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DEVELOPMENT OF AN ENZYME–LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTING ANTIBODY TO *BRUCELA SUIS* IN PORCINE SERA

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ABSTRACT

To overcome the problems often encountered with the serodiagnosis of swine brucellosis, 546 matched lymph nodes and serum samples were collected from slaughter swine and the sensitivity and specificity of an ELISA method were compared with the conventional serodiagnostic tests for brucellosis. Sera with an optical density ratio in the ELISA of greater than 0.18 were considered suspicious or positive. A total of 66 animals were culture positive and the ELISA was shown to have a sensitivity of 85% compared with 62%, 42% and 38% and a specificity of 78% compared with 88%, 95% and 92% in the RBPT, SAT and CFT respectively. The apparent lover specificity of the ELISA compared to the conventional tests was considered mainly due to difficulties in demonstrating active infection and the much lower threshold of detectable antibody and not due to heterospecific, predominantly Igm antibody causing false positive reactions.

INTRODUCTION

Swine brucellosis is widespread throughout South America and Southern Asia (Matyas and Fujikura, 1984). It is a serious pathogen in man most commonly encountered in meat workers (Ray, 1980). Recently a high reactor rate in slaughter swine in Indonesia was reported (Priadi *et al.*, 1985) and in abattoir workers (Authors' unpublished work).

Many serological tests have been developed to detect brucella infected animals (Brindley-Morgan, 1967), of these the Rose Bengal Plate Test (RBPT), the Serum Agglutination Test (SAT) and the Complement Fixation Test (CFT) are routinely used (Alton *et al.*, 1975). Recently Enzyme-linked Immunosorbent Assay (ELISA) methods have been described for the diagnosis of brucellosis in cattle (Heck *et al.*, 1982, I—Ming Chen *et al.*, 1984, Cargill et al. 1984) and sheep (Ris *et al.*, 1984, Spencer and Burgess, 1984 and Worthington *et al.*, 1984). The ELISA was superior to the conventional methods for detecting infected animals but some lack of specificity was reported (Heck *et al.*, 1982, Cargill *et al.*, 1984). Other advantages of the ELISA, particularly over the CET is its ability to detect all classes of antibody, particularly the 1gG isotypes at minute levels (Nielsen *et al.*, 1983). Furthermore, the ELISA is a relatively simple primary binding direct method, unlike the CET which depends on to highly labile biological reagents suffers from and the occurence of anti-complementary serums.

The conventional methods for the serodiagnosis of swine brucellosis are limited by heterospecific antibody indistinguishable from anti-brucella IgM in the blood of non-infected swine. Furthermore, some infected swine are serologically non-responsive, or titre rapidly disappear following spontaneous recovery (Deyoe and Manthei, 1975). Thoen *et al.* (1980) reported an ELISA for swine brucellosis that was more effective in detecting infected animals than either the CFT or the card test, the REPT equivalent. Thus to investigate the sensitivity and specificity of an ELISA for the detection of brucella infection in swine, a serological and bacteriological study has undertaken in slaughter swine.

MATERIALS AND METHODS

Sample Collection from slaughter swine

All pigs were exotic breeds raised in Java. Most, if not all the males had been castrated. After electorcution individual pigs were eartagged to allow later identification, the sex noted and blood collected. Lymph nodes were collected into individual plastic bags then placed in dry ice immediately and returned to the laboratory and stored at—85°C until culture. The serum was separated by centrifugation and stored at—20°C until testing.

Bacteriology

Lymph nodes were thawed, fat tissues removed, and sliced into small pieces and mace-

rated in 5 volumes of phosphate buffer saline pH 7.2 in a stomacher (Colworth Laboratories, England). The macerated tissues were cultured on serum dextrose agar supplemented with bacitracin, polymixin B sulphate and cycloheximide. Representative colonies were subcultured, identified and biotyped by conventional methods (Alton *et al.*, 1975).

Conventional Serological Methods

Sera were tested by the Rose Bengal Plate Test (PBPT) (Anon, 1980) and the Complement Fixation Test (CFT) (Anon., 1977). The Serum Agglutination Test (SAT) was performed as a 5—well test in microtitre plates using 25 microlitre volumes of sera with a 1/5 dilution of Milk Ring Test Antigen (CSL, Victoria) as an antigen and adjusted to give a titre equal to that obtained with the tube test using the International Standard *B. abortus* serum. The results were expressed in International Unit of Antibody-European Method (Alton *et al.*, 1975).

