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Evaluation of an indirect enzyme-linked immunoassay for presumptive serodiagnosis of *Brucella ovis* in sheep

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Abstract

An indirect enzyme-linked immunoassay (IELISA) for the detection of antibodies to *Brucella ovis* was evaluated. Relative to the complement fixation test (CFT) the sensitivity of the IELISA was 96.3% and the specificity was 99.6%. The sensitivity and specificity obtained in this study were comparable to ELISAs and CFTs of other studies in which *B. ovis* isolation was used for evaluation making it a choice to replace current serological tests such as the agar gel immunodiffusion test and the CFT.

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1. Introduction

Brucella ovis is one of the common causes of epididymitis in rams and a rare cause of abortion in ewes and neonatal mortality in lambs. The disease has been reported in many countries including Canada (OIE, 2000). Although considered non zoonotic, there are economic consequences of the disease that include replacing rams with reduced fertility, the loss of rams with high genetic value and the cost of repetitive serological testing to eliminate the disease.

Current serological tests for the detection of antibodies to *B. ovis* are the agar gel immunodiffusion test (AGID), the complement fixation test (CFT) and various indirect enzyme-linked immunoassays (IELISA). The sensitivity of the AGID ranges from 54.2 to 100% depending on how the antigen and agar gel were prepared (Worthington et al., 1984, 1985; Marin et al., 1989; Robles, 1998; Cerri et al., 2000). However, the specificity remained the same at 100% no matter the antigen or the gel preparation method. Disadvantages of the AGID include low sample capacity, long turn around times, labour intensive work and cost, making the test difficult to use in eradication programs, surveys or disease outbreaks. The CFT has good sensitivities and specificities as shown in Table 1, however, disadvantages include frequent anti complementary results (Searson, 1982), prozone phenomena (Marin et al., 1989), incompatibilities with haemolysed sera (Worthington et al., 1984, Marin et al., 1989), serum inactivation (Marin et al., 1989), labour intensity, cost,

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Table 1
Comparison of CFT sensitivities and specificities for the detection of serum antibodies to *B. ovis*

Sensitivity (%)	Specificity (%)	Reference
98.68 (n =75)[a]	100 (n = 44)[b]	Cerri et al., 2000
97.5 (n = 79)[c]	96.0 (n = 175)[b]	Cho and Niilo, 1987
92.7 (n = 83)[c]	100 (n = 83)[b]	Marin et al., 1989
88.89 ± 11.85 (n = 28)[c]	99.69 ± 0.42[d] (n = 675)	Vigliocco et al., 1997

[a] *B. ovis* experimentally infected rams.
[b] Brucella-free rams.
[c] Rams from which *B. ovis* had been isolated.
[d] 95% confidence limits are measure of uncertainty.

false positives (Searson, 1982; Lee et al., 1985) and false negatives (Burgess and Norris, 1982; Searson, 1982; Lee et al., 1985). Up to 25% false negative rate is possible depending on the CFT method used (Searson, 1982).

The IELISA described in this paper has larger test capacity, same day turn around time, no difficulty with haemolysed sera, is less labour intensive and less costly in comparison to the AGID and the CFT. As well, the IELISA can be semi-automated or automated to expedite the process. Various ELISAs have been developed with good sensitivities and specificities as shown in Table 2. However, high background levels and detection of antibody to *B. melitensis* and other organisms due to cross reactivity with outer membrane proteins (OMPs) as a result of antigen preparation methods were problematic in some of these ELISAs. The IELISA described in this paper has low background activity and is less likely to cross react with other organisms due to antigen preparation methods.

Table 2
Comparison of ELISA sensitivities and specificities for the detection of serum antibodies to *B. ovis*

Sensitivity (%)	Specificity (%)	Reference
100 (n = 75)[a]	100 (n = 44)[b]	Cerri et al., 2000
100 (n = 79)[c]	99.4 (n = 175)[b]	Cho and Niilo, 1987
97 (n = 33)[d]	84 (n = 39)[b]	Nunez-Torres et al., 1997
97.6 (n = 83)[c]	100 (n = 83)[b]	Marin et al., 1989
96.43 ± 6.8 (n = 28)[c]	100 (n = 675)[b]	Vigliocco et al., 1997

[a] *B. ovis* experimentally infected rams.
[b] *B. ovis* free.
[c] Rams from which *B. ovis* had been isolated.
[d] From bacteriologically confirmed infected animals.

The relative sensitivity and specificity of the IELISA were comparable to the actual IELISA sensitivities and specificities shown in Table 2.

