# THE EVALUATION OF SEROLOGY AND BACTERIOLOGY FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN SOUTH SULAWESI

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## ABSTRACT

Sulaiman, I., B.E. Patten, and P. Darmadi. 1993. The evaluation of serology and bacteriology for the diagnosis of bovine brucellosis in South Sulawesi. *Penyakit Hewan* 25 (46A): 29-33.

Serum plus the supramammary, prefemoral, prescapular, retropharyngeal and mesenteric lymph nodes were obtained from 627 cattle slaughtered at the city abattoirs. The sera were tested by the Rose Bengal Plate Test (RPBT), Complement Fixation Test (CFT) and Enzyme-linked Immunosorbent Assay (ELISA), and lymph nodes were examined bacteriologically. A total of 93 sera were positive in one of the serological tests and 68 *B. abortus* isolates were obtained from the lymph nodes from these positive cattle. Lymph nodes from 83 cattle negative in all of the serological tests were examined bacteriologically and 5 *B. abortus* isolates were obtained. Four of the 5 isolates from the negative cattle were typed as Strain 19 whereas only 13 of the 68 isolates from serologically positive cattle were Strain 19 isolates.

**Key words:** Serology, bacteriology, bovine brucellosis, South Sulawesi

## ABSTRAK

Sulaiman, I., B.E. Patten dan P. Darmadi. 1993. Evaluasi secara serologi dan bakteriologi untuk diagnosis brucellosis di Sulawesi Selatan. *Penyakit Hewan* 25 (46A): 29-33.

Serum sapi dan limfoglandulla diambil dari 627 sapi yang dipotong di Rumah Potong Hewan. Uji Rose Bengal (RBPT), Uji pengikatan komplemen (CFT), dan Uji Enzyme-Linked Immunosorbent Assay (ELISA) digunakan untuk pengujian serum. Sedangkan limfoglandulla yaitu supramammaria, prefemoralis, prescapularis, retrofaringealis, dan mesenterialis diperiksa secara bakteriologis. Limfoglandulla dari 93 ekor sapi yang menunjukkan positif serologis dan limfoglandulla dari 83 ekor sapi yang menunjukkan negatif serologis dikultur untuk pemeriksaan bakteriologi. 68 dari 93 ekor positif serologis diisolasi *B. abortus*, empat diantaranya diidentifikasi *B. abortus* strain 19.

**Kata kunci:** Serologi, bakteriologi, brucellosis sapi, Sulawesi Selatan

## INTRODUCTION

Bovine brucellosis was first detected in South Sulawesi in 1977 in the Kabupaten Sidrap. The outbreak was confirmed on the basis of serological tests and the bacteriological culture of cows which had aborted from which field strain *B. abortus* was isolated (Crowther pers. comm.). In the period from 1977 to 1987 the number of cases of brucellosis increased significantly in several Kabupatens, mainly in those areas adjacent to the first infected area. In 1988 an eradication and control program for brucellosis was conducted in all kabupatens in South Sulawesi. The program consisted of a sero-survey to obtain epidemiological data on the prevalence and distribution of brucellosis, plus control and eradication using a *B. abortus* strain 19 vaccine (Pusvetma, Surabaya, Indonesia) with reduced dose (DGLS, pers.comm.). The Rose Bengal Plate Test (RPBT) as a screening test and the Complement Fixation Test (CFT) as a definitive test.

The CFT was the only test approved to determine infected cattle (DGLS, pers.comm). However, haemolysed or contaminated sera received from the field (Sulaiman, unpublished), resulted in difficulties in confirming the sero-status of the animal sampled.

Ideally, the test of choice will identify most infected animals and result in low false negative rate. The enzyme linked immunoSorbent assay (ELISA) has been reported to have higher sensitivity than the RBPT and CFT (Sutherland and Den Hollander, 1986). Since 1990, an ELISA has been used to detect bovine brucellosis reactors at BPPH Wilayah VII, Maros. This study compares the sensitivity and specificity of the RBPT, CFT and ELISA based on the isolation of *B. abortus* from matched lymph nodes.

