

Indirect Enzyme-Linked Immunosorbent Assay Test Kit Development for Specific Antibody Detection Against *Brucella abortus* in Cattle

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ABSTRACT

The smooth lipopolysaccharide (SLPS) antigen, prepared from *Brucella abortus* strain 99 was used in an indirect Enzyme-Linked Immunosorbent Assay (iELISA). The data were expressed as optical density and generated using computer software. The positive results were accepted at the cut-off values of ≥ 40 percent positivity (PP). The strong positive serum control (C++), weak positive serum control (C+), negative serum control (C-), and conjugate control (Cc) were used to optimize the test kit. The iELISA was optimization and standardization for antigen concentration, test material dilution and detection system dilution by checkerboard titration. The statistic evaluation using data from sera from 317 infected and 5,300 non-infected cattle revealed that the sensitivity (Se) is 99.369 (98.497-100.0)%, specificity (Sp) is 99.887 (99.796-99.977)%, accuracy (Ac) is 99.858% and Kappa value is 0.987. The iELISA test kit was subsequently validated by testing 9,877 field serum samples submitted to laboratory. The complement fixation test was used as gold standard. The results revealed that the relative Se is 99.026 (97.929-100.0)%, relative Sp is 98.547 (98.308-98.787)%, relative Ac is 98.562% and Kappa value is 0.804 when cattle sera were tested. The components of this iELISA test kit consisted of antigen, control sera, and essential diluents for 800 tests. This test showed high sensitivity, specificity, and accuracy and could be performed in mass. Therefore, this iELISA test kit might be an appropriate tool for brucellosis control in Thailand.

Key words: test kit, iELISA, Brucellosis

INTRODUCTION

Brucellosis, which is globally widespread, is an important infectious zoonotic disease caused by *Brucella* spp. Among the entire range of species, *B. abortus*, *B. melitensis*, and *B. suis* have been reported as highly pathogenic bacteria for humans (OIE, 2004). Since one of the measures for brucellosis control is identification of the infected

herds by exploiting a serological diagnosis, several conventional serological tests have been developed and available for rapid diagnosis of brucellosis. However, all of those assays have some limitations; thus a definitive diagnosis usually required more than one single test (OIE, 2004). In Thailand, the serological tests that have been used for brucellosis diagnosis in cattle and buffaloes include Rose Bengal test (RBT), EDTA-tube

agglutination test (EDTA-TAT), and complement fixation test (CFT). From previous study, an iELISA was developed for the diagnosis of bovine brucellosis (Chaichanasiriwithaya *et al.*, 2001). The developed iELISA may be used either for the screening or confirmation of brucellosis. The iELISA techniques are more sensitive than CFT (Uzal *et al.*, 1995; Nielsen *et al.*, 1996; Tittarelli *et al.*, 2005). The aim of this study was to develop an iELISA test kit for detecting the antibody against *B. abortus* and test a large number of field sera.

MATERIALS AND METHODS

Antigen preparation. *B. abortus* strain 99, obtained under JICA Project, was grown onto agar medium, harvested according to the protocol suggested by OIE (2004). The smooth lipopolysaccharide (SLPS) was extracted from heat-killed *B. abortus* strain 99 cells (Nielsen *et al.*, 1996; Chaichanasiriwithaya *et al.*, 2001; OIE, 2004) and used as the antigen. In brief, the heat-killed bacteria were extracted with 90% phenol (v/v). The phenol phase was then harvested and precipitated with 1% methanol-saturated sodium acetate. The pellet was collected after centrifugation at 6,000x g for 15 minutes at 4°C and resuspended in 80 ml of distilled water. The supernatant was collected and mixed with trichloroacetic acid at ratio 1:20 (w/v). The resuspended mixture was then centrifuged at 10,000x g for 10 minutes at 4°C. The supernatant containing SLPS was collected and diluted with distilled water, lyophilized, weighed and reconstituted to 1.0 mg/ml.

Control serum samples. Various dilutions of positive sera were used to determine the optimal dilution used in the developed iELISA test kit. These selected dilutions included (1) a strong positive control serum (C++), which produced the optical density (OD) at approx.1.0 within 10 minutes, (2) a weak positive control

serum (C+), which gave rise the OD just above the cut-off value, and (3) a negative control serum (C-), which was pooled sera from known *B. abortus* negative. The positive control serum used in this study was selected from infected animals that *B. abortus* was confirmed by isolation.

