EURASIAN
JOURNAL OF VETERINARY SCIENCES
www.ejvs.selcuk.edu.tr

RESEARCH ARTICLE

Serodiagnosis of *Brucella canis* infection in dogs by a dipstick enzyme immunoassay

Uckun Sait Ucan[1]*, Zeki Aras[1], Ahmet Semacan[2]

Özet

Uçan US, Aras Z, Semacan A. Köpeklerde *Brucella canis* enfeksiyonunun dipstik enzim immunoassay ile teşhisi. ***Eurasian J Vet Sci, 2010, 26, 2, 109-112***

Amaç: Köpeklerin brusellozunun teşhisi bakteriyolojik, serolojik ve moleküler yöntemlerle yapılmasına rağmen pratisyen veteriner hekimlerin hızlı ve güvenilir bir teste ihtiyaçları vardır. Bu çalışmada hızlı serolojik tanı için Dipstik EIA geliştirilmesi amaçlandı.

Gereç ve Yöntem: Test, Çabuk Lam Aglütinasyon ve indirekt ELISA testleri ile karşılaştırıldı.

Bulgular: Testin, iELISA'ya göre %82 sensitivite ve %95 spesifiteye sahip olduğu belirlendi.

Öneri: Testin yüksek spesifitesinden dolayı saha şartlarında tarama testi olarak kullanımı önerilir.

Abstract

Ucan US, Aras Z, Semacan A. Serodiagnosis of *Brucella canis* infection in dogs by a dipstick enzyme immunoassay. ***Eurasian J Vet Sci, 2010, 26, 2, 109-112***

Aim: A rapid and reliable test is needed by the veterinary practitioners although the canine brucellosis can be diagnosed by bacteriological, molecular and serological methods. By this study, developing a dipstick EIA for a rapid serodiagnosing the infection was aimed.

Materials and Methods: The test was compared with Rapid Agglutination Test iELISA.

Results: Sensitivity and specificity of the test were 82% and 95%, respectively when iELISA was evaluated as gold standard.

Conclusion: Since the test has a good degree of specifity it is recommended as a screening test in the field.

[1]Department of Microbiology, [2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Selcuk University, Campus, 42075, Konya, Turkey

Received: 26.04.2010, Accepted: 07.08.2010

*usucan@selcuk.edu.tr

Anahtar kelimeler:*B. canis*, EIA, iELISA, köpek

Keywords: *B.canis*, EIA, iELISA, dog

► Introduction

Brucella canis (B. canis) is a causative agent of canine brucellosis. It causes abortus in females and infertility and testicular atrophy followed by epididymitis in males (Lisle and Carmichael 1974, Alton et al 1988, Arda et al 1997). The infection was first reported in dogs with abortus history in United States of America by Carmichael in 1966 (Alton et al 1988). The disease has a worldwide distribution (Diker et al 1987, Alton et al 1988, Öncel et al 2005, Uçan et al 2009). By the studies conducted in Turkey so far, prevalence of the infection has been reported to be varying between 6.3% and 21.5% (Diker et al 1987, Öncel et al 2005, Uçan et al 2009). The definitive diagnosis of brucellosis consists in the isolation of the *B. canis* by microbiological culture of blood. However this method is time-consuming, expensive, need more experience and risky for the laboratory personnel (Lisle and Carmichael 1974, Nielsen et al 1985, Alton et al 1988, Lucero et al 2002). Clinical canine brucellosis is commonly confirmed by serology. Serologic tests used for diagnosing canine brucellosis include followings: Rapid Agglutination Test (RAT), 2-Mercaptoethanol Tube Agglutination Test (2-Me-TAT), Agar Gel Immunodiffusion Test (AGID), modified Micro Plate Agglutination Test (MMPAT) and ELISA (Damp et al 1973, Alton et al 1988, Lucero et al 2002). One of the most common tests used among these is the agglutination test despite its lower sensitivity (Lisle and Carmichael 1974). Immunoassays have higher sensitivities and specifities by comparison with agglutination tests regarding diagnosing canine brucellosis (Nielsen et al 1985, Lucero et al 2002, Barrouin-Melo et al 2007).

A practical, economic and highly sensitive assay for sero-diagnosing *B. canis* infection in dogs in the field is needed. The objective of this study was to develop an enzyme immunoassay on GelBond for the serological diagnosis of canine brucellosis.