## MATERIALS AND METHODS

### Specimens

Blood and lymph nodes for this study were obtained from 627 cattle at the time of slaughter at the Ujung Pandang abattoir, South Sulawesi. The age of the cattle ranged from 6 to 10 years old and the animals were from

provinces in South Sulawesi where *Brucella* strain 19 (S19) vaccination had been undertaken at least 10 to 12 months previously. Five pairs of lymph nodes namely, retropharyngeal, prescapular, prefemoral, supra-mammary, and mesenteric lymph nodes, were collected for bacteriological examination from each animal.

**Serology**

Blood samples were collected in 10 mL plastic tubes and placed on wet ice. On return to the laboratory the blood was allowed to clot at room temperature, and the serum collected and stored at -20°C until used. The sera were tested by the RBPT, CFT and ELISA.

*RBPT*

The standard procedure for the RBPT (Anonymous, 1980), using a Rose bengal stained *Brucella* antigen supplied by CSL Ltd, Melbourne, Australia (CSL) was used. The agglutination reaction was graded as 0, 1+, 2+, and 3+, with a reaction of >0 considered positive.

*CFT*

The warm micro-CFT as described by Alton *et al.* (1975) using CFT *Brucella* antigen, haemolysin, and guinea-pig complement supplied by CSL was used. The CFT was considered positive where there was 50% haemolysis at a serum dilution of 1:4 or greater.

*ELISA*

The ELISA procedure was performed according to the method of Sudibyo and Patten (1989) with slight modification. *B. abortus* lipopolysaccharide (LPS) antigen and horseradish peroxidase conjugated rabbit anti-bovine IgG were supplied by the FAO/IAEA Animal Production Division, Austria. Reference positive and negative sera were supplied by Balitvet, Bogor, Indonesia.

A 96-well round bottomed polystyrene microtitre plate (Nunc, Denmark) was coated overnight at 4°C with 50 µL/well *B. abortus* LPS at a 1:2400 dilution in 0.1 M carbonate buffer, pH 9.6. Test sera and negative sera were diluted 1:200 in phosphate buffered saline pH 7.4 containing 0.05% Tween 20 (PBST) in duplicate; test sera were added to wells A1-A2 up to wells H9-H10. A control positive serum was diluted by 2-fold dilution from 1:2000 to 1:64000, then each dilution was added to wells A11-A12 up to F11-F12. Wells G11-G12 and H11-H12 contained control negative serum and diluting buffer, respectively. The plate was incubated for 1 hour at 37°C, washed 4 times with PBST and then 50 µL of anti-bovine IgG conjugate, diluted 1:3000 in PBST, was added per well, incubated similarly and washed. A 1 mM solution of 2,2' azino-di-(3 ethylbenthiazoline sulphonic acid (ABTS) with 50 µL/10 ml 3% $H_2O_2$ in citrate buffer pH 4.6 was used as substrate. The plates were incubated at room temperature for approximately 30 min. with shaking and then read at 405 nm using a Multiskan II platereader (Flow Laboratories, UK).

The "cut-off" level was defined as the optical density of a 1:32,000 dilution of positive reference serum (J. Searson. pers. comm.). The optical density readings were converted into ELISA units (EU) using a computer program (Platereader V3.2, Regional Veterinary Laboratory Benalla, Australia) which calculated the EU values from a standard curve produced from the control serum dilutions. The 1:2000 dilution of the control serum was assigned a value of 1024 EU, the 1:32,000 cut-off serum a value of 64 EU and the conjugate control an EU value of 0. Test samples which produced EU values of 64 or greater were regarded as positive.

**Bacteriology**

Lymph nodes were collected in an aseptic manner at the abattoir and placed into virgin zip-lock plastic bags and stored on wet ice. All of the nodes from cattle which were positive by any serological test, and the lymph nodes from a randomly selected number of cattle which were serologically negative, were cultured for *B. abortus*.