Indirect ELISA system. All of the assay parameters were optimized and standardized; the sample dilution and the reagents used in this assay similar to those previously described (Nielsen *et al.*, 1996; Chaichanasiriwithaya *et al.*, 2001; OIE, 2004). Briefly, 100 µl of SLPS (1 µg/ml in carbonate buffer, pH 9.6) was dispensed into each well of 96-well polystyrene plates (Nunc maxysorb) and incubated at 4°C for 18 hours or 37°C for 1 hour. One hundred microlitres of serum samples and control sera were incubated at room temperature for 30 minutes. The Recombinant-Protein G-Peroxidase (HRP-rec-Protein G) conjugate were incubated with the bound antibodies at room temperature for 30 minutes. The plate was washed 4 times in between each step. The ABTS peroxidase-substrate was incubated with HRP-rec-Protein G for 10 minutes at room temperature and then the reaction was stopped by using 1 % SDS. The OD was observed by an ELISA reader (LabSystems Multiskan MS) at the wavelength of 414 nm using reference filter at 492 nm.

Samples for validation of the kit. A total of 5,300 negative sera were collected from cattle that had no previous history or serological evidence of *B. abortus* infection of at least 3 years. Positive sera were 317 samples, selected from infected herds which were classified as *B. abortus* positive by isolation and/or serological methods. Field sera used as the test samples were 9,877 samples submitted for serological diagnosis of brucellosis to National Institute of Animal Health (NIAH), Bangkok, and from three Regional Veterinary Research and Development Centers (RVRDCs), located in Lampang, Khon Kaen, and Nakhon Si Thammarat provinces. The

complement fixation test with the positive cut off titer $\geq 1:5$ was used as standard method.

Internal quality control (IQC). The internal controls consisting of C++, C+, C-, and conjugate control (Cc), were included in all plates as control data for analyzing the test samples. The control sera were tested to determine specific limits of the acceptance. The results of each run were used to establish the initial upper control limit (UCL) and lower control limit (LCL) of acceptability. Standard deviations (SD) by mean of dispersion and means (\bar{X}) were analyzed in which the value $\bar{X} \pm 2$ SD was selected for UCL and LCL. The control data were summarized and produced a type of control chart called “Shewhart Chart” for the acceptance. The acceptable criteria of C++, C+, C- and Cc were considered from measuring the OD and percent positivity (PP) values of the 4 replicates. The OD and PP of each control in individual microplate were accepted as 3 or 4 of 4 values within UCL and LCL range as followed the protocol of Joint FAO/IAEA (1994) and Crowther *et al.* (2006).

Data management. The manipulation and analysis of OD data were performed by importing the OD file into a suitably configured spreadsheet. The Excel software was used to build a template for iELISA format of the plate reader outputs. The OD value was then interpreted as PP as follows.

$$PP = \frac{\text{Replicate OD value of test sample}}{\text{Median OD value of C++ control}} \times 100$$

Statistics

Determination of the cut-off value. The positive and negative serum samples were tested and used for creating frequency distribution. Receiver Operator Characteristic (ROC) curve analysis was used as the supplement to determine the cut-off value (Nielsen *et al.*, 1996; Dawson and Trapp, 2004a).

Sensitivity (Se), Specificity (Sp) and Accuracy (Ac). Se, Sp and Ac of the tests were calculated with respect to the *Brucella* - infected and free groups (OIE, 2004). Alternative method for evaluation of iELISA test kit must be employed in the absence of a gold standard (Pouillot *et al.*, 2002). Thus, for comparison, the relative Se and Sp with respect to that of CFT were tested and calculated from the cattle sera submitted to NIAH and RVRDCs.

Kappa (K) value. The kappa statistic was used to measure the agreement between two tests. It was used as a measure to estimate the agreement of iELISA and gold standard method (isolation) and iELISA with CFT (Dawson and Trapp, 2004b).

Production of a kit. The components of the kit was set up and optimized for good performance. The shelf-life of the reagents was tested every 2 months for a period of 16 months.

RESULTS

Optimization of SLPS antigen, control sera and reagents. The antigen in each bottle was optimized for 10 plates and kept as freeze-dried. The positive serum was selected from infected cattle with titer 1:148.5 IU in the tube agglutination test while the negative serum did not contain *Brucella* antibodies. The weak positive control serum prepared by mixing positive serum in negative serum at ratio 1:12 and showed the ODs above the cut-off value. The LCL and UCL of C++, C+, C- and Cc were obtained as OD at 0.807-1.199, 0.458-0.77, 0.092-0.22, and 0.045-0.109, and PP at 81-120%, 46-77%, 9-22% and 4-11%, respectively (Table 1).

Determination of the cut-off values. The PP values from the frequency distribution and ROC curves of *Brucella* antibodies positive and negative serum samples were used for the estimation of cut-off values for iELISA which was 40 PP (Figure1 and Table 2).

Internal quality control (IQC). The IQC was observed and accepted by repetitive testing 5 plates/time for 30 times. The mean value obtained from each replicate was in the range of selected LCL and UCL.