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA was performed as a modification of the method described in the Manual, Enzyme-Linked Antiglobulin Test for Bovine Brucellosis, CSIRO Division of Animal Health, Parkville, Victoria, Australia.

Flat-bottomed microelisa plates (Dynatech 192A Imulon) were coated with 50 ul of a 1/50 dilution of the supernatant from a 10 times normal strength *Brucella abortus* antigen (CSL Victoria). Plates were incubated at 4^0 C overnight, washed 3 times with phosphate buffer saline containing 0.05% Tween 20 (PBST) before use. Test sera were diluted 200—fold in PBST, tested in duplicate, 50 microl in each of 2 wells. Each plate included 2—fold dilutions from 1/50 to 1/25,600 of a standard positive serum. The plates were incubated at room temperature for 1 hour then washed 3 times with PBST. Then 50 ul of a 1/500 dilution of Rabbit Anti-Swine IgG light and heavy chain horseradish peroxidase (RASW IgG H + L/PO, Nordic) conjugate was added to each well and the plates incubated for 1 hour at room temperature. The substrate was purified 5 amino salicylic acid (5—AS) used at a final concentration of 0.5 mg/ml. 1% H_2O_2, was added at 0.1 ml per 10 ml of 5 —AS. After 25 minutes incubation, the dark brown colour change was quantified by measuring the optical density at 450 mm using a single channel microelisa plate reader (Dynatech, Australia). As small plate to plate and day to day variations in colour development can occur, the reaction was expressed as the optical density ratio (ODR) of the mean optical density of the test sera to the standard positive control serum at a 1/200 dilution in each plate. An ODR of 0.18 was arbitrarily chosen as the threshold reaction, above which a serum could be considered suspicious or positive.

RESULTS

Isolation of *Brucella suis*

From 546 animals slaughtered at Kapuk and Surabaya abattoirs, a total of 103 *B. suis* isolates were obtained from 66 infected pigs. The frequency of isolation from submaxillary (SMLN), mediastinal (MDLN), retropharyngeal (RPLN) and mesenteric (MLN) lymph nodes is shown in Table 1. From 12 of the 21 infected animals sampled at Kapuk abattoir, *B. suis* was isolated from two or more sets of LN. From 15 of the 45 infected animals sampled at Surabaya, *B. suis* was isolated from both SMLN and RPLN. The isolates were all identified as *B. suis* biotype 1.

Table 1. The frequency of *Brucella suis* isolation from various lymph nodes collected from slaughter swine

Kapuk abattoir 37 isolates from 21 animals			Surabaya abattoir 61 isolates from 45 animals	
RPLN	MDLN	MLN	RPLN	SMLN
19 (51.4%)	12 (32.4%)	6 (16.2%)	27 (45.0%)	33 (55.0%)

Serological Tests

In the RBPT the reactions were recorded as 3, 2, 1 or negative. CFT titres of 1/4 and SAT titres of 60 IH were considered as positive. The results shown in Tables 2 and 3 compare the rusult of the CFT, SAT and ELISA at each of the RBPT reactions. Of the 66 culture positive animals, 56 were detected by ELISA.

In contrast, only 41, 27 and 25 animals were detected by the RPBT, SAT and CRT respectively (Table 2). Twenty culture positive animals had a RBPT reaction of 3 and these

animals were all detected by the ELISA, nineteen were detected by CFT and 7 were detected by SAT. Fourteen culture positive animals had a RBPT reaction of 2. All 14 were detected by ELISA, but only 5 by CFT and 6 by SAT. Seven culture positive animals had RBPT reactions of 1. All 7 were detected by ELISA but 0 and 3 of the animals were detected by CFT and SAT respectively. Most importantly, from the remaining 24 of the total of 66 culture positive animals, 15 were detected by ELISA and only 1 each by the CFT and SAT respectively and non by the RBPT. Thus 10 culture positive animals were not detected in any test, but the ELISA was clearly superior having a sensitivity of 84.8% compared with 62.1%, 37,9% and 40.9% for the RBPT, CFT and SAT respectively.

The comparative serological results for the 480 animals which were culture negative are presented in Table 3. A similar pattern of results to those recorded in the culture positive animals was observed with 58 animals reacting positively in the RBPT, 36 and 38 in the CFT and SAT respectively. One hundred and three animals had an ODR in the ELISA of 0.18. The specificity of the ELISA, calculated from the number of sera from culture negative animals giving a negative reaction, was slightly lower (78.0%) compared with the RBPT (87.9%), CFT (92.5%) and SAT (92.1%).

