Indian Journal of Animal Sciences 78 (4): 357–359, April 2008

Evaluation and comparison of recombinant Omp 28 antigen in indirect ELISA for sero-prevalence study of canine brucellosis

V C MATHUR[1], D K SINHA[2], P CHOUDHURY[3], HARI SHANKAR[4] and S K SRIVASTAVA[5]

Indian Veterinary Research Institute, Izatangar, Uttar Pradesh 243 122 India

Received: 25 February 2007; Accepted: 17 October 2007

Key words: Canine brucellosis, Indirect ELISA, Recombinant Outer membrane protein 28 (rec. Omp 28), Sonicated antigen

Canine brucellosis caused by *Brucella canis* is an insidious disease, therefore, many infected dogs appear clinically normal. This disease is zoonotic in nature, hence, close contact between people and infected dogs increase the risk of transmission; however, its impact on public health is probably underestimated due to lack of reporting and inadequate diagnostic services (Lucero *et al.* 2002, 2005). The recombinant antigen based diagnostics have never been performed for diagnosis of canine brucellosis. In the present study, *B. melitensis* recombinant outer membrane protein 28 (rec. Omp 28) was evaluated and compared as antigen in indirect enzyme- linked immunosorbant assay (I-ELISA) with hot saline extract (HSE) and sonicated antigen (SA) as well as with the conventional Rose Bengal plate test (RBPT).

Antigens: The standard culture of *B. canis* Mex 51 was procured from *Brucella* laboratory, Div of Veterinary Public Health, IVRI, Izatnagar. The culture was tested for purity and then propagated on potato infusion agar on large scale as described by Alton *et al.* (1975) for preparation of antigens.

The HSE and SA were prepared as per Zoha and Carmichael (1982b). The *B. melitensis* rec. Omp 28 cloned in pGEMT vector and expressed in pProc vector was kindly provided by Division of Bacteriology and Mycology, IVRI, Izatnagar.

Raising of anti-B. canis *serum*: Healthy dogs (2) were inoculated heat killed sonicated *B. canis* cells (1×10^9) in Freund's incomplete adjuvant intramuscularly. A booster was given on day 14. One week after the booster, about 20 ml bloods was withdrawn intravenously, serum was separated, pooled and stored at –20°C in aliquots.

Collection of dog serum samples: Dog serum samples were collected from different sources. About 5 ml of blood was collected from dog in a sterile syringe and transferred to a sterile test tube, which was kept in slanted position for about 2 h at room temperature. Then the tube was kept overnight in refrigerator (4–6°C). Thereafter, centrifuged at 3000 rpm for about 15 min. Serum separated was collected in sterile eppendorf and after adding 0.01% sodium azide, the sample was kept at –20°C.

Rose Bengal plate test (RBPT): It was performed as per Alton *et al.* (1975).

Indirect enzyme- linked immunosorbant assay (I-ELISA): It was done as per Nielson *et al.* (1984) by using antigens namely HSE, SA and rec. Omp 28 with slight modifications. The optimum concentration of antigen for coating the plate as well as serum dilution was attained by checkerboard titration following the method of Kumar *et al.* (1985).

The chi square analysis was carried out using Microsoft Excel 2003 to assess the significance between the tests results. Relative sensitivity and specificity of the tests, Kappa statistics to know the agreement between the tests and concordance percentage were estimated as per the methods of Mac Diarmid and Hellstrom (1987), Perrin and Surean (1987) and Thrusfield (1997), respectively.

Dog serum samples (787) were collected and tested by RBPT, HSE ELISA, SA ELISA and rec. Omp ELISA. The overall sero-prevalence of canine brucellosis based on three ELISA employed were rec. Omp ELISA (12.3%), SA ELISA (8.3%), RBPT (7.6%) followed by HSE ELISA (4.3%). Significant differences were observed between sero-prevalence rates estimated by all tests except between SA ELISA and RBPT where no significant difference was observed. The rec. Omp ELISA had a highest relative sensitivity while HSE ELISA exhibited the maximum relative specificity in comparison with RBPT (Table 1).

The antibodies against *Brucella* can be detected at 2 weeks post-infection. These are developed against the cell wall and cytoplasmic protein of *Brucella*. In general, antibodies against the lipopolysaccharide (LPS) appear earlier (Moon *et al.* 1994) and disappear shortly after bacterimia has ceased,

Present address: [1]I.F.S (P),Indira Gandhi National Forest Academy, Dehradun.

[2]Senior Scientist, [4]Principal Scientist and In-Charge, Epidemiology Section, CADRAD; [3] Senior Scientist, [5]Emeritus Scientist, Division of Bacteriology and Mycology.

Table 1. Relative sensitivity and specificity of the three ELISA as compared to the standard Rose Bengal plate test for canine brucellosis

Test	Relative sensitivity (%)	Relative specificity (%)
HSE ELISA	26.66	98.30
SA ELISA	30.00	99.10
Rec. Omp ELISA	56.66	92.70

whereas antibodies against the cytoplasmic antigen tend to persist longer-from 6 months to a year and allow the detection of chronic cases (Zoha and Canmichael 1982 a, b; Carmichael *et al.* 1984).

