Available online at www.sciencedirect.com
ScienceDirect
Research in Veterinary Science 83 (2007) 340–346
RVS
www.elsevier.com/locate/rvsc

Diagnosis of canine brucellosis by ELISA using an antigen obtained from wild *Brucella canis*

Stella Maria Barrouin-Melo [a,*], Fernando Padilla Poester [b], Marcos Borges Ribeiro [c], Adriano Costa de Alcântara [c], Paulo Henrique Palis Aguiar [a], Ivana Lúcia Nascimento [c], Robert Eduard Schaer [c], Roberto Meyer Nascimento [c], Songelí Menezes Freire [c]

[a] *Departamento de Patologia e Clínicas, Escola de Medicina Veterinária, Universidade Federal da Bahia, Av. Adhemar de Barros, 500, Salvador, BA, CEP 40170-110, Brazil*
[b] *Instituto de Pesquisas Veterinárias Desidério Finamor (FEPAGRO), Estrada do Conde, 6000, Eldorado do Sul, RS, CEP 92990-000, Brazil*
[c] *Laboratório de Imunologia e Biologia Molecular, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Vale do Canela S/N, Salvador, BA, CEP 40110-100, Brazil*

Accepted 19 February 2007

Abstract

An indirect ELISA test was developed for the diagnosis of *Brucella canis* infection in dogs. A bacterial whole cell extract was used as a solid phase antigen, using *B. canis* isolated from an infected animal. Sera from culture-positive and healthy negative animals were used as internal reference controls. The cut-off point was determined by a mathematical formula for a statistically valid value, which defined the upper prediction limit, based on the upper tail of the *t*-distribution of 21 negative control sera readings, for the confidence level of 99.5%. The sensitivity and specificity of the ELISA test were 95% and 91%, respectively. The ELISA test showed a significant concordance index ($K = 0.84$) with the agar gel immunodiffusion test. The reliability of the ELISA for the detection of infected animals was established by a double blind study testing 280 sera provided by serum banks from different diagnostic and research institutions and analyzed by ROC Curve.

Keywords: *Brucella canis*; ELISA; Serodiagnosis; Canine brucellosis

1. Introduction

Canine brucellosis is a zoonotic bacterial infection whose clinical diagnosis is difficult to perform (Berthelot and Garin-Bastuji, 1993; Mateu-de-Antonio et al., 1993; Carmichael and Shin, 1996). Human infections have been reported, being laboratory technicians and dog owners the most exposed to *Brucella canis* (Godoy et al., 1977; Lucero et al., 2005). The disease has a worldwide distribution and represents a great cause of economic losses in breeding kennels (Carmichael, 1966; Wanke, 2004). Despite serological tests are routinely used, the blood culture is considered the only definitive test for the diagnosis of canine infection (Carmichael and Shin, 1996; Baldi et al., 1997; Wanke, 2004). Clinical canine brucellosis is commonly confirmed by different serological tests, which include: (i) tube agglutination test (TAT); (ii) rapid plate agglutination test (RPAT), both with or without addition of 2-mercaptoethanol; and, (iii) agar gel immunodiffusion

Abbreviations: *ELISA, enzyme-linked immunosorbent assay; AGID, agar gel immunodiffusion; TAT, tube agglutination test; B. canis, Brucella canis; B. ovis, Brucella ovis*; OD, optical density

* Corresponding author. Tel.: +55 71 3263 6705; fax: +55 71 3263 6718.
E-mail address: barrouin@ufba.br (S.M. Barrouin-Melo).

0034-5288/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2007.02.006

test (AGID), using antigens prepared either with *B. canis* or *Brucella ovis* (Serikawa et al., 1989; Baldi et al., 1994, 1997; Carmichael and Shin, 1996; Lucero et al., 2002). Cross-reactions are common, occurring with bacteria like *Pseudomonas aeruginosa*, mucoid *Staphylococcus* spp., and *Bordetella bronchiseptica* among others, producing false positive results (Johnson and Walker, 1992; Mateu-de-Antonio et al., 1993; Baldi et al., 1994; Carmichael and Shin, 1996).

Tests like the counterimmunoelectrophoresis (Myers and Varela-Diaz, 1980), the indirect immunofluorescence, the complement fixation test (Schlemper and Vaz, 1990; Johnson and Walker, 1992; Carmichael and Shin, 1996) and the enzyme-linked immunosorbent assay (ELISA) (Berthelot and Garin-Bastuji, 1993; Baldi et al., 1994) have shown to be effective for the diagnosis of *B. melittensis*, *B. ovis* and *B. abortus*.