Microbiological culture was undertaken according to the procedure as described by Corner *et al.* (1984) with modification. The lymph nodes were dipped into 70% ethanol and flamed for a few seconds before the tissue surrounding the nodes was removed. Each node was cut into small pieces and homogenised using an electric blender. The homogenised tissue was placed into a 25 mL sterile universal bottle containing 10 mL of sterile nutrient broth and mixed using a vortex mixer. The homogenised tissue fluid was streaked onto *Brucella* selective agar consisting of Trypticase soy broth (BBL, USA.) with 1.5% agar containing *Brucella* selective supplement (*Brucella* antibiotic Supplement SR083, Oxoid, UK.) and 5% bovine serum. One mL of the homogenised mixture was inoculated into 9 mL selective broth medium containing Trypticase soy broth with *Brucella* selective supplement (Oxoid, UK) and 5% bovine serum. Both the plate and the broth were incubated at 37°C in 8 to 10% $CO_2$ in air for 5 days. After

5 days the plate and broth were examined for any growth. If any growth was detected in the broth culture then 1 mL was subcultured onto a selective agar plate which was re-incubated in the same manner as above. The selective plates were examined every 3 days and were discarded after 3 weeks incubation if no suspicious *B. abortus* colonies were detected.

Any suspicious *B. abortus* colonies were selected and sub-cultured onto *Brucella* selective agar, 10% sheep blood agar (Oxoid Columbia agar base with 10% defibrinated sheep blood) and MacConkey agar plates (Oxoid, UK) for morphological and biochemical identification tests. Bacterial isolates were classified as *B. abortus* if they were Gram negative coccobacilli, not acid fast, oxidase positive, catalase positive, agglutinated with mono-specific anti-A serum, produced no growth on MacConkey agar, produced no haemolysis on blood agar, were non motile and urease positive.

*B. abortus* isolates were biotyped according to their sensitivity to basic fuchsin and thionine dyes, and their agglutination with mono-specific anti-A or mono-specific anti-M according to the procedure as described by Alton *et al.* (1975). Dyes were incorporated in trypticase soy agar with 5% bovine serum, at the following concentrations, thionine (BDH Chemicals Ltd., UK.) 1:25,000, 1:50,000, and 1:100,000 and basic fuchsin (BDH Chemicals Ltd., UK.) 1:50,000 and 1:100,000.

*B. abortus* cultures which were sensitive to thionine but resistant to basic fuchsin at a concentration of 1:50,000, and reacted only with mono-specific anti-A serum, were classified as *B. abortus* biotype 1 (Bio1). *B. abortus* Bio1 were further classified as strain 19 (S19) according to their growth on agar containing 1 mg/mL erythritol (BDH Chemicals Ltd. UK.) and agar containing penicillin 5 u/mL.

Mono-specific anti-A and anti-M serum and control *Brucella* cultures (*B. abortus* 544, *B. abortus* S19, *B. melitensis* M16) were kindly provided by The Central Veterinary Laboratory, [illegible]ge, *United Kingdom*.

## RESULTS

As shown in Table 1, 93, or 14.8%, of the 627 serum samples collected from the slaughtered cattle were positive in either the RBPT, CFT or ELISA. A total of 68 *B. abortus* isolates were obtained from lymph nodes from sero-positive cattle. In addition, 5 out of 83 sets of lymph nodes from sero-negative cattle produced *B. abortus* isolates after culture.

Sixty-two of the serum samples were positive in all 3 serological tests from which 61 isolates of *B. abortus* were obtained. Four samples were positive in both the ELISA and RBPT from which 2 isolates of *B. abortus* were cultured. Thirteen samples were positive in the ELISA only, from which 4 isolates of *B. abortus* were cultured and 1 isolate of *B. abortus* was cultured from 14 samples positive only in the RBPT.

*B. abortus* was isolated from tissues from all cattle with CFT titres of 16 or greater. *B. abortus* was not isolated from lymph nodes from 1 animal with a CFT titre of 4 and from 1 with a CFT titre of 8. *B. abortus* was isolated from all tissues from cattle with EU of 256 or greater and was also isolated from cattle with EU <64. However, the organism was not isolated from the 4 cattle with EU ranging between 128 to 255.

Table 1. *B. abortus* serology and culture results from abattoir samples

| Serology result | | | Number sero-positive | Culture type[1] | | | |
|---|---|---|---|---|---|---|---|
| RBPT | CFT | ELISA | | Bio1 | S19 | AT | Total |
| + | + | + | 62 | 48 | 11 | 2 | 61 |
| + | - | + | 4 | 2 | 0 | 0 | 2 |
| + | - | - | 14 | 1 | 0 | 0 | 1 |
| - | - | + | 13 | 2 | 2 | 2 | 4 |
| - | - | - | - | 1 | 4 | | 5 (83)[2] |
| Total | | | 93 | 54 | 17 | 2 | 73 |

[1] *B. abortus* - Bio1, biotype 1; S19, strain 19; AT, atypical biotype
[2] The number subjected to bacteriological culture from 534 sero-negative cattle

The sensitivity and specificity of the serological tests is shown in Table 2. The sensitivity of the RBPT was 85%, the CFT was 83% and the ELISA was 93%. The CFT had the highest specificity although it had the lowest sensitivity. The RBPT was more sensitive than the CFT but less sensitive than ELISA.