Validation of the test kit. The Se, Sp, Ac and Kappa (K) values were determined using the isolation of *B. abortus* as the gold standard and the relative Se, Sp, Ac and K values were undertaken from the comparison data of iELISA and CFT from 4 laboratories. The results of Se, Sp, Ac, and K values were 99.369 (98.497-100.0)%, 99.887 (99.796-99.977)%, 99.858%, and 0.987, respectively, whereas the relative Se, Sp, Ac, and K values were 99.026 (97.929-100.0)%, 98.547 (98.308-98.787)%, 98.562%, and 0.804, respectively.

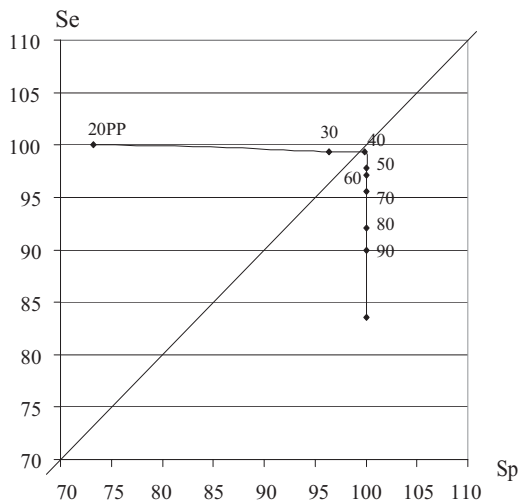


Figure 1 Receiver – operator characteristic (ROC) curve of iELISA test performance.

Table 1 Acceptance criteria for lower control limit and upper control limit of iELISA kit.

Control values	Strong positive	Weak positive	Negative control	Conjugate control
No of tests	1000	1000	1000	1000
Max OD	1.335	0.817	0.251	0.136
Min OD	0.750	0.413	0.084	0.040
Mean of OD	1.00284	0.614149	0.156018	0.076563
SD	0.098401	0.078128	0.031574	0.016017
X \pm 2SD	1.003 \pm 0.196	0.614 \pm 0.156	0.156 \pm 0.064	0.077 \pm 0.032
LCL-UCL : OD	0.807 - 1.199	0.458 - 0.77	0.092 - 0.22	0.045 - 0.109
LCL-UCL : PP	81 - 120	46 - 77	9 - 22	4 - 11

Table 2 Distribution of percent positivity (PP) cut off value in the iELISA for cattle brucellosis diagnosis in comparison to *B. abortus* isolation.

Cut off value PP (%)	<i>B. abortus</i> isolation		iELISA	
	Positive	Negative	Sensitivity (%)	Specificity (%)
10	317	224	100.0	4.23
20	317	3881	100.0	73.23
30	315	5103	99.37	96.28
40	315	5294	99.37	99.89
50	310	5300	97.79	100.0
60	308	5300	97.16	100.0
70	303	5300	95.58	100.0
80	292	5300	92.11	100.0
90	285	5300	89.91	100.0

Test kit components. The amount of each component in the kit was adequate for testing 800 samples. There were 12 items in one set. The conjugate and diluents for the test system was stored at 4°C. The antigen, C++, C+, and C- were prepared in freeze-dried condition. The shelf-life of the kit reagents was over a period of 1 year.

DISCUSSION

The control program for brucellosis in Thailand has been implemented for more than 20 years. The national control program has been strengthened since 2000. Identification of infection and removal of infected animals from the herd was the policy strategies. The diagnostic tools employed for serological diagnosis in control program and surveillance should be highly sensitive, more specific, practical and economical.

In this study, iELISA was performed following the previous study (Chaichanasiriwithaya *et al.*, 2001). The test was optimized, standardized, and validated so that it became a ready-to-use test kit. A cut-off was established using *B. abortus* isolation as the gold standard and the diagnostic performance was maximized. The cut-off calculated by percent positivity was 40 PP, which improved the specificity of the test (99.89%) over the cut-off 30 PP (96.28%) whereas Se was 99.37%. High sensitivity and specificity iELISA may be used alone as the diagnostic tool to detect brucellosis in low prevalence area and false serological positive present (Mainar-Jaime *et al.*, 2005).

The 9,877 serum samples were submitted for the diagnosis of brucellosis. The iELISA showed a relative sensitivity and specificity of 98.026% and 99.547% compared to CFT, as a standard method. Based on the Kappa value of this kit, it indicated a good agreement with CFT. The staff of 4 laboratories, participating the study, were trained and performed for 3 times with the results of LCL and UCL being in the acceptable range.