► Materials and Methods

Blood Serum Sample

A number of 135 canine sera including two positive and eight negative control sera from serum bank of Microbiology Department of Microbiology, Faculty of Veterinary Medicine was used in the study. Sera were kept at –20^{0}C until use.

Source of antigens

Antigens were prepared from *B. canis* NCTC 10854 and *Brucella ovis (B. ovis).*

Serology

Antigen from *B. ovis* was used in the Dipstick Enzyme Immunoassay (Dipstick EIA) and indirect ELISA (iELISA). For the RAT, *B. canis* was served as antigen.

Rapid Agglutination Test (RAT)

Antigen was prepared according to protocol by Lisle and Carmichael (1974). *B. canis* NCTC 10854 was subcultured on the media (pepton 10 g, NaCl 5 g, agarose 20 g, distilled water 1000 ml) at 37^{0}C for 24 hr. Following incubation period colonies were picked up by using 20 ml of PBS and spun at 10.000 g for 20 min. The pellet was washed again two times with the same amount of PBS as above. The pellet was then resuspended at 125 g/L with PBS, filtrated through a piece of sterile gauze and inactivated by keeping in 56^{0}C for 1 hr. The antigen suspension was added with stain solution (2 g brillant gren, 1 g crystal violet, 300 mL distilled water) giving 6 ml/L final concentration. A further filtration by using sterile glass wool was performed and thiomersal was added with a final concentration of 0.01% then kept at 4^{0}C in dark bottles until use.

By trying different ratios (1/1, ½, 1/3) of sera and antigens by various quantities (40, 20, 10, 5 ml) in the test, the best reaction was detected to occur by using 20 µl of each antigen/serum for 2 min shaking. This amount of the reagent and serum was used in testing all the sera.

Dipstick Enzyme Immunoassay (Dipstick EIA)

Antigen was prepared as described elsewhere (Myers et al 1972) with minor modifications. Briefly, *B. ovis* was sub-cultured on Blood Agar Base (Oxoid, CM0271) with 5% horse serum (Oxoid, SR0035) at 37^{0}C for 24 h in microaerophilic atmosphere. After incubation, colonies were picked up by 50 ml of phosphate buffered solution PBS and filtered through a piece of sterile gauze. The filtrate was washed 3 times with PBS and the pellet was re-suspended in 10 ml PBS and sterilized at 120^{0}C in autoclave. After cooling the suspension was spun at 12000 g for 20 min at 4 ^{o}C. Supernatant was divided into volumes of 500 µl and kept at -20^{0}C until use for dipstick EIA and iELISA as antigen. Protein concentration of the antigen was determined by the method "DC protein assay" (Cat No. 500-0116, Bio-Rad Lab., USA). Dipstick EIA was standardized and run as described previously (Nielsen et al 1985). Optimum concentration for antigen as well as anti-dog IgG Horse Radish Peroxidase Conjugate (HRPC) and serum dilutions were determined by checker-board titration method. To cover dipstick with an antigen, a quantity of 25 µg antigen was added to mixture of 1.25 ml of antigen solution including 0.5% Bovine Serum Albumin (BSA), 0.06 M Carbonate buffer (pH 9.5) and 1.25 mL of agarose solution (0.06 M carbonate buffer, 0.4% agarose (pH 9.5). A marker was used to draw a line at the 30 mm at hydrophilic side of the GelBond (Cambrex Bio Science Rockland, Inc. Cat No. 53734) cut 85 x 100 mm. The antigen-agarose mixture above (premelted and cooled to 60 ^{o}C) was poured ontoGelBond, allowed to gel ensuring did not spread beyond the width and then dried with

hot air. The GelBond was then cut into 4 mm x 85 mm strips and stored in stoppered plastic containers at 4 °C. The dipstick EIA was performed in 5 numbers of glass tubes with dimensions 12 x 75 mm as follows: 25 µl of serum was added to the 2.5 ml of dilution solution (PBS with 1% BSA, 0.05% Tween 20). Dipstick carrying antigen was placed into first tube and kept for 15 min. The dipstick was then transferred to the tube 2 for first wash. The tube was slightly shaked by hand for 5 min. The dipstick was placed in tube 3 containing 1.5 ml of conjugate (HRPC Sigma, A-9042) diluted 1/4000 for 10 min. Re-wash was performed in another tube. To allow substrate-chromogen reaction develop, the dipstick was kept in the tube 5 that contained 1.5 mL TMB (Sigma, SIT-0440) for 10 min. The reaction was ended by adding stop solution (2M, H_2SO_4).The dipstick was removed and colour intensity of the tube last in sequence was assessed by an ELISA reader at 450 nm (MWGt Lambda Scan 200 Biotek Inc, USA). A colur scale was constructed by readings from sera of both positives and negatives on the basis of values from iELISA.

