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SHORT COMMUNICATION

Development and Comparative Evaluation of a Competitive ELISA with Rose Bengal Test and a Commercial Indirect ELISA for Serological Diagnosis of Brucellosis

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Abstract The development of a competitive ELISA for the detection of brucella-specific antibodies in bovines is described. Anti-brucella guinea pig serum was used as a source of competing antibodies. Lipo-polysaccharide purified from inactivated *B. abortus* S19 culture was used as antigen for the development of the assay. Sera from cattle were used in the competitive ELISA, rose bengal test and a commercial indirect ELISA. The following cattle sera were tested: (i) known positive sera ($n = 80$) (ii) known negative sera ($n = 100$) and (iii) field sera ($n = 1184$). Based on the receiver operating characteristics curve analysis and frequency distribution of the percentage of inhibition, 30% inhibition was considered the cut-off for positive and negative results. The sensitivity and specificity estimate on comparison with the commercial indirect ELISA was 94.87 and 92.12% respectively. The competitive ELISA described is a simple method for the routine screening of animal sera for detecting Brucella-specific antibodies.

Keywords *Brucella abortus* · Brucellosis · Competitive ELISA

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Sero-epidemiology of brucellosis is currently done by employing the Rose Bengal card test (RBT) [1] and the buffered antigen plate agglutination test (BPAT) [2]. The World Organization for Animal Health (OIE) has approved an indirect ELISA (i-ELISA) for testing serum and milk [1, 3, 4]. Commercial kits employing i-ELISA are expensive for routine screening of dairy herds in developing countries. In such cases developing c-ELISAs with polyclonal sera would be an alternative method [5]. This paper describes the results of a competitive ELISA (c-ELISA) and comparison with i-ELISA and RBT.

Anti-brucella specific polyclonal sera raised in rabbits and guinea pigs were used for the c-ELISA. LPS from formalin-inactivated *Brucella abortus* S19 (*B. abortus* S19) bacterial cells was extracted using the procedure described previously [6]. The RBT was done as described elsewhere [2] using Rose Bengal stained *B. abortus* antigen (Indian Veterinary Research Institute, Izatnagar) to screen sera for presence of antibodies to *Brucella* sp. The indirect ELISA (Veterinary Laboratories Agency, Weybridge, UK) was performed according to the manufacturer's instructions.

The following types of sera samples were used for tests: (i) Known positive sera ($n = 80$). These bovine sera tested positive in RBT and i-ELISA. (ii) Known negative sera ($n = 100$). These bovine sera tested negative by both RBT and i-ELISA. (iii) Bovine sera ($n = 1184$) collected from various farms were included as test samples. These samples also contained 35 days post vaccination sera samples from cattle vaccinated with brucella vaccine. These were used to compare the performance of the c-ELISA with RBT and i-ELISA.

The brucella c-ELISA was performed as follows: The optimal concentration of antigen (LPS from *B. abortus* S19) and dilution of the anti-brucella rabbit and guinea pig

serum were determined by performing a checker board titration of the different antigens against the immune rabbit and guinea pig sera. Immune rabbit sera at the appropriate dilution were coated on to 96-well micro-titer ELISA plates (Nunc®ImmunoMaxisorp™, Nunclon, Germany) using carbonate buffer (pH 9.6). After overnight incubation the plates were washed and brucella LPS in phosphate buffered saline containing 3% skim milk powder (SM-PBST) was added. The plates were incubated at 37°C for 1 h. After incubation, the plates were washed, test samples added in duplicates and the plates were incubated at 37°C for 1 h. The plates were then washed followed by addition of anti-brucella guinea pig sera at appropriate dilution prepared in a blocking buffer containing 2.5% normal rabbit serum and 5% normal bovine serum. Anti-guinea pig IgG HRPO conjugate (Sigma, USA) at 1:2000 dilution in blocking buffer was added to the plates and incubated at 37°C. Binding of secondary antibody was detected by adding 3,3′,5,5′ tetra-methylbenzidine and hydrogen peroxide mixture. The percentage of inhibition (PI) was calculated as follows: PI = [100 − (OD value of test sample) × 100)]/(OD value of serum control).

A frequency plot was made from the PI derived for the known positive and negative samples. The cut-off was derived by calculating the mean PI of all the negative samples and the standard deviation from the mean value. Initial optimal estimates of the criteria between positive and negative reactions (the cut-off values) were determined by receiver operating characteristics (ROC) analysis [7]. The repeatability of the assay was checked using Cochran's Q statistics [8]. The specificity and sensitivity estimates, the kappa value and the likelihood estimates were calculated using a Bayesian model [9] comparing the results of the c-ELISA and i-ELISA. The sensitivity and specificity were 98.75 and 100% for the known positive and negative bovine sera at 30% PI.