The HSE of rough brucellae is mainly composed of rough-lipopolysaccharide (R-LPS) and Omp and regarded to be specific for rough and smooth brucellae (Myers *et al.* 1972). An ELISA using *B. canis* R-LPS antigen resulted in sensitivity and specificity values of 95.8 and 98.8%, respectively, with dog sera (Nielsen *et al.* 2004). This finding is in concordance with our results where we found HSE ELISA, based on cell wall antigens of *B. canis* Mex 51 to be more specific and sensitive. The high specificity may be attributed to the fact that infection specific for rough *Brucella* only would have been detected.

The sonicated antigen contains protein fractions of cytoplasm (Baldi *et al.* 1994) and has been found more sensitive (Wanke 2004). In agreement with finding of these 2 workers, SA ELISA, in this study, detected second highest positive cases, highlighting its sensitive nature. This indicated that this antigen may also detect species other then *B. canis* and natural infection of dogs with other species of *Brucella* has been extensively reported (Baek *et al.* 2003, Brooks 2006).

ELISA based on Omp, extracted from *B.* melitensis, appear to be sensitive to antibodies to *B. canis, B. abortus, B. suis* and *B. melitensis* (Hunter *et al.* 1986). Amongst outer membrane proteins, Omp of 28 kDa (earlier nomenclature BP26) molecular weight is of diagnostic importance and was reported to be the immunodominant antigen in infected sheep, goats, cattle and human. While Lindler *et al.* (1996) have reported this protein to be an outer membrane protein; Cloeckaert *et al.* (1996) found this protein to be localized

Table 2. Concordance between serological tests of canine brucellosis

Test 1	Test 2	Concordance (%)
HSE ELISA	SA ELISA	90.59
HSE ELISA	Rec. Omp ELISA	88.31
HSE ELISA	RBPT	93.60
SA ELISA	Rec. Omp ELISA	88.56
SA ELISA	RBPT	89.40
Rec. Omp ELISA	RBPT	89.50

Table 3. Value of Kappa statistic for different tests of canine brucellosis

Test 1	Test 2	Kappa value
HSE ELISA	SA ELISA	0.21*
HSE ELISA	Rec. Omp ELISA	0.25*
HSE ELISA	RBPT	0.35*
SA ELISA	Rec. Omp ELISA	0.39*
SA ELISA	RBPT	0.24*
Rec. Omp ELISA	RBPT	0.40*

*Inference–Fair agreement.

exclusively intracellular as a soluble protein. The greater concordance of SA ELISA with rec. Omp ELISA (Table 2) support the findings of Cloeckaert *et al.* (1996) which was further strengthened by greater agreement (Kappa statistics) between these 2 (Table 3). It may be due to the presence of 28 kDa protein in the SA.

In this study rec. Omp 28 based ELISA proved to be extremely sensitive compared to all other tests used, which is in concordance with the findings of Prasad (2004). Increased sensitivity may lead to false positive results but out of 3 antigens used in ELISA, the quantity of rec. Omp 28 was the minimum (125 ng/well) required for detecting the positive sample in comparison to HSE and SA (each 750 ng/well). Hence one can conclude that the analytical sensitivity of rec. Omp ELISA is far greater than HSE and SA ELISA. This rec. Omp ELISA presents a few other advantages also. With regard to antigen production, cultivating *Brucella* may be dangerous for workers and is also a slow process which takes three days, whereas cultivating recombinant *E. coli* is safe, takes only 16–18 h and gives high yield production of rec. Omp 28 protein (Zygmunt *et al.* 2002). This also makes the test cost effective in long run. The stability of the protein makes it suitable for field use (Prasad 2004). From this study, it can be inferred that *B. melitensis* rec. Omp 28 is a potential antigen for diagnosis of canine brucellosis.

SUMMARY

Brucella melitensis rec. Omp 28 was evaluated and compared as antigen in I-ELISA with HSE and SA as well as with RBPT. Dog serum samples (787) were collected and tested. A sero-prevalence of 12.3, 8.3, 7.6 and 4.3% of canine brucellosis was recorded by rec. Omp ELISA, SA ELISA, RBPT, HSE ELISA, respectively. Significant differences were observed between sero-prevalence rates estimated by all tests except between SA ELISA and RBPT. The rec. Omp ELISA had the highest relative sensitivity while HSE ELISA exhibited the maximum relative specificity in comparison with RBPT. The present study advocated to use rec. Omp 28 antigen based ELISA for diagnosis of canine brucellosis.

ACKNOWLEDGEMENT

The authors are thankful to the Director, IVRI, the Joint

Director, CADRAD and I/C *Brucella* laboratory, Division of Veterinary Public Health, IVRI, for providing necessary facilities to carryout this work. The financial support provided to the first author in the form of fellowship by the Deemed University, IVRI, Izatnagar, is duly acknowledged.

REFERENCES

Alton G C, Jones L M and Peitz D E. 1975. *Laboratory Techniques in Brucellosis.* 2nd edn, pp. 149–54. WHO, Geneva.

