THE USE OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DIAGNOSIS OF BRUCELLOSIS IN CATTLE IN INDONESIA

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) has been developed to detect antibody to *Brucella abortus* in bovine serum. The results of testing 2,819 bovine serums by ELISA, Rose Bengal plate test (RBPT) and complement fixation test (CFT) were compared. 21.7% (611) serums were positive in all tests. A further 0.5% (15) were positive only in the RBPT, and 2.5% (70) only in ELISA, no serum was positive in the CFT only. Of the 616 serum samples positive in RBPT and CFT, 99.2% (611) were positive in the ELISA. Of the 2,142 serums negative in the RBPT and CFT, 96.7% (2,072) were negative in the ELISA. More positive reactors were detected in the ELISA than in the RBPT and CFT. The ELISA method described is simple and generally much easier to perform for a large number of serums and is possibly the most reliable, consistent test for use in any eradication programme for bovine brucellosis undertaken in Indonesia.

ABSTRAK

Enzyme-linked immunosorbent assay (ELISA) sedang dikembangkan untuk mendeteksi antibodi terhadap *Brucella abortus* yang terdapat dalam serum sapi. Sebanyak 2.819 serum sapi diuji dengan ELISA, aglutinasi cepat Rose Bengal dan uji pengikatan komplemen. Sebanyak 21,7% (611) serum memberikan reaksi positif terhadap *Brucella abortus* pada semua uji. Selanjutnya 0,5% (15) serum bereaksi positif hanya pada uji aglutinasi Rose Bengal dan 2,5% (70) bereaksi positif pada ELISA saja. Tidak ditemukan yang hanya bereaksi positif pada uji pengikatan komplemen. Dari 616 serum yang bereaksi positif pada uji Rose Bengal dan pengikatan komplemen, 99,2% (611) memberikan reaksi positif pada ELISA. Dari 2.142 serum yang bereaksi negatif pada uji Rose Bengal dan pengikatan komplemen, 96,7% (2.072) bereaksi negatif pada ELISA dan 3,3% bereaksi positif ELISA. Dengan ELISA dapat ditemukan serum bersifat reaktor positif lebih banyak dibandingkan dengan uji yang lain. Uji ELISA yang dikembangkan lebih sederhana dan lebih mudah dikerjakan untuk memeriksa sampel serum dalam jumlah yang besar. Di samping itu, ELISA memberikan hasil yang akurat dan dapat dilakukan secara konsisten dalam usaha pemberantasan brucellosis pada sapi di Indonesia.

INTRODUCTION

Brucellosis is an infectious disease of animals and man caused by organisms of the genus *Brucella* (Gillespie and Timoney, 1981). The diagnosis of brucellosis in domestic animals is achieved by the use of serological tests, allergic tests and by isolation and identification of the organism (Fensterbank, 1986). However the identification of brucellosis in a large number of animals is usually based on serological methods. (Nielsen *et al.*, 1988).

Bovine brucellosis in Indonesia occurs in Java, Sumatra, Kalimantan, Sulawesi and Nusa Tenggara. The diagnosis of brucellosis in Indonesia is usually made by the Rose Bengal plate test (RBPT), serum agglutination test (SAT) and complement fixation test (CFT). The CFT has been proved to offer higher specificity than SAT for the serological diagnosis of bovine brucellosis (Stemshorn *et al.*, 1985).

Various ELISA methods have been reported for the detection of serum antibodies to *Brucella abortus*. The methods all were found to be specific and to have equal or greater sensitivity than RBPT or CFT (Byrd *et al.*, 1979; Ruppanner *et al.*, 1980; Heck *et al.*, 1981; Heck *et al.*, 1982).

This paper compares ELISA for the detection of antibodies to *B. abortus* with RBPT and CFT for the diagnosis of brucellosis in cattle.

MATERIALS AND METHODS

Bovine Serum Samples

Two thousand eight hundred and nineteen serum samples were received from Jakarta, West Java, South Sulawesi and East Nusa Tenggara.

Rose Bengal Plate Test (RBPT)

The RBPT antigen was a heat killed *B. abortus* strain 19 cell suspension, stained with Rose Bengal (Davies, 1971). The packed cell volume was adjusted to 8% by the use of the packed cell volume method and the pH was adjusted to 3.65 (Davies, 1971). The standard method described for the diagnosis of bovine brucellosis was applied (Anon., 1980).

Complement Fixation Test (CFT)

The CFT antigen, haemolysin and guinea-pig complement were supplied by the Commonwealth Serum Laboratories (CSL), Melbourne, Australia. The warm micro CFT procedure as described by Alton *et al.* (1975) was followed. Two fold serum dilutions from 1:4 to 1:128 inclusive were used for the test, 1:2 serum dilution was used as an anti-complementary control. A reaction producing 50% haemolysis (+2) at serum dilution of 1:4 or greater was classified as positive.