Of the 73 *B. abortus* isolates, 54 were typed as Bio1 and 17 were typed as S19. Nine of the Bio1 isolates did not require $CO_2$ for growth and 2 did not utilise urea. Six of the 17 S19 isolates grew on erythritol agar. Four of the 5 isolates from sero-negative cattle were *B. abortus* S19.

Two *B. abortus* isolates were resistant to thionine at a concentration of 1:25.000 and to basic fuchsin. One of the isolates reacted with monospecific anti-A serum and 1 reacted weakly with monospecific anti-M. One did not

produce $H_2S$ and both isolates did not require supplementary $CO_2$ for growth. The 2 isolates were classified as atypical biotypes (AT).

The isolation rate for *B. abortus* from the various lymph nodes is shown in Table 3. *B. abortus* was isolated mainly from the supramammary and retropharyngeal lymph nodes. The lowest isolation rate was observed from the mesenteric lymph node.

**Table 2.** Sensitivity and specificity of the RBPT, CFT and ELISA

| Serology | | Culture | | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|---|
| Method | Result | Positive | Negative | | |
| RBPT | Positive | 64 | 16 | | |
| | Negative | 9 | 83 | 88 | 84 |
| | Total | 73 | 99 | | |
| CFT | Positive | 61 | 2 | | |
| | Negative | 12 | 98 | 84 | 98 |
| | Total | 73 | 100 | | |
| ELISA | Positive | 68 | 13 | | |
| | Negative | 5 | 86 | 93 | 87 |
| | Total | 73 | 99 | | |

**Table 3.** Tissue distribution of *B. abortus* isolates from lymph nodes collected from abattoir slaughtered cattle

| Lymph Node[1] | Culture positive | |
|---|---|---|
| | No. | % |
| Retropharyngeal | 42 | 58 |
| Supramammary | 45 | 61 |
| Prefemoral | 20 | 27 |
| Prescapular | 21 | 28 |
| Mesenteric | 10 | 12 |

[1]Seventy three sets of tissues were examined

## DISCUSSION

This study revealed that the sensitivity of ELISA was greater than the RBPT and the CFT. Sutherland and Den Hollander (1986) reported that, under experimental conditions, there was some similarity between ELISA and CFT results from cattle vaccinated with S19, but ELISA gave positive results for a longer period than CFT. While the RBPT has a higher sensitivity than the CFT this may be due to haemolysis of sera, with subsequent anti-complementary reactions, or because of high ratio of specificity of IgG2 to IgG1 that results in the tendency of the CFT to prozone (McNaught *et al.*, 1977). The effect of anti-complementary or prozone effects on CFT results has been reported in several studies (Plackett and Alton, 1975; McNaught *et al.*, 1977). In Indonesia, it is frequently not possible to re-sample animals due to limited man-power and financial resources. In this study animals were collected from the abattoirs where it is obviously not possible to re-sample animals. As it is necessary to re-sample and re-test an anti-complementary serum to determine whether an animal is *Brucella* positive and so in the field situation described the CFT may not be a completely suitable test.

In this study, *B. abortus* was most frequently isolated from the supramammary and retropharyngeal lymph nodes and least from the mesenteric lymph node. Hornitzky and Searson (1986) also reported that the supramammary and retropharyngeal nodes were the most likely to yield *B. abortus* on culture. *B. abortus* Bio1 was the biotype most frequently isolated, however 23% of isolates were S19. Fourteen *B. abortus* S19 isolates were obtained from cattle with positive serology results and 4 from sero-negative cattle.

