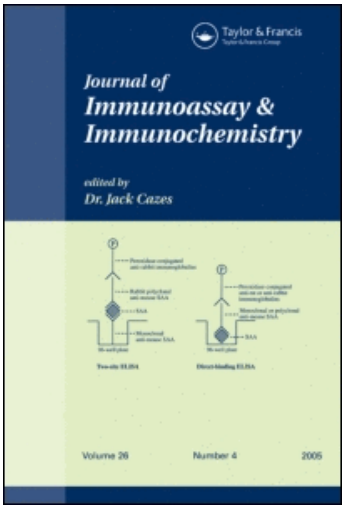

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Detection of Ovine Antibody to *Brucella ovis* by Indirect Enzyme Immunoassay

K. Nielsen[a]; P. Smith[a]; W. L. Yu[a]; X. Rojas[b]; B. Perez[c]; S. Conde[d]; L. Samartino[d]; C. Robles[e]
[a] Ottawa Laboratories (Fallowfield), Canadian Food Inspection Agency, Ottawa, Ontario, Canada [b] Institute of Microbiology, University of Southern Chile, Valdivia, Chile [c] SAG, Santiago, Chile [d] INTA, Buenos Aires, Argentina [e] INTA, Bariloche, Argentina

To cite this Article Nielsen, K. , Smith, P. , Yu, W. L. , Rojas, X. , Perez, B. , Conde, S. , Samartino, L. and Robles, C.(2007) 'Detection of Ovine Antibody to *Brucella ovis* by Indirect Enzyme Immunoassay', Journal of Immunoassay and Immunochemistry, 28: 3, 243 — 250
To link to this Article: DOI: 10.1080/15321810701454789
URL: http://dx.doi.org/10.1080/15321810701454789

Journal of Immunoassay & Immunochemistry, 28: 243–250, 2007

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810701454789

Detection of Ovine Antibody to *Brucella ovis* by Indirect Enzyme Immunoassay

K. Nielsen, P. Smith, and W. L. Yu
Canadian Food Inspection Agency, Ottawa Laboratories (Fallowfield), Ottawa, Ontario, Canada

X. Rojas
Institute of Microbiology, University of Southern Chile, Valdivia, Chile

B. Perez
SAG, Santiago, Chile

S. Conde and L. Samartino
INTA, Buenos Aires, Argentina

C. Robles
INTA, Bariloche, Argentina

Abstract: Because some batch-to-batch variation in the preparation of rough lipopolysaccharide (RLPS) from *Brucella ovis* has been experienced, several protocols were tested to establish the most reliable method for detection of antibody in indirect enzyme immunoassay. An early version of the assay gave a performance index (PI = sum of optimum percent sensitivity and percent specificity, determined by receiver operator characteristic analysis) of 198.6. This assay used RLPS from *B. ovis* as the antigen and a monoclonal antibody specific for bovine IgG_1 heavy chain-enzyme conjugate for detection. This was not repeatable using other batches of antigen. Newer versions of the assay generally had decreased sensitivity values, giving PIs of 193. Use of a recombinant protein A/G-enzyme conjugate did not improve the PI (PI = 190), giving reduced specificity and higher sensitivity. The final version used *B. abortus* RB51 RLPS as the antigen and protein A/G-enzyme

Address correspondence to K. Nielsen, Canadian Food Inspection Agency, Ottawa Laboratories (Fallowfield), 3851 Fallowfield Road, Ottawa, Ontario, Canada K2H 8P9. E-mail: nielsenk@inspection.gc.ca

conjugate for detection, giving a PI of 197. Because of the batch uniformity of the *B. abortus* RB51 RLPS and the versatility of the protein A/G-enzyme conjugate, the latter version appears to be the most useful for diagnostic serology.