The preliminary observation for relative Se, Sp, and Ac of this iELISA kit were 100, 96.54-98.34%, and 96.68-98.42%, respectively (Wong-wong, 2006; Chumek and Srikawkheaw, 2007). The software for iELISA interpretation was designed to ensure stringent laboratory precision and normality among the network of laboratories and to some extent eliminate possible human errors. This iELISA test kit was capable for detecting antibodies to smooth-typed *Brucella* spp. in several species of hosts, because a recombinant Protein G conjugated with horseradish peroxidase was selected as the detection reagent for this system (Kramsky *et al.*, 2003; OIE, 2004).

CONCLUSION

The iELISA test kit described here can be considered as a cost-effective, sensitive and specific diagnostic tool for large-scale brucellosis surveys in the control program and surveillance of brucellosis in Thailand. However, the test kit should be further evaluated as a diagnostic tool in various species of animals for brucellosis control.

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LITERATURE CITED

- Chaichanasiriwithaya, W., R. Kanitpun, M. Ekgat, S. Thammasart and S. Wongkasemjit. 2001. Development of ELISA to diagnosis of bovine brucellosis, p. 1-11. *In Proceeding of the 27th Conference of Veterinary Sciences*. The Thai Veterinary Medical Association. Bangkok, Convention Hall, Sofitel Central Plaza Hotel, Bangkok, 24-26 October

- Chumek, P. and N. Srikawkheaw. 2007. Comparison of validation method in antibody detection against *Brucella abortus* in bovine serum by RBT, EDTA-TAT, and ELISA. **Thai-NIAH eJournal**: <http://www.dld.go.th/niah>, 1(3): 180-188.
- Crowther, J.R., H. Unger and G.J. Viljoen. 2006. Aspects of kit validation for tests used for the diagnosis and surveillance of livestock diseases: producer and end-user responsibilities. **Rev. sci. tech. Off. Int. Epiz.** 25(3): 913-935.
- Dawson, B. and R.G. Trapp. 2004a. ROC curves, p. 314-315. In **Basic & Clinical Biostatistics**. 4th ed. Mc Graw-Hill Companies, Inc.
- Dawson, B. and R.G. Trapp. 2004b. Measuring agreement between two people or methods, p. 118-119. In **Basic & Clinical Biostatistics**. 4th ed. Mc Graw-Hill Companies, Inc.
- Joint FAO/IAEA Programme, Animal Production and Health. 1994. **Brucellosis test kit : Indirect enzyme immunoassay for detection of bovine antibody to *Brucella abortus***. Bench Protocol Version-BRA 1.3 Wagramerstrasse 5, P.O. Box 100. A- 1400 Vienna, Austria.
- Kramsky, J.A., E.J.B. Manning and M.T. Collins. 2003. Protein G binding to enriched serum Immunoglobulin from non domestic hoofstock species. **J. Vet. Diagn. Invest.** 15: 253-261.
- Mainar-Jaime, R.C., P.M. Munoz, M.J. de Miguel, M.J. Grillo, C.M. Marin, I. Moriyon and J.M. Blasco. 2005. Specificity dependence between serological tests for diagnosing bovine brucellosis in *Brucella*-free farms showing false positive serological reaction due to *Yersinia enterocolitica* O:9. **Can. Vet. J.** 46(10): 913-916.
- Nielsen, K., D. Gall, W. Kelly, A. Viglicco, D. Henning and M. Garcia. 1996. **Immunoassay development application to enzyme immunoassay for the diagnosis for brucellosis**. Agriculture and Agri-Food monograph. A 62-45/1996E. ISBN 0-662-24163-0.
- Pouillot, R., G. Gerbier and I. A. Gardner. 2002. "TAGS", a program for the evaluation of test accuracy in the absence of a gold standard. **Prev. Vet. Med.** 53(1-2): 67-81.
- Tittarelli, M., A. Giovannini, A. Conte, M. Di Ventura, D. Nannini and V. Caporale. 2005. The use of homologous antigen in the serological diagnosis of brucellosis caused by *Brucella melitensis*. **J. Vet. Med. Series B.** 52(2): 75-81.
- World Organisation for Animal Health (Office International des Epizooties, OIE). 2004. Principles of validation of diagnostic assays for detection diseases, p. 21-29. In **Manual of standard tests and vaccines for terrestrial animal (mammals, birds and bees)**, 5th ed., Paris, France.
- Wong-wong, N. 2006. Antibody detection titer against bovine brucellosis by enzyme-linked immunosorbent assay. **Northern Animal Health News.** 14(4): 50-55.
- Uzal, F.A., A.E. Carrasco, S. Echaide, K. Nielsen and C.A. Robles. 1995. Evaluation of an indirect ELISA for the diagnosis of bovine brucellosis. **J. Vet. Diagn. Invest.** 7(4): 473-475.