The animals in this study had been vaccinated at least 10 to 12 months previously (Dinas Peternakan, pers.comm.). As the animals were 6 to 10 years old, a positive serological response to S19 vaccine may be expected to last for up to 12 months in at least 50% of the vaccinated animals (Nielsen and Duncan, 1990). In this study, however, 76% of animals from which S19 was isolated were sero-positive suggesting that the animals may have been previously vaccinated or infected, with a more sustained secondary antibody response being produced by vaccination. Duffield *et al.* (1984) also isolated *B. abortus* S19 9 to 12 months after S19 vaccination from cattle which were positive by the CFT. As S19 vaccinated animals are not marked or identified in Indonesia, the presence of S19 infected cattle may cause problems in undertaking epidemiological sero-surveys and in the control or eradication of bovine brucellosis.

In this study, some of the *B. abortus* S19 isolates grew on agar containing erythritol. Erythritol-utilising S19 isolates have previously been recovered from cattle which have aborted following vaccination with a reduced dose of S19 vaccine (Beckett and MacDiarmid, 1985) and it has been reported by Alton *et al.* (1988) that many S19 isolates may tolerate erythritol although they give typical S19-associated reactions in other biochemical reactions. It was not possible to determine whether any of the animals sampled in this study had aborted either before or after vaccination.

The fact that *B. abortus* was not isolated from 8 of 13 cattle with EU values ranging from 64 to 127 resulted in a lower specificity for the ELISA. This EU range, which is within one dilution of the cut-off value, can be considered "dubious" (Patten, pers. comm.) and the animal would normally be re-sampled, where possible. *B. abortus* was not isolated from 2 cattle with positive RBPT, CFT and ELISA serology results. This may be because the animals had overcome the infection or because of very low numbers of organisms which were not detected with the bacteriological techniques used.

Although the ELISA has greater sensitivity than conventional tests, it may detect more false positive animals due to S19 vaccination, especially in herds where vaccinated cattle are not marked or identified. A combination of serological tests may still be necessary in the first stage of eradication program where *B. abortus* S19 vaccine is used to protect animals.

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## REFERENCES

ALTON, G.G., L.M. JONES and D.E. PIETZ. 1975. Laboratory techniques in Brucellosis 2nd edn. W.H.O. Geneva, Switzerland.

ALTON, G.G., L.M. JONES R.D. ANGUS and J.M. VERGER. 1988. Techniques for the brucellosis laboratory. I.N.R.I. Paris, France.

ANONYMOUS. 1980. Standardised Rose Bengal test for bovine brucellosis. *Aust. Vet. J.* 56: 555.

BECKETT, F.W. and S.C. MACDIARMID. 1985. The effect of reduced-dose *Brucella abortus* strain 19 vaccination in accredited dairy herds. *Br. Vet. J.* 141: 507-514.

CORNER, L.A., G.G. ALTON and H. IVER. 1985. An evaluation of a biphasic medium for the isolation of *Brucella abortus* from bovine tissues. *Aust. Vet. J.* 62: 187-189.

DUFFIELD, B.J, T.A. STREETON and G.A. SPINKS. 1984. Isolation of *Brucella abortus* from supramammary lymph nodes of cattle from infected herds vaccinated with low dose strain 19. *Aust. Vet. J.* 61: 411-412.

HORNITZKY, M. and J. SEARSON. 1986. The relationship between the isolation of *Brucella abortus* and serological status of infected, non-vaccinated cattle. *Aust. Vet. J.* 63: 172-174.

MCNAUGHT, D.J., R.J. CHAPPEL, G.S. ALLAN, J.A. BOURKE and B.A. ROGERSON. 1977. The effects of IgG2 and of antigen concentration on prozones in the complement fixation test for bovine brucellosis. *Res. Vet. Sci.* 22: 194-197.

NIELSEN, K. and R.J. DUNCAN. 1990. Animal Brucellosis. CRC Press, Boston, USA.

PLACKETT, P. and G.G. ALTON. 1975. An indirect haemolysis test (IHLT) for bovine brucellosis. *Aust. Vet. J.* 52: 136-140.

SUDIBYO, A. and B.E. PATTEN. 1989. The use of an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of brucellosis in cattle in Indonesia. *Penyakit Hewan* 21: 18-21.

SUTHERLAND, S.S. and L. DEN HOLLANDER. 1986. Comparison of an enzyme-linked immunosorbent assay using monoclonal antibodies and a complement fixation test for cattle vaccinated and infected with *Brucella abortus*. *Vet. Microbiol.* 12: 55-64.