Keywords: Ovine antibody, Brucella ovis, Indirect enzyme immunoassay

INTRODUCTION

Serological diagnosis of *B. ovis* infection in sheep has primarily been done using a hot saline extract antigen in agar gel immunodiffusion (AGID) or a whole cell antigen in a complement fixation test (CFT).[1] The CFT is a prescribed test for international trade while AGID and enzyme immunoassays are alternative tests.[1] Because of the inherent problems with anticomplementary activity of rough antigens, the *B. ovis* cellular antigen has a short half-life, possibly due to shedding of RLPS into the liquid phase and direct activation of complement by RLPS. The hot saline extract used for the AGID is a mixture of antigens but largely RLPS.[2] This antigen works well except some confusion arises due to the occurrence of multiple precipitin lines. As a result, each hexagonal set-up consists of antigen in the central well and two wells containing reference serum in order to determine if identity between precipitins in the reference serum and the test sera exist. The AGID is a very labour intensive test that does not lend itself to semi-automation. A number of indirect enzyme immunoassays (IELISA) have been developed. The function of the IELISA depends on the suitability of the antigen and the detection system used. As is the case with most IELISAs, a variety of reagents have been applied to the detection of antibody to *B. ovis* with various results but general concurrence that the IELISA was more sensitive and less prone to problems than the CFT and the AGID. In general, most antigens used in early IELISAs were extracted with hot saline, solvents and/or detergents and, as such, would contain RLPS as part of the antigenic mixture. Most assays used polyclonal anti-sheep immunoglobulins specific for IgG heavy chain, IgG heavy and light chain (that is, would react with all immunoglobulin classes), protein G or protein A/G, conjugated with enzyme.[2–26] Other antigens, including a cytosol protein preparation and purified cytosol proteins, Omps as well as recombinant protein antigens have been used.[5,6,13–17,26] However, these antigens provided little if any advantage over the RLPS antigen in IELISA. In addition, *B. canis* RLPS[18,20,27] and *B. abortus* RB51 RLPS[20] have been used. An advantage of using the *B. abortus* RB51 is that it is relatively easy to obtain cells for preparation of RLPS. It is also advantageous to use protein G or protein A/G enzyme conjugates as the IELISA may then also be used for the diagnosis of canine brucellosis and detection of antibody to rough *B. abortus*.[20]

This communication compares the RLPS prepared from *B. ovis* and *B. abortus* RB51 as well as a monoclonal antibody and protein A/G enzyme conjugates for the detection of antibody to *B. ovis* in sheep.

EXPERIMENTAL

Serum Samples

Sheep from South American flocks with clinical evidence of brucellosis were bled and positive sera, based on agar gel immundiffusion or complement fixation tests were selected for the study. Initially 163 samples were used, however, additional samples were added as they became available. Negative sera, initially 403 but additional samples were added as they became available, were collected randomly from Canadian flocks. All sera were taken off the clot and frozen at -20°C until tested. Because only a small amount of some sera was available, not all tests were performed with all sera.

Preparation of Antigens

B. ovis (ATCC 25840) and *B. abortus* RB51 cells suspended in 0.15 M NaCl were heat killed at 80°C for 90 minutes followed by freeze drying. Rough lipopolysaccharide (RLPS) was extracted from dry cells by the method of Galanos et al.[28] Briefly, 5 gm (dry weight) of cells were extracted with 320 ml of petroleum ether: chloroform: phenol at a ratio of 8:5:2 with constant sonication for 5 min. After stirring for an additional 15 min. the cells were pelleted by centrifugation at $10000 \times g$ for 10 min at 4°C. The extraction procedure was repeated and the two supernatant solutions were pooled. The petroleum ether and chloroform were evaporated in a chemical hood and the remainder was dialized against water to remove the phenol. This resulted in a dense white precipitate. The RLPS material was freeze dried.

Serological Tests

Polystyrene 96-well plates (NUNC 692620) were coated with 100 μL of 5.0 μg RLPS/mL of 0.06M carbonate buffer, pH 9.6 overnight at ambient temperature. The plates were then frozen. For use, plates were thawed and equilibrated at room temperature for about 60 minutes, washed 4 times with 0.01M PO_4 containing 0.15M NaCl and 0.05% Tween 20, pH 7.2 (PBST). For *B. ovis* RLPS assays, 100 μL of serum diluted 1:40 in PBST containing 15 mM EDTA and 15 mM EGTA, pH 6.3 for 30 min was added to each well. For *B. abortus* RB51 RLPS assays, 100 μL of serum diluted 1:20 in 50 mM EDTA and 50 mM EGTA was added to each well. After 4 washes with PBST, 100 μL

monoclonal antibody specific for bovine IgG_1 (M23)-or recombinant protein A/G conjugated with horseradish peroxidase,[20] appropriately diluted in PBST, were added for 60 min. Substrate and chromogen (H_2O_2 and TMB, 75 μL or H_2O_2 and ABTS, 100 μL) in 0.05M citrate buffer, pH 4.5 were added to each well and allowed to develop for 10 min. after 4 washes with PBST. Finally, if TMB was used as the chromogen, 75 μL 0.1M H_2SO_4 was added to each well to stop enzyme conversion and the resultant colour development was assessed in a spectrophotomter at 450 nm. If ABTS was used as the chromogen, optical density values were determined at 414 nm.