Enzyme-Linked Immunosorbent Assay (ELISA)

Crude lipopolysaccharide (LPS) from *B. abortus* was prepared according to the method of Plackett and Stewart (1986). The final dialysed solution was dispensed into 2 ml aliquots and freeze dried in tared vials. Each freeze-dried vial contained approximately 20 mg by weight of crude LPS.

ELISA was performed as described by the CSIRO Division of Animal Health, Parkville, Victoria, Australia (Plackett and Stewart, 1986), with slight modification. Polystyrene 96 well, round bottomed micro plates were coated with 50 µl per well of *B. abortus* LPS antigen and incubated overnight at 4°C. The coating solution was discarded and the plates were washed 4 times with PBS containing 0.05% Tween – 20 (PBST) at room temperature. Test serums were diluted 1:200 in PBST, 50 µl of each sample was added into wells in duplicate. Positive and negative control serums were included in each plate. The plates were incubated at room temperature for 1 hour with shaking and then washed 4 times with PBST. Subsequently, 50 µl of the optimum dilution of sheep antibovine IgG horseradish peroxidase (SAB IgG, Silenus Laboratories Pty. Ltd., Victoria, Australia) in PBST with 0.2% casein was added to each well and incubated for 1 hour at room temperature with shaking. The plates were then washed 4 times as above. One hundred microliters of substrate solution [1 mM 2,2-azino (3 ethyl-benzithiazoline sulphonate acid) (ABTS) with 2.5 mM H_2O_2 in 0,1 M citrate/phosphate buffer pH 4.2] was added into each well and the plates were shaken at room temperature for up to 60 minutes. Plates were read by means of a Micro-ELISA reader (Titertek Multiskan) at a double wavelengths (414 nm and 492 nm). The "cut-off" level was defined as the optical density of a 1:32,000 dilution of positive reference control serum supplied by courtesy of Dr. J. Searson, Department of Agriculture, District Veterinary Laboratory, Wagga-Wagga, New South Wales, Australia. This "cut-off" was arbitrarily assigned as a titre of 64 ELISA units.

RESULT AND DISCUSSION

Antigens and conjugates were titrated to determine the optimum concentration for use under the condition stipulated for the test. The optimum concentration of the antigen was found to be a 1:10,000 to 1:15,000 dilution which represents a concentration of approximately 1 µg/ml. This is similar to the dilution of crude alkali treated *B. abortus* antigen as recommend by Plackett and Stewart (1986). At the optimum dilution of antigen, a 1:2,000 dilution of positive reference control serum had a dual wavelength absorbance at 415 nm/492 nm (A415/492) of > 2.0 and the 1:32,000 "cut-off" dilution had a mean A415/492 of 0.640 ± 0.130 (Fig.1). A control serum produced at Balitvet Bogor was titrated and compared to the titration of the positive reference control serum. A dilution of 1:3,600 of the Balitvet control (S15. 8/3/87) serum had an A415/492 equivalent to that of the 1:32,000 dilution of the reference control serum. The Balitvet control serum when titrated at a starting dilution of 1:300 and with a cut-off dilution of 1:3,600 would be suitable for use in the assay. The optimum conjugate dilution when titrated against the antigen and positive reference control serum was found to be equivalent to that recommended by the manufacturer.

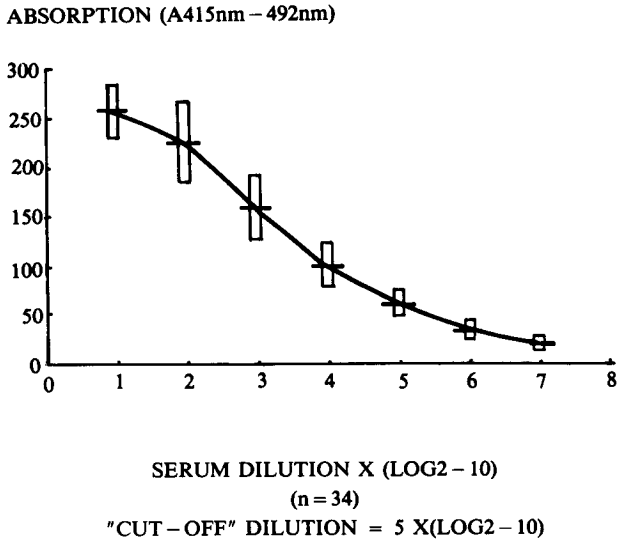

Figure 1. Titration of *B. abortus* positive serum (NSW positive control)

The optimum cut-off value used for the test was that determined by Dr. J. Searson (personal communication) which is the absorbance of the 1:32,000 dilution of the positive control reference serum. This cut-off was determined after extensive testing in NSW Australia and is applicable to non-vaccinated cattle, the cut-off value for vaccinated cattle is the A415/492 of a 1:16,000 dilution of the positive control reference serum (J. Searson, personal communication). The value for the optimum cut-off may require revision once local data is accumulated from *B. abortus* culture positive animals.