Several authors have recommended the use of ELISA for the detection of anti-*B. canis* antibodies in dogs, but variable results have been reported. Johnson and Walker (1992), using *B. canis* cell wall antigens, obtained equivalent specificity but lower sensitivity when compared with TAT. Mateu-de-Antonio et al. (1993) obtained 95.6% of specificity and 93.8% of sensitivity in an ELISA test using as antigen an extract prepared from a less mucoid variant of *B. canis*. Baldi et al. (1994, 1997), using as antigen a purified cytoplasmic protein (p18), and Letesson et al. (1997) using recombinant cytoplasmic proteins (p15, p17 and p39), all common proteins in the genus *Brucella*, reported sensitive and specific ELISAs for canine and cattle brucellosis, respectively.

In Brazil, the acidified antigen test (rose bengal), the 2 mercaptoethanol test and the complement fixation test (CFT) are currently used tests for the serodiagnosis of smooth *brucellae* (Brasil, 2001). The AGID test is the most widely used in routine serodiagnosis of rough *brucellae*. Presently there is only one commercially available AGID kit in the country, which uses a heat saline soluble *B. ovis* extract as antigen (Schlemper and Vaz, 1990; Keid et al., 2004). Agglutination tests using whole bacterial cells, AGID and ELISA tests with diverse antigen preparations, from cell fractions to recombinant proteins, have been the most quoted tests in reports worldwide for current serological diagnosis of the infection (Ebani et al., 2003; Lucero et al., 2005; Wanke, 2004; Wanke et al., 2006).

None of the referred serological methods are diagnostically conclusive, being the ELISA test still considered the most specific and sensitive. Furthermore, the use of an antigen, which is easy to prepare and capable to provide a test with good sensitivity and specificity, would improve surveillance studies of canine brucellosis. The test would allow as well the identification of potentially infected individuals. The present study describes the application of an antigen prepared from a *B. canis* which was isolated from an infected dog and used for the standardization of an indirect ELISA test.

2. Materials and methods

2.1. Bacterial strain and antigen preparation

A gram negative bacteria was isolated on blood agar (96 h incubation at 37 °C, with no CO_2 requirement), from the placenta and fetuses of a bitch with positive AGID test for *B. canis* that aborted between 42nd and 45th days of gestation (Vargas et al., 1996). The small non hemolytic colonies isolated presented a white-greyish color with a rough morphology as demonstrated by the purple color of the colonies after the plates being flooded with crystal violet dye (White and Wilson, 1951). No growth occurred on Mac Conkey agar. The organism gave positive results for catalase, oxidase, nitrate reduction, urease and negative results for citrate, Voges Proskauer and methyl red. No H_2S production or glucose utilization were observed. The bacteria agglutinated with anti-R sera and with acriflavin with no agglutination with anti-A or -M monospecific sera (Alton et al., 1988). Based on these results the bacterium was classified as *B. canis*.

For the antigen preparation, a heat soluble bacterial extract was obtained following the method described by Myers et al. (1972), with minor modifications. Briefly, the culture was transferred to 5 mL of Brucella Broth (BBL Microbiological Systems, Cockeysville, USA), cultured for 48 h at 37 °C, and expanded in Roux flasks containing 100 mL of Brucella Agar (Difco Laboratories, Detroit, USA) under aerobiosis, for 48 h at 37 °C, as described by Carmichael and Bruner (1968). Bacterial cells were harvested with 50 mL of sterile PBS (phosphate buffered saline; 150 mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, 9 mM $Na_2HPO_4 \cdot 12H_2O$, pH 7.4) and inactivated by heat (1 h, 56 °C). The suspension was filtered through sterile gauze and washed three times by centrifugation (3,500 × g, 10 min) in PBS. The pellet was then re-suspended in 10 mL of PBS and autoclaved at 120 °C under 1.5 Atmospheres for 20 min. The cells were then centrifuged at 12000 × g for 20 min, at 4 °C. The supernatant, collected and stored in 200 μL aliquots at −20 °C, was used as the ELISA solid phase antigen. The protein concentration of the antigen was determined by the method of Lowry et al. (1951).