2. Materials and methods

2.1. Ovine serum samples

Negative samples from Canada (n = 1620) were defined as those from animals with no clinical or epidemiological evidence of *B. ovis* infection.

Serologically positive samples (n = 81) were defined as those from infected flocks which were positive (≥1/5 dilution) on the CFT. A CFT reaction of 50% hemolysis at a dilution of 1/5 (15.6 international complement fixation test units, Alton et al., 1988) or higher was considered positive.

Controls consisted of a strong positive serum from a sheep from which *B. ovis* was isolated, a weak positive serum from a sheep from which *B. ovis* had been isolated, a negative serum from a negative animal with no clinical or epidemiological evidence of *B. ovis* infection and a conjugate control that contains all the assay components except serum. This control was included to ensure that the diluent buffer performed correctly and did not influence the results (Gall and Nielsen, 2002). If contaminated or improperly prepared, the buffer could influence test results.

2.2. Complement fixation test (CFT)

The CFT as described by Samagh and Boulanger (1978) was modified using *B. ovis* antigen and *B. ovis* serum controls. Samples greater than or equal to a 1/5 dilution were considered positive.

2.3. Indirect ELISA (IELISA) for detection of antibody in serum

The IELISA was a modification of the method reported by Vigliocco et al. (1997). Briefly, the *B. ovis* rough lipopolysaccharide (rLPS) was prepared as described by Galanos et al. (1969). Polystyrene microplates (Nunc™ non treated, non tissue culture 269620) were passively coated with 100 μl of 1 μg/ml of *B. ovis* rLPS dissolved in 0.06 M carbonate buffer, pH 9.6 at 20 °C (ambient temperature). The next day,

the plates were frozen at −20 °C. Before use, the microplates were thawed and washed four times with 0.01 M phosphate buffered saline containing 0.05% Tween 20, pH 7.2 (PBST). Immediately, following the wash cycle 100 μl volumes of controls and test sera diluted 1/200 in PBST containing 0.015 M ethylenediamine tetraacetic acid (EDTA) and ethylene glycol bis(ß-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were added to each microplate and incubated for 30 min at 25 °C. Following incubation, the microplates were washed 4 times with PBST and 100 μl of appropriately diluted murine monoclonal antibody specific for an epitope of bovine IgG_1 (which cross reacts extensively with sheep IgG (Henning and Nielsen, 1992) conjugated with horseradish peroxidase was added to each microplate and incubated for 60 min at 25 °C. After incubation, the microplates were washed 4 times with PBST and substrate/chromogen (100 μl of 0.05 M citrate buffer, pH 4.5 containing 0.5 μl 3% hydrogen peroxide (H_2O_2) and 2.5 μl 0.040 M azino-ethylbenz-thiaoline-sulfonic acid (ABTS) per well) was added and the microplates shaken for 10 min prior to reading at 414 nm in a spectrophotometer.

2.4. Data handling and analysis

The results of each serum control and sample tested by the IELISA were expressed as a percentage (positivity (%)) of the test sample optical density (OD) reading of the mean of the positive control included on each microplate at the 10 min development time. Percent positivity (P (%)) was calculated as follows:

$$P\,(\%) = \frac{\text{OD of the test sample}}{\text{mean OD of the positive control}} \times 100$$

Using receiver operating characteristics (ROC), the data was analysed (Schoojans et al., 1995). This analysis determined the optimal cutoff value between the defined positive ($n = 81$) and negative ($n = 1620$) data to achieve the optimal relative sensitivity and specificity estimates. In addition, the area under the curve (AUC) was determined indicating the accuracy of the IELISA.

The positive predictive value (PPV) and negative predictive value (NPV) were calculated as follows (Medcalc, 1998):

$$\text{PPV} = \frac{\text{sensitivity} \times \text{prevalence}}{\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})}$$

$$\text{NPV} = \frac{\text{specificity} \times (1 - \text{prevalence})}{(1 - \text{sensitivity}) \times \text{prevalence} + \text{specificity} \times (1 - \text{prevalence})}$$