Table 2. The number of sera from 66 culture positive swine reacting in each serological test

Reaction	RBPT	CFT ⩾ 1/4	SAT ⩾60 IU	ODR > 0.18	ELISA Mean ± SD ODR		Range
3	20	19	17	20	0.94	0.14	0.56 — 1.16
2	14	5	6	14	0.85	0.18	0.49 — 1.09
1	7	0	3	7	0.81	0.21	0.29 — 1.01
Neg	25	1	1	15	0.75	0.25	0.05 — 0.95
Total	66	25	27	56			

Table 3. The number of sera from 480 culture negative swine reacting in each serological test

Reaction	RBPT	CPT ⩾ 1/4	SAT ⩾60 IU	ODR > 0.18	ELISA Mean ± SD ODR		Range
3	24	23	24	24	0.97	0.13	0.72 — 1.21
2	14	9	10	14	0.84	0.20	0.31 — 1.06
1	20	4	4	20	0.77	0.27	0.14 — 1.03
Neg	422	0	0	45	0.11	0.08	0.02 — 0.99
Total	480	36	38	103			

DISCUSSION

Brucella suis was most commonly isolated from the RPLN and SMLN in slaughter animals. In many cases there was a very heavy growth of the organism. These animals constitute a major source of contamination of the abottoir environment and infection of the workers which has already been observed at the Kapuk abattoirs.

On sensitivity of a serological test is defined as the percentage of culture positive animals with a positive test. The ELISA detected 85% of the culture positive animals tested thus giving 15% false negative reactions. On the other hand the RBPT gave 38% false negative reactions, with the SAT and CFT proving even less sensitive. Thus the ELISA is considerably more efficient in detecting *B. suis* infected animals. Our test failed to detect 10 (15%) of the culture positive animals and these results are consistent with the ELISA described by Thoen *et al.* (1980) which failed to detect 7.5% *B. suis* culture positive swine and that by Heck *et al.* (1982) which failed to detect 6% of *B. abortus* culture positive cattle. Of the 10 infected animals not detected by our ELISA, infection may have occurred the preslaughtered period before any serological response had occurred, or their titres fallen below detectable levels (Deyoe and Manthei, 1975).

The specificity of a test is defined as the percentage of non-infected animals with a negative test and gives an indication of the number of false positive reactions. Whilst there was a higher rate of apparent false positive reactors in the ELISA compared to the conventional tests, this may be attributable to the difficulty in demonstrating active infection, through undetectable numbers of infecting organisms and limited range of lymph nodes sampled. Much-higher false positive reactor rates were described by Cargill *et al.* (1985) in a *Brucella abortus* ELISA suggesting further that cultural methods cannot be relied upon to prove absence of infection. There is also the question of animals recovering spontaneously from active infection (Deyoe and Manthei, 1975) but remaining seropositive. Furthermore many animals in heavily infected environments may seroconvert without active infection having occurred.

The amount of IgG antibody required to react in a *B. abortus* ELISA is several thousand times less than that required in the conventional tests (Allan *et al.*, 1975, Nielsen *et al.*, 1983). This would account for the increased sensitivity of the ELISA over the conventional tests as much lower levels of antibody will be detected. Similarly a lower specificity may be the result. Thus a compromise is required where enhanced sensitivity may be associated with an "apparent" loss in specificity.

In a study by Brewer *et al.* (1983), pigs experimentally infected with *B. abortus* showed steady serological responses measured by ELISA over a 60 day observation period. In contrast, at certain points, considerable fluctuations were observed in the conventional tests. At such points "apparent" false positives would be detected by the ELISA.

Heterospecific antibody of the IgM class may also be responsible for false positive reactions in the conventional tests (Deyoe and Manthei, 1975). As the IgG conjugates normally used in the ELISA are highly specific (Hammerberg and Schuring, 1984, Brewer *et al.*, 1983) then it is unlikely that heterospecific IgM antibodies considered would be detected in our system.

The ELISA method described is simple, quick and more effective in identifying infected animals which are not detected in the conventional tests. It is the most reliable and consistent test for use as a herd screening test or to test individual animals in control or eradication programmes for swine brucellosis.