2.2. Sera

Twenty positive sera from animals with clinical signs, positive bacterial isolations and AGID-positive tests for brucellosis test were used as reference. Ten negative serum samples from adult healthy animals, obtained in a kennel with no history or evidences of canine brucellosis (such as reproductive disorders associated to fever, lymphadenopathy or other clinical signs) were tested negative by the AGID and used as negative reference. These reference sera were used for the calculation of specificity and sensitivity of the ELISA test. Twenty one samples from healthy 6 months old animals, kept in a research center were used

for calculation of the cut-off for the ELISA tests and as negative control sera for western blotting assays. These sera were kindly supplied by The Oswaldo Cruz Foundation (FIOCRUZ-Bahia). Two hundred and eighty (280) serum samples of unknown serology status for *B. canis* were obtained from serum banks of different research institutions. These 280 field sera were tested together with the reference sera in a double blind testing, for determination of the kappa values for concordance evaluation between the positive results obtained by ELISA and AGID.

2.3. Agar gel immunodiffusion test – AGID test

All sera were tested using a commercial kit (Tecpar-Brazil) according to the manufacturer's instructions.

2.4. Indirect ELISA protocol

The indirect ELISA was standardized and performed as described elsewhere (Mateu-de-Antonio et al., 1993; Carpenter, 1997) with modifications. Optimum antigen concentration, as well as peroxidase-conjugated anti-dog immunoglobulin and sera dilutions were determined by serial titrations and incubations were performed in a humid chamber. Briefly, flat bottomed polystyrene 96-well microtiter plates (Corning Inc., New York, USA), were sensitized overnight at 4 °C with 50 μL/well of antigen, diluted to 5 μg/mL in 0.05 M sodium carbonate pH 9.6. The plates were washed five times with PBS containing 0.05% (v/v) Tween-20 (PBS-T). Each well of the antigen-coated plates were blocked with 200 μL of 5% skimmed milk in PBS-T and incubated at 37 °C for 1 h. After five washes, 100 μL of control and test sera samples, diluted 1:1000 in PBS-T containing 1% (v/v) of skimmed milk, were added to each well in duplicate. The plates were sealed and incubated at 37 °C for 1 h. After five washing cycles with PBS-T, 100 μL of anti-dog IgG conjugated with peroxidase (Sigma, St Louis, USA), at a dilution of 1:10 000 in PBS-T, were added to each well, and the plates incubated at 37 °C for 1 h. After five washings as described above, the color reaction was developed by adding 50 μL/well of a solution containing 1.0 mg/mL of o-phenylenediamine dihydrochloride (OPD; Sigma, St Louis, USA) in 0.05 M citrate buffer (pH 4.0) with 0.04% (v/v) H_2O_2. The plates were incubated in the dark for 15 min, at room temperature. Color development was halted by the addition of 25 μL/well of 4N H_2SO_4. The absorbance measurements were made at 492 nm, using an automatic ELISA plate reader (550, Bio-Rad, Life Sciences, Hercules, USA). Samples with an absorbance value equal to or higher than the cut-off value were considered as positives.

2.5. SDS–PAGE and Western blotting

The antigen was solubilized in sample buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using 12% polyacrylamide gels and analysed with Coomassie blue (2.5% brilliant blue in 50% methanol, 10% acetic acid) staining. For Western blotting, electrophoresed antigen was transferred to nitrocellulose membranes by using wet procedure. Unbound sites on the membranes were blocked with 5% skimmed milk in PBS-T, at 4 °C, by incubation overnight. The membranes were cut into 0.5 cm wide strips after being washed five times with PBS-T. Each of 20 positive and 20 negative control sera, diluted 1:100 in PBS-T with 1% of skimmed milk were added to individual strips, which were incubated at 37 °C for 1 h. Following, the strips were washed five times with PBS-T and blots developed with peroxidase labeled anti-dog IgG (Sigma, St Louis, USA), diluted at 1:500 in PBS-T, during 1 h at 37 °C. After five washing steps, the strips were immersed in a substrate solution, made by a 1:5 dilution of a 0.3% 4-Cl-α-naphtol in methanol and Tris-saline buffer (0.05 M Tris–HCl, 0.2 M NaCl, pH 7.2), with 0.04% (v/v) H_2O_2, and the reaction developed in darkness, at room temperature, for 10–15 min. A last washing step was performed once with distilled water.