Table 1. Test results from Dipstick EIA and iELISA.

Dipstick EIA	iELISA		
	Positive	Negative	Total
Positive	27	5	32
Negative	6	97	103
Total	33	102	135

Indirect Enzyme Linked Immunosorbent Assay (iELISA)

iELISA was standardized and performed as described by Mateu-de-Antonio et al (1993). To determine optimum concentrations for antigen, anti-dog IgG HRP (Sigma) and sera dilutions, a checkerboard method was employed. 100 µl antigen of which protein quantity was 1.5 µg/mL homogenized in 0.05 M carbonate-bicarbonate buffer (pH:9.6) was dispersed into wells of microplates (Immulon II, Nunc C bottom, 446612) kept overnight at 4 °C. Then, the microplates were washed with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH:8) three times. Following a washing step, 200 µl of post coat solution made of 1% BSA was added and waited for 1 h at 37 °C followed by three times washing. A further incubation at 37°C for 1 h was proceeded by dispersing 100 µL of sera (both control and test) diluted at 1/300 with diluting solution that consisted of PBS with 1% BSA and 0.05% Tween 20. The wells were then washed 3 three times again. The conjugate (1/4000) in diluting buffer was added to the wells and incubated for 1 hr at 37°C. After washing as above, 100 µL of TMB substrate were put and incubated for 10 min at room temperature. The reaction was stopped by adding 100 µL of stop solution (2M H_2SO_4) and adsorbance values were read at 450 nm by ELISA-reader (MWGt Lambda Scan 200 Bio-Tek Inst. Inc. USA). Figures obtained from samples that equal to or more than cut-off value (1.750) were considered as positive.

Statistics

Data were analyzed by t-test by Microsoft Office Excel 2007. Significance level was set at $p<0.05$. Interassay variation analyses were made as described by Nielsen et al (1985).

► Results

Optimal concentrations for antigen, conjugate and serum dilution were 1 µg/mL, 1/4000 and 1/200, respectively. Corresponding figures for iELISA were 1.5 µg/mL, 1/4000 and 1/300, respectively. Thirty nine (28.9%) out of 135 sera samples gave positive reaction using *B. canis* antigen by RAT. When the same samples were also tested by iELISA, positivity was lower (24.4%). 6 out of 33 positive sera by iELISA were false negative by dipstick EIA (Table). By interassay variation analysis, 13.9%, 10.2% and 5.9% levels of variation were detected when strong positive, weak positive and negative (6 sera from each) used. Correlation coefficient was 0.413 ($p<0.05$). Sensitivity and specifity of the dipstick EIA were 82% and 95%, respectively when the iELISA was considered as gold standard.

► Discussion

Canine brucellosis is a zoonotic bacterial infection (Arda et al 1997). Its clinical diagnosis is difficult to perform (Barrouin–Melo et al 2007). Canine brucellosis due to *B.canis* in Turkey has occurred in kennels as reported before (Diker et al 1987, Öncel et al 2005, Uçan et al 2009).

There have been a limited number of laboratories to support pet clinics in most of the parts of Turkey for years. This undoubtedly leads to clinicians to decrease quality of the health services they give. Barrouin-Melo et al (2007) has noted that a rapid test to be used in clinics on diagnosis of *B. canis* infection was necessary. Since canine brucellosis seems to have a nationwide distribution and represents a public health concern (Diker et al 1987, Öncel et al 2005, Uçan et al 2009) the veterinary practitioners are in need of some simple and ready to use techniques or kits for their routine use in diagnosis of diseases like canine brucellosis in the field.

For epidemiological purposes, two tests can together be used for determining prevalence of an infection; a screening test and a definitive test. Although more commonly used, rapid agglutination tests have low sensitivity and are recommended to rather be used as screening tests (Lucero et al 2005, Barrouin–Melo et al 2007, Uçan et al 2009). In this study, a common screening test, RAT was used for determining negatives since its sensitivity is known to be quite low.