Data

Each 96-well plate contained duplicate samples of strongly positive control serum as well as a weakly positive and a negative serum and a conjugate control (no serum). All data was converted to % positivity (%P), calculated relative to the strongly positive serum using the following formula:

$$\%P = \text{optical density}_{\text{test}} / \text{average optical density}_{\text{strong positive control}} \times 100$$

The data was analysed using MedCalc software[29] to determine the optimum cutoff values and the % sensitivity and % specificity using that cutoff value. The performance index (PI) was calculated by adding the % sensitivity and the % specificity values.

RESULTS

The IELISA using the original preparation of *B. ovis* RLPS and the M23 monoclonal antibody enzyme conjugate gave sensitivity and specificity values of 98.8 and 99.8%. This result could not be duplicated using several batches of *B. ovis* RLPS prepared by the same procedure, giving sensitivity and specificty values between 99.4 and 93.8%. The loss in specificity was somewhat remedied by using the latter *B. ovis* RLPS antigen and protein A/G enzyme conjugate, however, this combination resulted in a loss of sensitivity (sensitivity and specificity values of 92.2 and 98.2%). Using *B. abortus* RB51 RLPS, the sensitivity and specificity values with protein A/G enzyme conjugate were 98.0 and 99.0%., very similar to the values obtained when M23 enzyme conjugate was used. These data are presented in Table 1.

DISCUSSION

There are inherent problems in producing suitable *B. ovis* antigen, especially antigens suitable for the CFT. It was initially thought that such antigen could

Table 1. Sensitivity and specificity values determined by receiver operator characteristic analysis to determine the optimal cutoff value between positive and negative serological reactions of sheep to *B. ovis* and *B. abortus* RB51 antigens using monoclonal antibody M23 - or protein A/G enzyme conjugates

	B. ovis, M23[a]	*B. ovis* M23[b]	*B. ovis*[a] PAG	RB51 M23	RB51 PAG
N+/−[c]	163/403	163/403	193/1089	265/405	293/1091
Cutoff (%P)	11	6	20	21	14
% sensitivity	98.8	99.4	92.2	98.5	98.0
% specificity	99.8	93.8	98.2	97.8	99.0
PI	198.6	193.2	190.4	196.3	197.0

[a]Original *B. ovis* antigen used in this assay.

[b]Newer lots of *B. ovis* antigen used for these assays.

[c]N = number of positive (+) and negative (−) sera, as defined in the Materials and Methods section, tested in each assay.

be used in enzyme immunoassay by extracting the immunodominant RLPS or other antigens. In this communication, we report problems with extraction of suitable RLPS from *B. ovis* cells that were unsuitable for CFT use. This problem was overcome by using *B. abortus* RB51 extracted RLPS with a minimal loss in overall assay performance (Table 1). In addition, while the use of protein G enzyme conjugate for antibody detection has been reported, the utility of the assay could be extended to detection of canine antibody to *B. canis* by using protein A/G. Thus, a suitable multispecies assay capable of detecting antibody to several *Brucella sp*. was developed. These results agree with others (18, 27) who compared *B. ovis* and *B. canis* antigens.

The standardization of serological tests has proven to be difficult. Primary binding assays in the main perform better than older conventional tests. However, all primary binding assays and in particular the ELISAs rely on several reagents. Because commercial kits are generally expensive, especially for high throughput laboratories, in-house developed reagents are in common use. This makes comparison of data between laboratories virtually impossible. Therefore, a standardized assay using an antigen that is relatively easy to prepare and an enzyme conjugate that is commercially available would be useful for harmonization of results. It is proposed that RLPS from *B. abortus* RB51 be used as the antigen. RLPS is relatively easy to prepare, requiring common inorganic solvents, a centrifuge, a household hair dryer and dialysis tubing for its preparation. As standardization of the antigen is partly based on weight, it is also necessary to have freeze drying apparatus available as well. This antigen can be used for detection of antibody to *Brucella sp.* in sheep, goats, cattle, dogs (18, 20) and probably also in man. A number of IELISAs use poly- or monclonal antibody enzyme conjugates. Most of these reagents are not commercially available and if they are, the

