose, can be determined experimentally (Chin and Turner 1990). As the immunological response of an infected organism is mainly directed towards the outer membrane of bacteria, these antigens are very interesting for serological diagnosis of infection. For example, the high level of ELISA reactivity to outer cell components in rams infected by *Brucella ovis*, may be explained by preferential bonding of antibody to native (LPS and proteins)

rather than to denaturated antigens (Chin 1983, Chin and Turner 1990). That is why special attention was paid to this here and chemical extraction of proteins was made on living cells of *Brucella canis*.

Enzyme immunoassays are applied in the diagnosis of many infectious diseases and it is one of the most widespread laboratory techniques. As a variant, dot ELISA is very successfully used in the diagnosis of bovine brucellosis. The obtained results, generally agree with the results obtained by classical ELISA, while disagreement was recorded in less than 1% of cases (Chand et al., 1990). ELISA is also successfully used in the diagnosis of brucellosis caused by *B. canis* (Mateu de Antonio et al., 1993; Serikawa, et al. 1989). Few studies have applied ELISA to detect *B. canis* antibody in dogs. Serikawa et al. (1989) developed a 2-antibody sandwich type ELISA. This ELISA was able to classify correctly 98,72 % of field samples that were negative by other serological methods and 80% samples that were positive. On the other hand, 48,64% sera with a suspected antibody titer that were culture negative were classified as positive.

In another study, indirect ELISA with antigens obtained from an M- variant of *Brucella canis* detected 93,75% positive and 95,65% negative sera. This indirect ELISA may be useful in the diagnosis of brucellosis in dogs. The hot saline extracted antigen (HSS) used in this test is mainly composed of R-LPS and proteins. Also, in this test 2 of 14 sera with antibodies to smooth *Brucella* only, were classified as positive. This is explained by the fact that HSS contains proteins which are common to S *Brucella* species. However, this is a very small percentage and irrelevant to this research (Mateu de Antonio, et al. 1993).

Our dot ELISA, showed a different number of positive results with each antigens. With the soluble sonic antigen, which is mainly composed of cytoplasmic proteins but also contains R-LPS, 10 positive sera were detected while with outer membrane proteins 7 positive sera were detected. This can be explained by the fact that sonication of bacteria resulted in a higher number of different proteins. No method was used to eliminate R-LPS and this antigen was accessible to antibodies of different specificity. On the other hand, fewer proteins were obtained by chemical extraction (about 11 different proteins) as determined by electrophoretic analysis (results are not shown). This may explain the lower sensitivity of outer membrane proteins.

Nitrocellulose used in this test as the solid surface instead of polystyrene plates, which enabled precise adsorption of cytoplasmic proteins, overcoming the problem already described. Of the examined sera, 12 had titres equal to or more than 1/100. Out of these 12, 10 sera showed positive reactions in the dot-ELISA with sonicated antigen. Out of the total number of examined sera, 22,72% had a positive reaction with sonic antigen, while with outer membrane proteins, 15,9% had a positive reaction. Three sera (6,82%) with agglutination titres of 1/100, 1/100 and 1/400 respectively did not give a positive reaction to outer membrane proteins. The examined sera were not treated with mercapthoethanol or heat, so in this test antibodies of G and M isotype were detected. Namely, using anti IgG (H+L) enables detection of the IgM isotype also because of the similarity of the light chains in these two classes of immunoglobulins (Mateu de Antonio et al. 1993).

The performance of dot-ELISA itself is easy because standardization is possible. Recording the reaction is also very simple and does not require an ELISA reader, although the estimation of weak positive reactions may be subjective. Adaptation of this method to plastic strips would allow performance and precise

diagnosis even in laboratories which are not equipped for classical immunoenzyme tests. Since out of 12 sera with an agglutination titre 1/100, only 2 had a negative reaction (4.54%) in dot-ELISA makes this test perspective. A more objective estimation as well as determination of the sensitivity and specifity of the test, requires the examination of a greater number of samples.

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DOT ELISA: BRZI METOD ZA SEROLOŠKU DIJAGNOSTIKU BRUCELOZE PASA

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SADRŽAJ

U radu je opisana tehnika dot-ELISA sa dva različito pripremljena antigena *Brucella canis*. Kao čvrsta faza za nanošenje antigena korišćena je nitrocelulozna membrana zbog većeg kapaciteta za vezivanje proteina. Testom je analizirano 40 seruma koji su prethodno ispitani brzim aglutinacionim testom i sporim aglutinacionim testom sa 2-merkaptoetanolom. Uzorci seruma su dobijeni od klinički sumnjivih pasa različitog pola, starosti i rase. Dodatna četiri seruma, dobijena od klinički zdravih životinja, služila su kao negativna kontrola. U ispitivanje je uključen i serum ženke sa visokim aglutinacionim titrom kod koje je izolovana *B. canis*. Od svih ispitanih seruma, 19 je imalo aglutinacioni titar > 1/50. Sa citoplazmatskim proteinima pozitivnu reakciju je dalo 10 seruma, a sa proteinima spoljašnje membrane 7. Svi pozitivni serumi u dot ELISA testu su imali aglutinacioni titar > 1/100.

Naše proučavanje je pokazalo da su citoplazmatski proteini *B. canis* u dot ELISA testu pouzdani i visoko specifični za brzu dijagnostiku bruceloze pasa.