Baek B K, Lim C W, Rahman M S, Hyun Kim C, Oluoch A and Kakoma I. 2003. *Brucella abortus* infection in indigenous Korean dogs. *Canadian Journal of Veterinary Research* **67**(4): 312–14.

Baldi P C, Wanke M M, Loza M E and Fossati C A. 1994. *Brucella abortus* cytoplasmic proteins used as antigens in an ELISA potentially useful for the diagnosis of canine brucellosis. *Veterinary Microbiology* **41**: 127–34.

Brooks WC. 2006. Brucellosis in dogs. http://www. Veterinary Partner.com

Carmichael L, Zoha S J and Flores-Castro R. 1984. Problems in the serodiagnosis of canine brucellosis: dog responses to cell wall and internal antigens of *Brucella canis*. *Development of Biological Standards* **56**: 371–83.

Clockaert A, Verger J M, Grayon M, Zygmunt M S and Grepinet O. 1996. Nucleotide sequence and expression of the gene encoding the major 25–kilodalton outer membrane protein of *Brucella ovis*: evidence for antigenic shift, compared with other *Brucella* species, due to a deletion in the gene. *Infection and Immunity* **64**: 2047–55.

Hunter S B, Bibb W F, Shih C N, Kaufmann A F, Mitchell J R and Mc Kinney R M. 1986. Enzyme-linked immunosorbent assay with major Outer Membrane Proteins of *Brucella melitensis* to measures immune response to *Brucella* species. *Journal of Clinical Microbiology* **24**(4): 566–72.

Lucero, N E, Escobar G I, Ayala S M and Lopez G. 2002. Sensitivity and specificity of an indirect enzyme-linked immunoassay for the diagnosis of *Brucella canis* infection in dogs. *Journal of Medical Microbiology* **51**(8): 656–60.

Lucero N E, Escobar G I, Ayala S M and Jacob N. 2005. Diagnosis of human brucellosis caused by *Brucella canis*. *Journal of Medical Microbiology* **54**(5): 457–61.

Kumar S, Band A H, Samantray J C and Dang N. 1985. A dot Enzyme linked immunosorbert assay for detection of antibodies against *Entamoeba histolytica*. *Journal of Immunological Methods* **83**: 125–33.

Lindler L E, Hanfield T L, Tall B D, Snellings N J, Rubin F A, Van de Verg L L, Hoover D and Warren R L. 1996. Cloning of a *Brucella melitensis* group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis. *Infection and Immunity* **64**(7): 2490–99.

Mac Diarmid S C and Hellstrom J S. 1987. An intradermal test for the diagnosis of brucellosis in extensively managed cattle herds. *Preventive Veterinary Medicine* **4**: 361–69.

Moon J S, Park Y H, Jung S C, Ku B G, Jang G C, Shin S S, Lee S L, Lee J M and Shin S J. 1994. Serological tests in canine brucellosis. *RDA Journal of Agriculture Science (Veterinary)* **36**: 614–21.

Myers D M, Jones L M and Varela-Diaz V M. 1972. Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by *B. ovis* and other *Brucella*. *Applied Microbiology* **23**: 894–902.

Nielsen K, Smith P, Conde S, Draghi de Benitez G, Gall D, Halbert G, Kenny K, Massengill C, Muenks Q, Rojas X, Perez B, Samartino L, Silva P, Tollersrud T and Jolley M. 2004. Rough lipopolysaccharide of *Brucella abortus* RB51 as a common antigen for serological detection of *B. ovis*, *B. canis*, and *B. abortus* RB51 exposure using indirect enzyme immunoassay and fluorescence polarization assay. *Journal of Immunoassay Immunochemistry* **25**(2): 171–82.

Nielsen K, Heck F, Waagner G, Stilla J, Rosenbaun B and Flores E. 1984. Comparative assessment of antibody isotype to *Brucella abortus* by primary and secondary binding assays. *Preventive Veterinary Medicine* **20**: 197–204.

Perrin P and Sureau P. 1987. A collaborative study of an experimental kit for rapid rabies enzyme immunodiagnosis (RREID). *WHO Bulletin* **65**: 489–93.

Prasad R. 2004. Evaluation of Recombinant OMP 28 protein of *Brucella melitensis* as a potential diagnostic agent. M.V.Sc. thesis submitted to Deemed University, IVRI, Izatnagar.

Thrusfield M. 1997. *Veterinary Epidemiology*. 2nd edn. ELST, UK.

Wanke M M. 2004. Canine Brucellosis. *Journal of Animal Reproduction Science* **82–83**: 195–207.

Zoha S J and Carmichael L E. 1982a. Properties of cell wall antigens of virulent *Brucella canis* and a less mucoid variant of reduced pathogenicity. *American Journal of Veterinary Research* **43**: 171–74.

Zoha S J and Carmichael L E. 1982b. Serological response of dogs to cell wall and internal antigens of *Brucella canis*. *Veterinary Microbiology* **7**: 35–50.

Zygmunt M S, Baucheron S, Vizcanio N, Bowden R A and Cloeckaert A. 2002. Single step purification and evaluation of a recombinant BP 26 protein for serological diagnosis of *Brucella ovis* infection in rams. *Veterinary Microbiology* **87**: 213–20.