cost is usually high. Therefore, this is another part of the assay that cannot readily be standardized. As a result, the use of protein A/G enzyme conjugate, a commercially available product, may be reasonable. Even though it is relatively expensive, the per test cost is reasonable due to the low quantity required. This scheme allows for standardization of the IELISA so that results between laboratories will be comparable and the process would be accelerated if the OIE was prepared to supply an international standard positive and negative serum, from each species, for each test.

Of the presented IELISA formats, the assay using *B. abortus* RB51 RLPS as the antigen and protein A/G enzyme conjugate resulted in the highest PI value and hence the most accurate, as well as the most readily standardizable serological test.

REFERENCES

1. *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Ovine epididymitis*; OIE: Paris, 2004, 589–597.
2. Riezu-Boj, J.; Moriyon, I.; Blasco, J.; Marin, C.; Diaz, R. Comparison of lipololysaccharides and outer membrane protein-lipopolysaccharide extracts in enzyme-linked Immunosorbent assay for the diagnosis of *Brucella ovis* infection. J. Clin. Microbiol. **1986**, *32*, 938–942.
3. Afzal, M.; Tengerdy, R.; Squire, P.; Ellis, R. Characterization of *Brucella ovis* lipopolysaccharide and its use for diagnosis of ram epididymitis by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **1984**, *20*, 1159–1164.
4. Cardenas, K.; Maki, L. Detection of antibody in rams with contageous epididymitis, using the enzyme-linked immunosorbent assay. Am. J. Vet. Res. **1986**, *47*, 738–739.
5. Cerri, D.; Ebani, V.; Pedrini, A.; Bassi, S.; Bey, R.; Andreani, E.; Farina, R. Evaluation of tests employed in serological diagnosis of brucellosis caused by *Brucella ovis*. New Microbiol. **2000**, *32*, 281–288.
6. Chin, J.; Pang, B.; Carrigan, M. Comparison of seroreactivity of rams with brucellosis in complement fixation test, whole cell ELISA and by imunoblotting. Vet. Microbiol. **1991**, *26*, 291–299.
7. Chin, J.; Pang-Turner, B. Profiles of serological reactivity against cytosoluble antigens of *Brucella ovis* in experimentally infected rams. J. Clin. Microbiol. **1990**, *28*, 2647–2652.
8. Chin, J. Comparison of different antigenic preparations for the detection of serum antibodies against *Brucella ovis* by ELISA. Austral. Vet. J. **1983**, *60*, 261–264.
9. Cho, H.; Niilo, L. Diagnostic sensitivity and specificity of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams. Canad. J. Vet. Res. **1987**, *51*, 99–103.
10. Estein, S.; Baldi, P.; Bowden, R. Comparison of serological tests based on outer membrane or internal antigens for detecting antibodies to *Brucella ovis* in infected flocks. Vet. Diag. Invest. **2002**, *14*, 407–411.