From a total of 2,819 bovine serums investigated, 661 (23.4%) were positive in RBPT, 632 (22.4%) were positive in CFT and 721 (25.6%) were positive in ELISA (Table 1). Fifteen (0.5%) of the 2,819 samples tested were positive to the RBPT only, 70 (2.5%) of the samples were positive in the ELISA only, and no serums were positive in the CFT only (Table 1). Of the 616 serum samples positive by RBPT and CFT, 99.2% (611) were positive by the ELISA. Of the 2,142 serums negative by RBPT and CFT, 96.7% (2,072) were negative by the ELISA (Table 1). Of the 2,158 serums which were negative in the RBPT, 16 (0.7%) were positive in the CFT and 80 (3.8%) were positive in the ELISA (Table 2).

The ELISA would appear to be more sensitive than the RBPT and CFT which agrees with the results presented by Cargill *et al.* (1985). They demonstrated that ELISA detected more positive reactors than CFT indicating that the ELISA was able to detect *B. abortus* antibodies in sera which were negative in the RBPT or CFT (Cargill *et al.*, 1985). Heck *et al.* (1982) also found that the ELISA was considerably more sensitive than the conventional methods for detecting antibody in serums from cows which were culture positive for *B. abortus*.

Byrd *et al.* (1979) and Ruppaneer *et al.* (1980) reported that the specificity of the ELISA test for identifying non-infected cows was 97% and 100%. It has been reported by Cargill *et al.* (1985) that in herds with chronic *B. abortus* infection, infected animals had high levels of IgG2. This class of immunoglobulin does not participate in complement fixation reactions and at high levels IgG2 can interfere with the ability of IgG1 to fix complement. CFT detects complement fixing IgG1 and any post-inactivation residual IgM in the serum (Mylrea & Fraser, 1976; Hobbs, 1985) whereas the enzyme conjugate used in the ELISA detected both the IgG1 and IgG2. The serums negative in the CFT, but positive in the ELISA in this study are possibly from chronically infected animals although the proportion of IgG1 and IgG2 in the serums was not determined.

In the diagnosis of brucellosis, serology is used only as an indicator of previous infection, and provides a rapid and efficient method of detecting infected animals so that they can be eventually eliminated from the herd, reducing the opportunity of infecting other in-contact animals.

In this study, if RBPT and CFT were used to indicate infection then 616 (21.9%) animals would have been slaughtered. However, 16 (0.5%) of the 2,819 animals tested would not be detected as they were RBPT negative. If RBPT and ELISA were used then 641 (22.8%) animals would have been slaughtered. However 16 (0.5%) CFT and ELISA positive and 70 (2.5%) ELISA positive animals would not have been slaughtered as they were all RBPT negative. If ELISA only was used then 727 (25.8%) animals would have been slaughtered, all except 70 (2.5%) of these animals would be either RBPT or CFT positive.

Further research is necessary to determine the infective status, particularly of the 70 animals positive

Table 1. Distribution of serological test result

RBPT	CFT	ELISA	Number of serums	%
+	+	+	611	21.7
+	+	−	5	0.2
+	−	−	15	0.5
+	−	+	30	1.1
−	−	−	2,072	73.5
−	−	+	70	2.5
−	+	+	10	0.3
−	+	±	6	0.2
Total 661(23.4%)	632(22.4%)	721(25.6%)	2,819	100%

RBPT : Rose Bengal plate test
CFT : Complement fixation test
ELISA: Enzyme-linked immunosorbent assay

Table 2. Comparison of ELISA, RBPT, and CFT for detecting antibody to *B. abortus* in bovine serum

RBPT		CFT		ELISA		
		Positive	Negative	Positive	Dubious	Negative
Positive	661	616	45	640	0	21
Negative	2.158	16	2,142	80	6	2,072

RBPT : Rose Bengal plate test
CFT : Complement fixation test
ELISA: Enzyme-linked immunosorbent assay

only in the ELISA. These animals were not readily accessible to perform the anamnestic test as reported by Corner *et al.* (1983) and were not slaughtered to allow collection of tissues for culture.

In the context of an eradication program, it would be appropriate to test samples by RBPT which is a rapid, easy, and low cost test which can be used in the field (Fensterbank, 1986). However, the test is subjective and results can vary greatly depending on the technician who is assessing the degree of agglutination and on the antigen being used (unpublished data). Subsequent confirmation of the RBPT result can then be performed using either a CFT or ELISA test. We would recommend the use of ELISA to detect those infected animals which for various reasons are not reacting in the CFT. Using this scheme a small number of non-infected animals are likely to be slaughtered however this number is likely to be low and is probably less of a problem than if infected, non-CFT reactive animals are left in the herd.

The ELISA test would appear to be an efficient and relatively easy test to perform which could take its place with RBPT for the screening of a large number of serums for the diagnosis of brucellosis. The test has a high sensitivity and is able to detect low levels of IgG1 and IgG2 whereas the CFT is only able to detect IgG1.

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