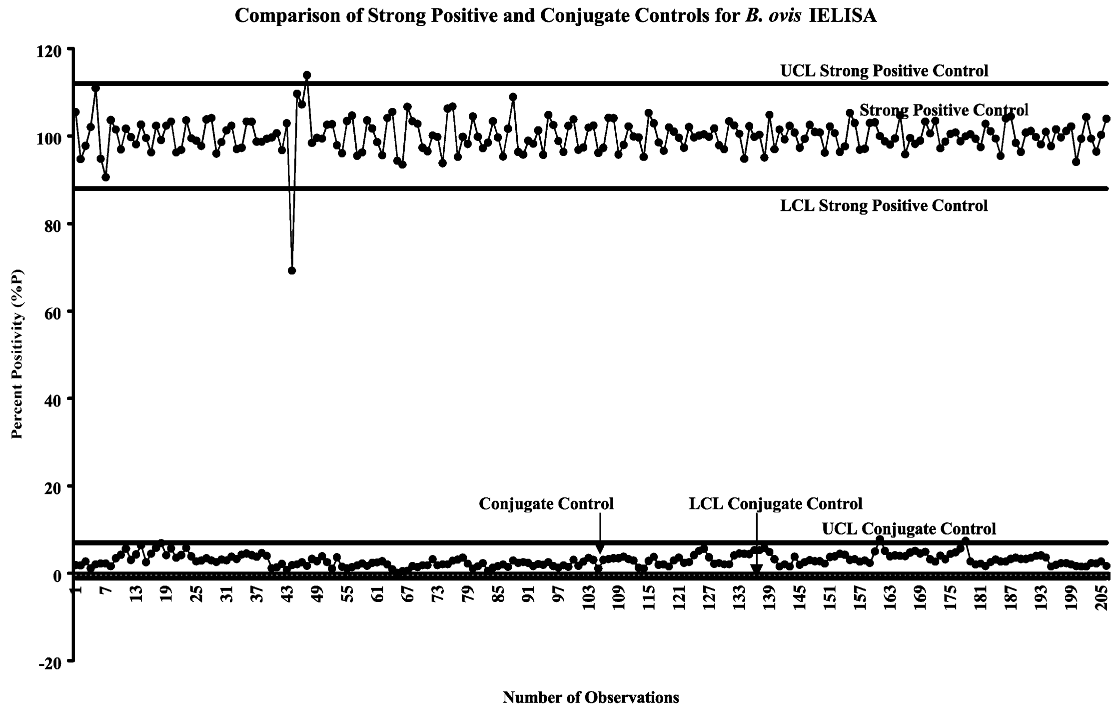
The ELISA software used for the IELISA was developed at the Animal Diseases Research Institute (ADRI) and is available upon request from W. Kelly, Ottawa Laboratory, Animal Diseases Research Institute (ADRI), Canadian Food Inspection Agency, Ottawa, Ont., Canada.

3. Results

The relative sensitivities and specificities of the IELISA at various cutoffs as calculated by ROC analysis software are shown in Table 3. The optimal cutoff as chosen by the software and indicated by the sum of the sensitivity and specificity values (196.10) was 11% positivity (P (%)) with relative sensitivity and specificity values of 98.8 and 97.3%, respectively. The cutoff of 14% P was chosen for testing resulting in relative sensitivity and specificity values of 96.3 and 99.6%, respectively. The AUC for this cutoff was 0.997 indicating that in more than 99% of the cases

Table 3
Comparison of relative sensitivities and specificities with 95% confidence intervals (95% C.I.) at various cutoffs for the IELISA

Cutoff (P (%))	Sensitivity (95% C.I.)	Specificity (95% C.I.)	Sum of sensitivity + specificity
>10	98.8 (93.3–9.8)	95.4 (94.2–96.3)	194.3
>11	98.8 (93.3–9.8)	97.3 (96.4–98.0)	196.1
>12	97.5 (91.3–99.6)	98.5 (97.8–99.0)	196.0
>13	96.3 (89.5–99.2)	99.1 (98.6–99.5)	195.4
>14	96.3 (89.5–99.2)	99.6 (99.1–99.8)	195.9
>15	95.1 (87.8–98.6)	99.7 (99.3–99.9)	194.8