2.6. Test performance determinations and statistical analysis

The cut-off value was calculated in according to a mathematical method as described by Frey et al. (1998), which takes in consideration the upper tail of the *t*-distribution of negative control sera readings, as expressed in the following equation

$$\text{Cut-off} = X + \text{SD}f \tag{1}$$

where X is the mean of independent sera readings and SD is the standard deviation, whose calculations were performed using Prism® software (GraphPad Software Inc.). The factor f is based on the number of negative controls and the confidence level $(1 - \alpha)$. The value of f used for calculation of the cut-off point in this study was of 2.932, and corresponded to the desired confidence level of 99.5% ($\alpha = 0.005$) and the sample size of 21 controls, since the sera of 21 healthy young dogs were used for this purpose (Frey et al., 1998).

Assay-to-assay variation was corrected for statistical analysis, in accordance with Zwirner (1996). The corrected OD (COD) of each serum sample was calculated by multiplying its mean OD value of readings by the correction factor (CF) between plates, as determined by the following equation:

$$\text{COD} = \text{OD} \cdot \text{CF} \tag{2}$$

where CF = \frac{Mean OD of the positive control sera from a reference plate}{Mean OD of the positive control sera from plate of the assay}.

To calculate the ELISA specificity and the sensitivity, 20 positive and 10 negative ELISA reference sera were analyzed in comparison with the same sera's reactivity by the AGID test. The following Eqs. (3) and (4) were applied, as described by Ferreira and Ávila (1996)

Specificity

$$= \backslash frac\{\text{True negative sera}\}\{\text{True negative} + \text{False positive sera}\} \quad (3)$$

Sensitivity

$$= \backslash frac\{\text{True positive sera}\}\{\text{True positive sera} + \text{False negative sera}\} \quad (4)$$

The results obtained by the indirect ELISA were compared with those obtained by AGID test using known reference and field samples of unknown serologic status. The reduced difference statistical test was used for comparison of the proportion of positive results between tests (Schwartz, 1987). The kappa index of concordance between methods was determined by testing 20 positive and 10 negative control sera, and the 280 sera from field, in double-blind tests (Armitage and Berry, 1994). Mean and standard deviation (SD) of corrected OD reading values of all sera were used for ROC Curve analysis (Griner et al., 1981).

3. Results

3.1. *B. Canis* antigen and standardization of the ELISA

Each Roux flask containing a *B. canis* culture yielded approximately 30 mg of bacterial extract, as determined by Lowry's method for protein quantitation. This antigen preparation showed a complex electrophoretic profile with various bands in the SDS–PAGE Coomassie blue-stained gel (Fig. 1). The Western immunoblotting analysis of the antigen using positive and negative reference sera showed a pattern of band recognition more consistently for polypeptides of 12, 18, 58 and 65 KDa, as represented in Fig 2.

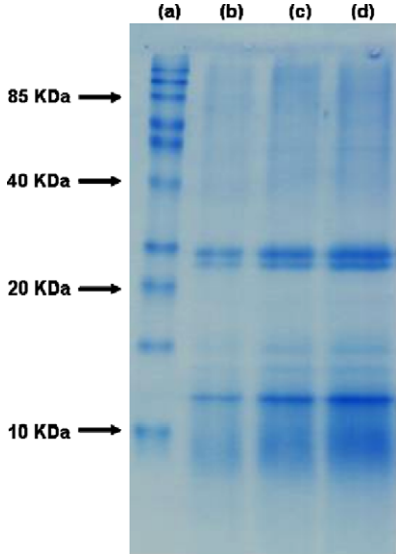

Fig. 1. Profile of *B. canis* antigen preparation in three different dilutions (lanes b, c and d) on a Coomassie blue-stained SDS–PAGE. Lane (a) contains the benchmark pre-stained protein ladder for molecular weight (Invitrogen) as indicated by numbers on the left.

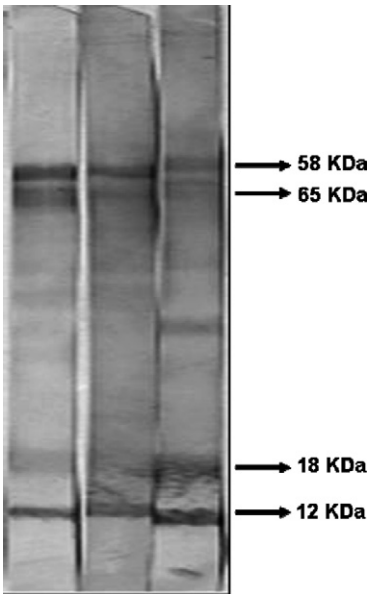

Fig. 2. Representative profile of the more consistently recognized bands by positive sera of *B. canis* antigen preparation on nitrocellulose immunoblot. The numbers to the right indicate the molecular weight of the protein bands.