Brucella c-ELISA results were compared with i-ELISA and RBT (Table 1). The repeatability was high (data not shown). Field sera samples ($n = 1184$) were tested by both c-ELISA and i-ELISA. The results were as follows: 359 sera samples were found positive by i-ELISA and 356 were positive by c-ELISA whereas with RBT only 221 samples were positive. A total of 814 samples were declared negative by all the three tests. Both the ELISAs had agreement on 345 samples. Out of 963 samples that showed negative results with RBT, 138 samples were positive by both the ELISAs. In case of the other 25 samples, there was disagreement between the two ELISAs. The higher number of positives may be due to the fact that ELISAs are more sensitive and specific. The statistical estimates for comparison of the brucella c-ELISA against the I-ELSIA are given in Table 2. The estimates reveal substantial to almost perfect agreement between the tests. The sensitivity and specificity estimate on comparison with I-ELISA were 92.3–97.5% and 90.3–94.0% respectively. Standardization and harmonization of serological tests used for the presumptive diagnosis of infectious diseases has always been difficult due to variability in reagents and subjective assessment [10]. However, development of such assays with monoclonal antibodies is time consuming and expensive. In addition, imported monoclonal antibodies and reagents are expensive and cannot be widely used. The c-ELISA described in this report may overcome these disadvantages. The result with our brucella c-ELISA shows that it can be used for routine screening of bovine sera and may reduce the dependence on imported, expensive commercial kits. The c-ELISA described here is a simple, suitable alternative for screening of bovine sera against brucellosis.

Table 1 Comparison of results obtained using competitive ELISA with RBT and indirect ELISA

Tests		Competitive ELISA		
		Positive	Negative	Total
RBT	Positive	207	14	221
	Negative	149	814	963
	Total	356	828	1184
Indirect ELISA	Positive	345	14	359
	Negative	11	814	825
	Total	356	828	1184

Bovine sera ($n = 1184$) were tested by each serological method

Table 2 Comparison of brucella c-ELISA with i-ELISA using Bayesian analysis ($n = 1184$)

Estimates	Values
Prevalance (%)	24.86
Sensitivity (%)	94.87 (92.3–97.5)
Specificity (%)	92.12 (90.3–94.0)
Acuracy (%)	93 (91.3–94.3)
Positive predictive value (%)	79.9 (75.6–84.3)
Negative predictive value (%)	98.2 (96.7–99.6)
Kappa 'κ'	0.819 (0.760–0.878)
Agreement	Substantial to almost perfect
Positive likelyhood	12.04 (9.52–15.23)
Negative likelyhood	0.06 (0.03–0.09)
Likelyhood ratio	766.67**
Performance index (sensitivity + specificity)	186.99

The estimates with 95% CI are given where ever applicable in parenthesis (** $P < 0.01$)

References

1. OIE Manual (2008) OIE Manual of standards for diagnostic tests and vaccines. Bovine brucellosis. OIE, Paris, pp 624–659
2. Morgan W, MacKinnon D, Lawson J, Cullen G (1969) The Rose Bengal plate agglutination test in the diagnosis of brucellosis. Vet Rec 85:636–637
3. Nielsen K, Gall D (1994) Advances in the diagnosis of bovine brucellosis: use of enzyme immunoassays. Gen Eng Biotech 14: 25–39
4. Vanzini V, Aguirre N, Valentini B et al (2001) Comparison of an indirect ELISA with the *Brucella* milk ring test for detection of antibodies to *Brucella abortus* in bulk milk samples. Vet Microbiol 82:55–60
5. Nielsen K, Kelly L, Gall D, Nicoletti P, Kelly W (1995) Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. Vet Immunol Immunopathol 46:285–291
6. Yi EC, Hackett M (2000) Rapid isolation of lipopolysaccharide and lipid A from gramnegative bacteria. Analyst 125:356–651
7. Schoonjans F, Zalata A, Depuydt CE, Comhaire FH (1995) MedCalc: a new computer program for medical statistics. Comput Methods Programs Biomed 48:257–262
8. Patil KD (1975) Cochran's Q test: exact distribution. J Am Stat Assoc 70:186–189
9. http://www.quantitativeskills.com/sisa/statistics/diaghlp.htm
10. Nielsen K, Smith P, Yu WL, Elmgren C, Nicoletti P, Perez B, Bermudez R, Renteria R (2007) Second generation competitive enzyme immunoassay for detection of bovine antibody to *Brucella abortus*. Vet Microbiol 124:173–177