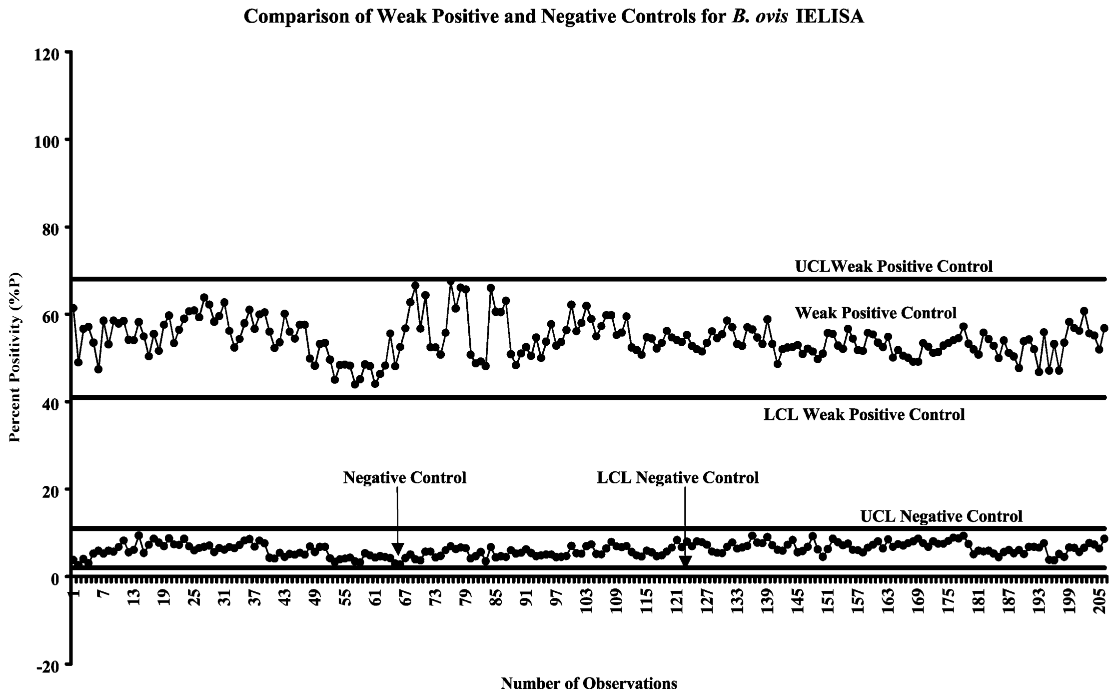


Fig. 1. The top chart compares the upper control limit (UCL) and lower control limits (LCL) for the strong positive serum and conjugate control. The bottom chart compares the UCL and LCL for the weak positive serum and the negative control serum. The UCL and LCL are 3 S.D. from the mean of each control.

Table 4
Comparison of positive predictive values and negative predictive values of the IELISA (cutoff = 14 *P* (%)) with a relative test sensitivity and specificity of 96.3 and 99.6%, respectively

Disease prevalence (%)	Positive predictive value	Negative predictive value
0.01	2.4	100.0
0.10	19.4	100.0
1.00	70.9	100.0
10.0	96.4	99.6
20.0	98.4	99.1

the animals were correctly identified (Schoojans et al., 1995).

Presented in Table 4 are the positive and negative predictive values for disease prevalences of 0.01, 0.1, 1, 10 and 20% using a cutoff of 14% and relative sensitivity and specificity values of 96.3 and 99.6%, respectively. As the disease prevalence increases, the positive predictive value (PPV) increases while the negative predictive value (NPV) remains relatively unchanged. A PPV is the probability of the disease being present when the test is positive and an NPV is the probability that the disease is not present when the test is negative.

The IELISA produced consistent results in repeated tests ($n = 206$) as shown in Fig. 1, except observations 45 and 48 which were outside the lower control limit and the upper control limit, respectively, of the strong positive control. The upper and lower control limits for each control were ±3 S.D. The percent coefficient of variation (CV (%)) for the positive, weak positive, negative and conjugate controls were 4, 8, 24 and 46%, respectively. Due to the higher CV (%) in the negative and conjugate controls, downward and upward trends are more easily detected as seen in Fig. 1.

The average mean value of the defined negative data ($n = 1620$) was 5% *P* and the mode value (Gall and Nielsen, 2001) was also 5% *P* ($n = 263$) indicating a low background activity. Mode is the value which occurs with the greatest frequency within a set of numbers. The low *P* (%) of both the negative and conjugate controls were 3–9 and 0–8% *P*, respectively, also suggesting low background activity.

4. Discussion

Relative to the CFT, the sensitivity of the IELISA for the selected cutoff of 14% *P* was 96.3% and was comparable to the actual sensitivities (proven infected) of the ELISAs shown in Table 2. Similarly, the specificity of the IELISA (99.6%) was comparable to the actual specificities (proven *B. ovis* free) shown in Table 2. The lower sensitivity was due to the introduction of bias when using the CFT as the standard for defining the positive reference sera. Use of another test to define the reference samples is permissible, if the other test approaches 100% (Baldock, 1988; Martin, 1977). Review of the data shown in Table 1 qualifies the CFT in this regard.