In order to determine the optimum concentration of antigen to be adsorbed to microtiter plates, previous assays were performed, using from 1 to 20 μg/mL in 0.05 M sodium carbonate pH 9.6. Best results were obtained with 5 μg/mL of bacterial extract in 50 μL (250 ng) of solution in each well. By serial titrations of positive control sera and peroxidase-conjugated anti-dog IgG antibody, the optimal dilutions for the indirect ELISA were established as being respectively 1:1000 and 1:10 000. The cut-off OD value, as determined by sera samples from 21 healthy 6 months old dogs, was 0.218.

3.2. Analysis of field sera by the indirect ELISA

A double-blind testing, performed with the indirect ELISA test, included 280 field sera from dogs with unknown history of brucellosis, the 30 reference positive and negative sera, and the 21 serum samples from young healthy dogs, totalizing 331 samples. In this study, it was found that 72 sera samples (26%) presented OD values above the cut-off, and were positive to ELISA. The OD values for the positive samples ranged from 0.218 to 1.115 (mean = 0.466; SD = 0.238) and for the negative samples ranged from 0.046 to 0.217 (mean = 0.108; SD = 0.042). The ratio between the OD value from the highest positive serum and the mean OD of the negative control sera was 10.32 (1.115/0.108), and was calculated according to Mateu-de-Antonio et al. (1993) and Baldi et al. (1994). The distribution of the OD frequencies among the field sera are shown in Fig. 3.

The sensitivity and the specificity of the indirect ELISA, determined by the comparative testing of positive and negative by the AGID, was of 95% and 91%, respectively.

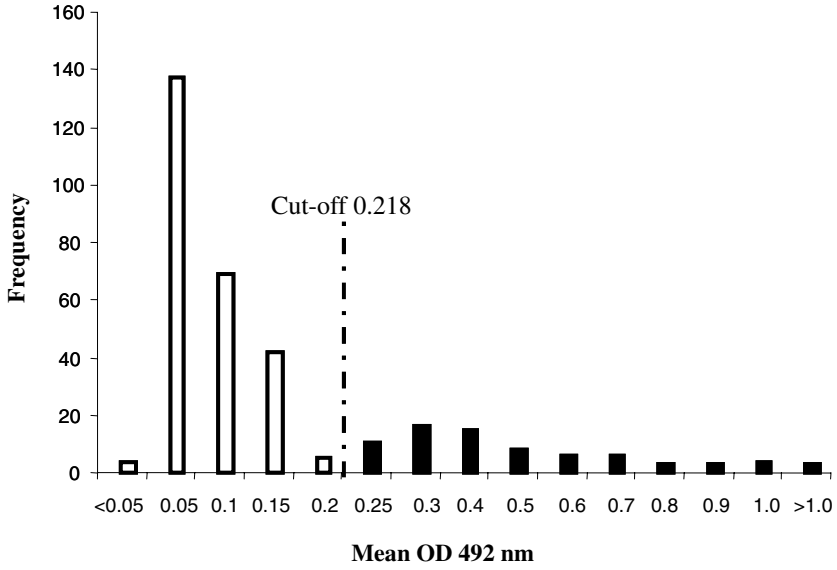

Fig. 3. Frequency distribution of the mean OD values of positive (■) and negative (□) canine field sera by indirect ELISA for detection of antibody against *Brucella canis*. All sera that had an OD above 0.218 (cut-off) were considered to be positive.

Assay-to-assay variation, as determined by each plate correction factor for repeated assays using the same 30 control sera, was 17%.

The ability to distinguish positive and negative sera to *B. canis* antigens by detection of antibody reactivity by AGID and by indirect ELISA was compared. These results were used to calculate the sensitivity, the specificity and the Kappa index of concordance between tests. The correlation between the ELISA and the AGID was highly significant, as expressed by the Kappa index of 0.84. The reduced difference test ($\epsilon = 1.64$; $p < 0.05$) also showed a significant test agreement.