REFFENCES

ALLAN, G.S., R.J. CHAPPEL, P. WILLIAMSON and D.J. MCNAUGHT. 1975. A quantitative comparison of the sentivity of serological tests for bovine brucellosis to different antibody classes. J. Hyg. Camb. *76*, 287 - 298.

ALTON, G.G., I.M. JONES and D. PIETZ. 1975. Laboratory techniques in Brucellosis. 2nd ed. Geneva WHO Monograph series No. 55.

ANONYMOUS. 1977. Standardised Complement Fixation Test for Bovine Brucellosis. Aust. Vet. J., *53* : 394.

ANONYMOUS. 1980. Standardised Rose Bengal Test for Bovine Brucellosis. Aust. Vet. J., *56*, : 555.

BREWER, R.A., F.A. STEWART and M.J. CORBELL. 1983. An indirect enzyme-linked immunosorbent assay for the detection of antibodies to *Brucella abortus* in porcine sera. Br. Vet. J. *139*, 495 - 500.

BRINDLEY-MORGAN, W.J. 1967. The serological diagnosis of Bovine Brucellosis. Vet. Rec. *80* No. 21, 612 - 620.

CARGILL, C., K. LEE and I. CLARKE. 1985. Use of an enzyme-linked immunosorbent assay in a bovine brucellosis eradication programme. Aust. Vet. J. *62* (2); 49 - 52.

DEYOE, B.I. and C.A. MANTHEL 1975. Brucellosis. Chap 25 in Diseases of Swine 4th Ed. HW Dunne and A.D. Leman Iowa State University.

HAMMERBERG, C. and G.G. SCHURIG. 1984. The culturing of peripheral blood lymphocytes in microelisa wells for the direct measurement of a specific antibody response. Vet. Immunol. and Immunopath. *7* : 139 - 152.

HECK, F.C., B.I. DEYOE and J.D. WILLIAMS. 1982. Antibodies to *Brucela abortus* in sera from Strain 19 vaccinated and non-vaccinated cows as determined by enzyme-linked immunosorbent assay and conventional serologic methods. Vet. Immunol. and Immunopaths, *3*, 629 - 634.

I-MING CHEN, C.O THOEN, D.E. PIETZ and R. HARRINGTON. (1984). Application of enzyme-linked immunosorbent assay for detection of Brucella antigens in vaginal discharge of cows. Am. J. Vet. Res. *45* : 32 - 34.

MATHYAS, Z. and T. FUJIKURA. 1984. Brucellosis as a world problem. Proc. 3rd Int. Symp. on Brucellosis, Algiers, 1983 S. Karger, Basel : 3 - 20.

NEILSEN, K.H., F.C. HECK, J.M. STILLER and B. ROSENHAUM (1983). Interaction of specifically purified isotypes of bovine antibody to *Brucella abortus in* the haemolysis in gel test and enzyme-linked immunosorbent assay. Rs. Vet. Sci. *35*, 14 - 18.

PRIADI, A., R.G. HIRST, U. CHASANAH, A. NURHADI, J.J. EMMINS, M. DAROJAT and M. SOEROSO 1985. Animal Brucellosis in Indonesia - *Brucella suis* infection detected by an enzyme-linked Immunosorbent assay. Proc. 3rd AAAP Animal Science Congress, Seoul, Korea, 507 - 509.

RAY, W.C. 1980. Brucellosis (due to *Brucella abortus* and *B. suis)* in CRC Handbook series in Zoonoses, Section A : Bacterial, Rickettsial and Mycotic Diseases. 99 - 223.

RIS, D.R., K.I. HAMEL and D.L. LONG. 1984. Comparison of an enzyme-linked immuno specific assay with the cold complement fixation test for the serodiagnosis of *Brucella ovis* infection. N.Z. Vet. J. *32* : 18 - 20.

SPENCER, T.I. and G.W. BURGESS, 1984. Enzyme-linked immunosorbent assay for *Brucella ovis* specific antibody in ram sera. Res. Vet. Sci. *36* : 194 - 189.

THOEN, C.O., MARY P. HOPKINS, AMY I. AMBRUST, R.D. Angus and D.E. Pietz. 1980. Development of an enzyme-linked immunosorbent assay for detecting anti bodies in sera of *Brucella suis* -infected swine. Can. J. Comp. Med. *44* : 294 - 298.

WORTHINGTON, R.W., W. WEDDELL and M.E. PENROSE. 1984. A comparison of three serological tests for the diagnosis of *B. ovis* infection in rams. N.Z. Vet. J. *32* : 58 - 60.