11. Ficapal, A.; Alonso-Urmeneta, B.; Valasco, J.; Moriyon, I.; Blasco, J. Diagnosis of *Brucella ovis* infection in rams with an ELISA using protein G as conjugate. Vet. Rec. **1995**, *137*, 145–147.
12. Gall, D.; Nielsen, K.; Vigliocco, A.; Smith, P.; Perez, B.; Rojas, X.; Robles, C. Evaluation of an indirect enzyme linked immunoassay for presumptive serodiagnosis of *Brucella ovis* in sheep. Small Rum. Res. **2003**, *48*, 173–179.
13. Goldbaum, F.; Leoni, J.; Wallach, J.; Fosatti, C. Characterization of an 18-kilodalton Brucella cytoplasmic protein which appears to be a serological marker of active infection in both human and bovine brucellosis. J. Clin. Microbiol. **1993**, *31*, 2141–2145.
14. Kittelberger, R.; Diack, D.; Vizcaino, N.; Zygmunt, M.; Cloeckaert, A. Characterization of an immuno-dominant antigen in *Brucella ovis* and evaluation of its use in an enzyme linked immunosorbent assay. Vet. Microbiol. **1998**, *59*, 213–227.
15. Kittelberger, R.; Hilbink, F.; Hansen, M.; Ross, G.; de Lisle, G.; Cloeckaert, A.; de Bruyn, J. Identification and characterization of immunodominant antigens during the course of infection with *Brucella ovis*. J. Vet. Diag. Invest. **1995**, *7*, 210–218.
16. Kittelberger, R.; Hansen, M.; Ross, G.; Hilbink, F.A. sensitive immunoblotting technique for the serodiagnosis of *Brucella ovis* infection. J. Vet. Diag. Invest. **1994**, *6*, 188–194.
17. Lee, K.; Cargill, C.; Atkinson, H. Evaluation of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams. Austral. Vet, J **1985**, *62*, 91–93.
18. Lopez, G.; Ayala, S.; Escobar, G.; Lucero, N. Use of *Brucella canis* antigen for detection of ovine serum antibodies against *Brucella ovis*. Vet. Microbiol. **2005**, *105*, 181–187.
19. Marin, C.; Alonso-Urmeneta, B.; Moriyon, I.; Perez-Gomez, S.; Blasco, J. Comparison of polyclonal, monoclonal and protein G peroxidase conjugates in an enzyme linked immunosorbent assay for the diagnosis of *Brucella ovis* in sheep. Vet. Rec. **1998**, *143*, 390–394.
20. Nielsen, K.; Smith, P.; Conde, S.; Draghi de Benitez, G.; Gall, D.; Halbert, G.; Kenny, K.; Massangill, C.; Muenks, Q.; Rojas, X.; Perez, B.; Samartino, L.; Silva, P.; Tollersrud, T.; Jolley, M. 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. J. Immunoassay Immunochem. **2004**, *25*, 171–182.
21. Nunez-Torres, E.; Diaz-Aparicio, E.; Hernandez-Andrade, L.; Trigo-Tavera, F.; Suarez-Guemas, F. Sensitivity and specificity of an ELISA as a screening test for the diagnosis of *Brucella ovis* in sheep. Rev. Latinoam. Microbiol. **1997**, *39*, 123–128.
22. Rahaley, R.; Dennis, S.; Smeltzer, M. Comparison of the enzyme-linked immunosorbent assay and complement fixation test for detecting *Brucella ovis* antibodies in sheep. Vet. Rec. **1983**, *113*, 467–470.
23. Spencer, T.; Burgess, G. Enzyme-linked immunosorbent assay for *Brucella ovis* specific antibody in ram sera. Res. Vet. Sci. **1984**, *36*, 194–198.
24. Vigliocco, A.; Silva Paulo, P.; Mestre, J.; Briones, G.; Draghi, G.; Tossi, M.; Nielsen, K. Development and validation of an indirect enzyme immunoassay for detection of ovine antibody to *Brucella ovis*. Vet. Microbiol. **1997**, *54*, 357–368.
25. Walker, R.; LeMaster, B.; Stellflug, J.; Biberstein, E. Use of enzyme-linked immunosorbent assay for the detection of antibodies to *Brucella ovis* in sheep: field trial. Am. J. Vet. Res. **1985**, *46*, 1642–1646.

26. Zygmunt, M.; Baucheron, S.; Vizcaino, N.; Bowden, R.; Cloeckaert, A. Single step purification and evaluation of recombinant BP26 protein for serological diagnosis of *Brucella ovis* infection in rams. Vet. Microbiol. **2002**, *87*, 213–220.
27. Lopez, G.; Escobar, G.; Ayala, S.; Lucero, N. Detection of antibodies to *Brucella ovis* in sheep milk using *B. ovis* and *B. canis* antigen. Vet. Microbiol. **2006**, *116*, 232–238.
28. Galanos, C.; Luderitz, F.; Westphal, O. A new method for extraction of R lipopolysaccharide. Eur. J. Biochem. **1969**, *9*, 245–249.
29. Scoonjans, F.; Zalata, A.; Depuydt, C.; Comhaire, F. MedCalc: a new computer program for medical statistics. Comput. Meth. Progr. Biomed. **1995**, *48*, 257–262.

Received November 8, 2006
Accepted December 30, 2006
Manuscript 3221