The overall diagnostic accuracy of the ELISA test was also evaluated by the ROC curve (receiver operating characteristics-SPSS) analysis, applied to the 280 field sera, using as standard references the absorbance values of the positive and negative control sera. For the selected cut-off value of 0.218, the ELISA test had both, specificity and sensitivity of 100%.

4. Discussion

In this study, we describe the development and evaluation of an indirect ELISA for the serodiagnosis of canine brucellosis. A bacterial whole cell extract was used as solid-phase antigen and sera from culture positive and healthy negative animals used as reference controls. In the double-blind analysis of the reactivity of canine field sera to *B. canis* antigens, the ELISA allowed a clear separation between positive and negative samples. In addition to the relatively good degree of agreement between ELISA and AGID, the results indicate that the ELISA is a specific and sensitive test for detection of anti-*Brucella* antibodies in canine sera.

The procedure used in the present work for obtaining the antigen preparation was based on the technique described by Myers et al. (1972), with some modifications. This technique may have accounted for the maintenance of some intact outer membrane proteins (OMPs) as well as cytosolic proteins, which are highly specific for the genus *Brucella* (Cloeckaert et al., 1990; Kittelberger et al., 1998; Wanke et al., 2002). Among four protein bands identified more consistently by *B. canis* positive sera by Western blotting in the present study, two low-molecular-weight bands, of 12 and 18 kDa, could be of particular interest for further studies. A cytoplasmic 18 kDa molecular weight *Brucella* protein has been identified previously as lumazine synthase and described as being highly antigenic in serological tests for canine brucellosis (Baldi et al., 1994, 1997; Goldbaun et al., 1999). On the other hand, low molecular mass protein fractions of *Brucella* outer membrane proteins are responsible for conferring good sensitivity in serological tests for diagnosis of recent infection (Cloeckaert et al., 1990; Wanke et al., 2002; Lopez et al., 2005). These features of the antigen preparation may explain the good sensitivity achieved by the ELISA test in our study, since the technique used to extract antigen from bacteria may influence the primary structure of the molecules and have an important role in its function.

Among various criteria reported for selecting negative serum groups used for determining the cut-off value, the serum collected from healthy dogs, before their puberty, under 6 months of age, was chosen. This criterion was based on the literature, which shows a higher probability of brucellosis be present in animals at reproductive ages than in ages before reproductive activity (Moore and Gupta, 1970; Carmichael and Joubert, 1988). The analysis of positive and negative sera provided by research institutions, as well as the use of field samples, made possible the comparative evaluation between the AGID test and the indirect ELISA.

The ELISA is a test that has the advantage of reducing interference of possible human errors and provides objective and readily measurable results due to the use of cut-off value and internal controls (Baldi et al., 1994; Troy et al., 1996). Despite the reduced overall sensitivity and specificity reported for the AGID test (Zwirner, 1996), the method was selected for comparison with the indirect ELISA test developed in the present study, because it is the official test for serological diagnosis of canine brucellosis in Brazil.

In terms of general sensitivity, ELISA techniques are superior to other tests, namely agglutination and AGID, commonly used for the serological diagnosis of *Brucella* infection (Johnson and Walker, 1992; Wanke et al., 2002; Wanke, 2004). High specificity and the capacity of detecting low levels of antibodies can be achieved with good antigen preparations, as shown by the results of the present study, in which the standardization of the indirect ELISA test was performed with sera dilutions of 1:1000. The agreement between AGID and ELISA was significant, as determined by the Kappa index of concordance of 0.84 between tests. The ELISA test specificity was of 91% and its sensitivity was of 95%. As the AGID results were taken as comparative standards, it is possible that these indexes of sensitivity and specificity may be underestimated. In fact, the ROC curve analysis of the test as it was performed showed 100% indexes for sensitivity and specificity. Studies regarding the reproducibility of the indirect ELISA are currently being carried out.

Despite the incidence of human infection by *B. canis* is not actually known (Wanke, 2004), the potential exposure to the bacteria represents a risk, particularly for laboratory or kennel workers, as well as dog owners, as reported elsewhere (Godoy et al., 1977; Carmichael and Shin, 1996; Lucero et al., 2005). It rises the needing for efficient and inexpensive tests, which are appropriate for use in disease control programs of public health, particularly in developing countries. The antigen preparation and its application in an indirect ELISA, as described in the present study, were found to be feasible, simple to prepare and of low cost. The bacteria used for antigen preparation were obtained from a *B. canis* culture, isolated from a local infected animal. In spite of efficient ELISA tests being described elsewhere (Mateu-de-Antonio et al., 1993; Baldi et al., 1994, 1997; Wanke et al., 2002), it must be emphasized that laboratory procedures for antigen purification, that can improve the quality of immunological tests, may require expensive reagents and technology. This aspect can be restrictive for a number of developing countries.