The diagnostic techniques most widely used are serological tests (Alton et al 1988, Arda et al 1997). Se-

rology provides rapid and practical methods on determining sero-prevalence of the infection (Alton et al 1988, Öncel et al 2005). The ELISA has been reported to be a specific and sensitive test for detection of anti-Brucella antibodies in canine sera (Barrouin-Melo et al 2007). In this study, iELISA was only used to measure sensitivity and specifity of the developed test. On the other hand, 39 out of 135 sera were positive by RAT. The corresponding figure for positives by dipstick EIA was 32 showing that dipstick EIA was more sensitive than RAT.

By antigen optimization trials, quantity of the antigen (1 µg/mL) necessary for coating GelBond in dipstick EIA assay was found to be lower than that (1.5 µg/mL) for iELISA. The immunological specificity (cross reactions with different microorganisms) of the dipstick EIA was not evaluated. Two major draw-backs of the conventional EIA is the need for using microplates and requirements for optical readers. There is no need for an optical reader to evaluate test results in the dipstick EIA. A total period of 45 min is enough to complete the assay. Because it has specificity of 95 % and is relatively simple to do, the dipstick EIA might be used by veterinary practitioners in examining canine brucellosis or even in programs for eradicating the diseases from a kennel. It could also be used for prebreeding examinations of the dogs since the disease is transmitted venerally.

► Conclusion

Although further study is needed to improve its immunological and epidemiological characteristics, the test developed here is found promising as screening test in the field.

► Acknowledgments

This research was supported by funds from KOSGEB (Project No:2008/4)

► References

Alton GG, Jones LM, Angus RD, Verger JM, 1988. Techniques for the Brucellosis Laboratory, Inra, Paris, France.

Arda M, Minbay A, Aydın N, Akay Ö, İzgür M, Leloğlu N, Kahraman M, Ilgaz A, Diker S, 1997. Özel Mikrobiyoloji, Medisan Yayınevi, Ankara, s: 110-124.

Barrouin-Melo AM, Poester FP, Ribeiro MB, Alcantra AD, Aguiar PHP, Nascimento IL, Schaer RE, Nascimento RM, Freire SM, 2007. Diagnosis of canine brucellosis by ELISA using an antigen obtained from wild Brucella canis. Res Vet Sci, 83, 340-346.

Damp SC, Crumrine MH, Lewis GE, 1973. Microtiter plate agglutination test for Brucella canis antibodies. Appl Microbiol, 25, 489-490.

Diker SK, Aydın N, Erdeğer J, Özyurt M, 1987. A serologic survey of dogs for brucella canis and Brucella abortus an evaluation of mercaptoethanol microagglutination test. AÜ Vet Fak Derg, 34, 268-277.

Lisle WG, Carmichael LE, 1974. A plate agglutination fort he rapid diagnosis of canine brucellosis. Am J Vet Res, 35, 905-909.

Lucero NE, Escobar GI, Ayala SM, Lopez G, 2002. Sensitivity and specificity of an indirect enzyme-linked immunoassay for the diagnosis of Brucella canis infection in dogs. J Med Microbiol, 51, 656–660.

Mateu-de-Antonio EM, Martin M, Soler M, 1993. Use of indirect enzyme linked immunosorbent assay with hot saline solution extracts of a variant (M-) strain of B. canis for diagnosis of brucellosis in dogs. Am J Vet Res, 54, 1043-1046.

Myers DM, Jones LM, Varela-Diaz VM, 1972. Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by Brucella ovis and other Brucellae. Appl Microbiol, 23, 894-902.

Nielsen K, Ballinger R, Stiller J, Rosenbaum, 1985. A "Dipstick" enzyme immunoassay for detection of antibody to Brucella abortus in cattle sera. Can J Comp Med, 49, 298-302.

Öncel T, Akan M, Sareyyüpoğlu B, Tel OY, Çiftçi A, 2005. Seroprevalence of Brucella canis infection of dogs in two provinces in Turkey. Turk J Vet Anim Sci, 29, 779-783.

Uçan US, Aras Z, Zorlutuna M, 2010. Detection of canine brucellosis by a rapid agglutination test using Rhizobium tropici as antigen. Revue Med Vet 161, 51-56.