Receiver operating characteristic analysis indicated an optimal cutoff for this test of 11% *P* with a sensitivity and specificity of 98.8 and 97.3%, respectively. Increasing the cutoff to 14% *P* greatly increased the specificity (99.6%) of this test reducing the likelihood of false positives which may be due to cross reactions from closely related epitope determinants from various sources (Velasco et al., 1997; Cerri et al., 2000).

Using the sensitivity and specificity of 96.3 and 99.6%, respectively, the negative and positive predictive values were calculated (Table 4) for different hypothetical prevalences of the disease. The positive predictive value increases substantially as the prevalence increased while the negative predictive value remained unchanged. In countries where the prevalence of the disease is high (10% or greater), this test would be very good and inexpensive in comparison to the AGID and CFT. A positive predictive value of 96.4% (Table 4) at 10% or greater prevalence indicates that more than 9 out of every 10 animals testing positive on the IELISA could have antibody to *B. ovis* infection. The negative predictive value remained virtually unchanged at different prevalence values indicating that animals testing negative on the IELISA were not infected with *B. ovis*. Since, the prevalence of *B. ovis* in Canada is unknown but considered very low, a high negative predictive value is expected because the majority of the animals tested will by definition be disease-free. The negative predictive value does not change dramatically with different prevalence values thus reducing the likelihood of false positives. Cross reacting organisms such as *Dichelobacter nodosus* (Whittington et al., 1996) may be responsible for some false positive reactions seen in serological tests. This bacterium is the causative organism for foot rot in sheep and is a reportable disease in western Canada (Alberta and British Columbia).

Unlike other ELISAs for *B. ovis* antibody detection, this version uses rLPS prepared according to the method described by Galanos et al. (1969). The rLPS which is relatively soluble at pH 9.6 at 1 μg/ml is passively coated onto the polystyrene matrix and is more purified than other antigen preparation techniques such as the hot saline (HS) extraction method which contains rLPS and outer membrane proteins (Riezu-Boj et al., 1986, 1990). These outer membrane proteins (OMPs) which are found on the surfaces of both *B. ovis* and rough *B. melitensis* cells could be responsible for cross reactivity with sera from *B. melitensis* infected or Rev 1 vaccinated sheep (Riezu-Boj et al., 1986, 1990; Marin et al., 1998). Another feature of this test is the murine monoclonal anti immunoglobulin conjugate specific for an epitope of bovine IgG_1. Other ELISAs use protein G conjugates or polyclonal rabbit anti-sheep IgG conjugates heavy and light chain specific that detect other antibody isotypes, such as IgM not necessarily due *B. ovis* infection but arising due to exposure to other microoganisms such as *Ochrobactrum anthropi* which cross reacts with sera from animals with natural and experimental brucellosis (Velasco et al., 1997). Antibody of the IgG_1 isotype is detectable in cattle 5–7 days after infection and it declines less than the other isotypes (Nielsen et al., 1996) in brucellosis. High background activity has been observed in other ELISAs and attributed to the use of polyclonal anti-sheep IgG conjugates heavy and light chain specific (Marin et al., 1989;1998). Attempts to reduce this high background by using protein G or monoclonal anti-ruminant IgG_1 conjugates have only proven only partially successful (Marin et al., 1998). The background activity of this IELISA is on average 5% *P* with a mode value of 5% *P*. The mode value represents 263 out of 1620 samples or 16.2% of the data. This low background may be due the combination of purified rLPS with no OMPs which lack species specificity (Plackett et al., 1989), murine monoclonal antibody specific for the epitope of bovine IgG_1 and the type of polystyrene 96-well microtitre plate used in this IELISA. Other ELISAs use high binding treated polystyrene microplates which may contribute to the high background levels (Nielsen et al., 1996) observed in these ELISAs by the attachment of OMPs and other proteins found in HS extracts. The current IELISA uses low binding, non tissue culture, untreated, less expensive polystyrene microplates.

Both, the AGID and CFT are labour intensive, costly to perform and have much longer turn around times than the IELISA. Unlike the IELISA both the AGID or CFT cannot test most haemolysed serum or samples that are unfit for use or contaminated. The IELISA is a primary binding assay that does not rely on secondary phenomena such precipitation (AGID) or fixing complement (CFT) and can therefore be performed with abused samples. In addition, unlike the AGID and CFT, the IELISA in this study has been tailored to be more specific by using highly purified reagents resulting in low background activity in contrast to other publications (Marin et al., 1998). The CFT is the prescribed test for international trade (OIE, 2000) despite its many disadvantages, including false positives and false negatives for ovine brucellosis as previously mentioned. In conclusion, the IELISA with equal or better test performance characteristics than the AGID or CFT should be considered as a prescribed test for international trade.

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