Taking into account the advantages of the indirect ELISA described herein, it can be concluded that the test might be sufficiently accurate to be recommended as a serological test for either, field sera screening or confirmation of clinical canine brucellosis. The assay, for detecting antibodies against *B. canis* antigens, may complement or offer an alternative to the available tools for diagnose the disease.

Acknowledgements

We gratefully acknowledge Prof. Thereza Martinez for assistance with bacterial techniques and Prof. Thereza Calmon for statistical counseling. We are also indebted to Dr. Moacir Paranhos (CPqGM-FIOCRUZ-BA), Dr. Carlos Chávez Olortegui (FUNED-MG), the Laboratório de Doenças Tropicais (HUPES-UFBA) and the Centro de Controle de Zoonoses–Prefeitura de São Paulo for providing the canine serum samples. This work was financially supported by the Brazilian National Council for Research and Technological Development (CNPq).

References

Alton, J.J., Jones, L.M., Angus, R.D., Verger, J.M., 1988. Techniques for the Brucellosis Laboratory. INRA, Paris.

Armitage, P., Berry, G., 1994. Statistical Methods in Medical Research, third ed. Blackwell Source, New York, p. 923.

Baldi, P.C., Wanke, M.M., Loza, M.E., Fossati, C.A., 1994. Brucella abortus cytoplasmic proteins used as antigens in an ELISA potentially useful for the diagnosis of canine brucellosis. Vet. Microbiol. 41, 127–134.

Baldi, P.C., Wanke, M.M., Loza, M.E., Monachesi, N., Fossati, C.A., 1997. Diagnosis of canine brucellosis by detection of serum antibodies against an 18 kDa cytoplasmic protein of *Brucella* spp. Vet. Microbiol. 51, 273–281.

Berthelot, X., Garin-Bastuji, B., 1993. Brucelloses canines. Le Point Veterinaire 25, 125–129.

Brasil, 2001. Departamento de Defesa Animal. Informações sobre o PNCBET. Ministério da Agricultura, Pecuária e Abastecimento, Brasília, Brasil. <http:www.agricultura.gov.br/sda/dda/programa.htm>.

Carmichael, L.E., 1966. Abortion in 200 Beagles. JAVMA 149, 1126–1966.

Carmichael, L.E., Bruner, D.W., 1968. Characterization of a newly recognized species of Brucella responsible for infectious canine abortions. Cornell Vet. 58, 579–592.

Carmichael, L.E., Joubert, J.C., 1988. Transmission of *Brucella canis* by contact exposure. Cornell Vet. 78, 63–73.

Carmichael, L.E., Shin, S.J., 1996. Canine brucellosis: a diagnostician's dilemma. Semin. Vet. Med. Surg. (Small Animal) 11, 161–165.

Carpenter, A.B., 1997. Enzyme-linked immunoassays. In: ROSE, N.R. (Ed.), Manual of Clinical Laboratory Immunology, fifth ed. ASM Press, Washington DC, pp. 20–29.

Cloeckaert, A., de-Wergifosse, P., Dubray, G., Limet, J.N., 1990. Identification of seven surface-exposed Brucella outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. Infect. Immun. 58, 3980–3987.

Ebani, V.V., Cerri, D., Fratini, F., Bey, R.F., Andreani, E., 2003. Serological diagnosis of brucellosis caused by *Brucella canis*. New Microbiol. 26, 65–73.

Ferreira, W., Ávila, S.L.M., 1996. Diagnóstico laboratorial das principais doenças infecciosas e auto-imunes. Guanabara Koogan, Rio de Janeiro; p. 302.

Frey, A., Canzio, J.D., Zurakowski, D., 1998. A statistically defined endpoint titer determination method for immunoassays. J. Immunol. Method 221, 35–41.

Godoy, A.M., Peres, J.N., Barg, L., 1977. Isolamento de *Brucella canis* em Minas Gerais Brasil. Arq. Esc. Vet. Univ. Fed. Minas Gerais 29, 35–42.

Goldbaun, F.A., Velikovsky, C.A., Baldi, P.C., Mortl, S., Bacher, A., Fossati, C.A., 1999. The 18-kDa cytoplasmic protein of Brucella

species – an antigen useful for diagnosis – is a lumazine synthase. J. Med. Microbiol. 48, 833–839.

Griner, P.F., Mayewski, R.J., Mushlin, A.I., Greenland, P., 1981. Selection and interpretation of diagnostic tests and procedures. Ann. Intern. Med. 94, 555–600.

Johnson, C.A., Walker, R.D., 1992. Clinical signs and diagnosis of *Brucella canis* infection. Comp. Cont. Educ. 14, 763–772.

Keid, L.B., Soares, R.M., Morais, Z.M., Richtzenhain, L.J., Vasconcellos, S.A., 2004. *Brucella* spp. isolation from dogs from commercial breeding kennels in São Paulo State, Brazil. Braz. J. Microbiol. 35, 161–166.

Kittelberger, R., Diack, D.S., Vizcaíno, N., Zygmunt, M.S., Cloeckaert, A., 1998. Characterization of an immuno-dominant antigen in *Brucella ovis* and evaluation of its use in an enzyme-linked immunosorbent assay. Vet. Microbiol. 59, 213–227.

Letesson, J.J., Tibor, A., Eynde, G., Wansard, V., Weynants, V., Denoel, P., Saman, E., 1997. Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. Clin. Diag. Lab. Immunol. 4, 556–564.

Lopez, G., Ayala, S.M., Escobar, G.I., Lucero, N.E., 2005. Use of *Brucella canis* antigen for detection of ovine serum antibodies against *Brucella ovis*. Vet. Microbiol. 105, 181–187.

Lowry, O.H., Rosembrogh, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lucero, N.E., Escobar, G.I., Ayala, S.M., 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.

Lucero, N.E., Escobar, G.I., Ayala, S.M., Jacob, N., 2005. Diagnosis of human brucellosis caused by *Brucella canis*. J. Med. Microbiol. 54, 457–461.

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

Moore, J.A., Gupta, B.N., 1970. Epizootiology, diagnosis and control of *Brucella canis*. JAVMA 156, 1737–1740.

Myers, D.M., Jones, L.M., Varela-Diaz, V.M., 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.

Myers, D.M., Varela-Diaz, V.M., 1980. Serological and bacteriological detection of *Brucella canis* infection of stray dogs in Moreno, Argentina. Cornell Vet. 70, 258–265.

Schwartz, D., 1987. Méthodes statistiques à l'usage des médecins et des biologistes, 10 ed. Médecine-Sciences Flammarion, Paris, pp. 218–223.

Schlemper, S.R.M., Vaz, A.K., 1990. Inquérito sorológico para brucelose canina por *Brucella canis* na região do planalto catarinense – Brasil. Rev. Bras. Med. Vet. 12, 8–12.

Serikawa, T., Iwaki, S., Mori, M., 1989. Purification of *Brucella canis* cell wall antigen using immunosorbent assay for specific diagnosis of canine brucellosis. J. Clin. Microbiol. 27, 837–842.

Troy, G.C., Becker, M.J., Greene, R.T., 1996. Proficiency testing of selected antigen and antibody tests for use in dogs and cats. JAVMA 209, 914–917.

Vargas, A.C., Lazzari, A., Dutra, V., Poester, F.P., 1996. Brucelose canina: Relato de caso. Ciência Veterinária 26, 305–308.

Wanke, M.M., 2004. Canine brucellosis. Anim. Reprod. Sci. 82–83, 195–207.

Wanke, M.M., Delpino, M.V., Baldi, P.C., 2002. Comparative performance of tests using cytosolic or outer membrane antigens of Brucella for the serodiagnosis of canine brucellosis. Vet. Microbiol. 88, 367–375.

Wanke, M.M., Delpino, M.V., Baldi, P.C., 2006. Use of enrofloxacina in the treatment of canine brucellosis in a dog kennel (clinical trial). Theriogenology 66, 1573–1578.

White, P.G., Wilson, J.B., 1951. Differentiation of smooth and non-smooth colonies of brucellae. J. Bacteriol. 61, 239–240.

Zwirner, N.W., 1996. ELISA. In: Margni, R.A. Imunología e Imunoquímica. fifth ed. Editorial Médica Panamericana., Buenos Aires; pp. 798–820.