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RESEARCH COMMUNICATION

Detection of *Brucella abortus* antigens by immunoperoxidase histochemical staining of lochia smears

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ABSTRACT

ALBERTS, J.N. & ERASMUS, J.A. Detection of *Brucella abortus* antigens by immunoperoxidase histochemical staining of lochia smears. *Onderstepoort Journal of Veterinary Research*, 62:147–150

An immunoperoxidase histochemical staining technique for the detection of *Brucella abortus* organisms in lochia of brucellosis-infected cows was described. In comparison with the standard culture technique, the former method resulted in 36% false positive and 2,3% false negative reactions, which represents a sensitivity of 82% and a specificity of 94%. False positive reactions are presumably the result of organisms which succumbed during transit. The feasibility of the test in the confirmation of brucellosis is discussed.

Keywords: *Brucella abortus*, immunoperoxidase histochemical staining, lochia

INTRODUCTION

Brucellosis caused by *Brucella abortus* remains an important cattle disease in some parts of the Republic of South Africa (Erasmus & Floor 1988; Erasmus 1995). The presence of large numbers of brucellae in lochia of infected cows at calving (Anon. 1977), but before expulsion of the placenta (McCaughey & Hanna 1973), indicates the suitability of this material for confirmation of an outbreak of brucellosis. The presence of brucellae in such a lochia sample can be demonstrated by culture on artificial media as well as by different staining techniques.

Staining of lochia smears by the conventional modified Ziehl-Neelsen (Stamp) technique (Alton, Jones, Angus & Verger 1988) is convenient, but experience is required for distinguishing organisms such as *Chlamydia* spp. and *Coxiella burnetii* from brucellae (Pienaar & Schutte 1994) when this method is employed (Anon. 1977). In known *Brucella*-infected herds in the Free State, an infected cow was identified on the basis of a positive lochia culture (Erasmus 1986). In this review, 16% Stamp-positive lochia smears were from non-infected animals (false positive tests) while 3,7% Stamp-negative smears were from infected animals (false negative tests) (Erasmus 1986). The high prevalence of false positive reactions could be attributed to organisms of this nature.

A direct fluorescent antibody test for the detection of *B. abortus* in lochia smears of cattle was described by Corbel (1973). Experiments in the Kroonstad veterinary laboratory, however, indicate that when this technique was employed, smears of lochia, transported in Stuart's transport medium (Oxoid), were not suitable for the detection of brucellae, because large globules of agar were present on the smears.

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Immunoperoxidase staining of *B. abortus* organisms on histopathological sections of infected lymph nodes was described previously (Meador, Tabatabai, Hagemoser & Deyoe 1986). As a minimum of approximately 10^6 colony-forming units of *B. abortus* per gram of tissue were needed to obtain a positive diagnosis after immunoperoxidase staining on histopathological sections of infected mouse spleen, this method appears to be a possible alternative for the demonstration of *B. abortus* organisms in lochia smears of infected cows. In this report the development of an indirect immunoperoxidase technique (IP) to identify *B. abortus* antigens in lochia smears is described and evaluated.

MATERIALS AND METHOD

Lochia samples

Material submitted for routine brucellosis diagnosis was used for this study. Lochia samples were received either in sterile containers or absorbed onto swabs and transported in Stuart transport medium. All samples were examined by bacteriological culture on *Brucella* medium base (Oxoid) containing *Brucella* selective supplement (Oxoid) and 10% bovine serum. Cultures were incubated in 10% carbon dioxide for up to 14 d at 37°C.

Preparation of antisera

Cultures of *B. abortus* strain 19 were inactivated by the addition of 0,5% formalin and kept at 37°C for 24 h and then at room temperature for 48 h. The concentrated cell suspension obtained was adjusted with normal saline solution, resulting in an absorbance of 0,3 at 540 nm. Rabbits were given two subcutaneous injections of the bacterin as follows: 1,0 mℓ of cells with Freund's complete adjuvant (Sigma), followed within 21 d by a 1,0-mℓ suspension of cells in Freund's incomplete adjuvant. Blood samples from these rabbits were collected by cardiac puncture 21 d after the final injection was given. These samples were tested by ELISA for the presence of antibodies to *B. abortus*. Rabbit IgG was prepared by precipitation in 33,3% saturated ammonium sulphate, followed by diethylaminoethyl (DEAE) cellulose ion-exchange chromatography with 0,02 M phosphate buffer (PBS), pH 7,2, as the elution buffer (Hudson & Hay 1989). Protein-containing fractions were pooled and stored at 4°C with 0,01% thiomersal as preservative.

Test procedure

Lochia smears were prepared on clean microscope slides and allowed to air-dry. After these smears had been fixed with acetone at room temperature for 2 min, they were rinsed twice in PBS. To inhibit non-specific binding, the smears were covered with PBS containing 1,0% goat serum and 3,0% bovine albumin (BSA) (Sigma) at room temperature for 10 min (Harlow & Lane 1988). After the last washing in PBS, rabbit anti-*B. abortus* IgG diluted 1:100 in 3,0% BSA-PBS was pipetted onto each smear. The reaction was allowed to develop in a moist atmosphere for 45 min at 37°C, after which the smears were washed in two changes of PBS containing 0,5% Tween 20 (PBST). Goat anti-rabbit IgG horseradish peroxidase conjugate (Zymed) diluted 1:150 in 3 % BSA-PBS was pipetted onto each smear. The smears were then incubated for 45 min at 37°C in a moist atmosphere. After the smears had been washed in two changes of PBST, they were allowed to react with freshly prepared aminoethylcarbazole (AEC) substrate at room temperature for 10 min. The reaction was stopped by washing them in distilled water. The preparations were then counterstained with Mayer's hematoxylin (Harlow & Lane 1988) for 4 min. After the surplus stain had been rinsed off in distilled water, the slides were dipped repeatedly into 30 mM of ammonium hydroxide until the stain turned blue (Harlow & Lane 1988). Then the smears were mounted in phosphate-buffered glycerol and examined microscopically.

Determination of the absolute specificity of the primary antibody

The absolute specificity of the *B. abortus* antibody was checked by performing IP staining on acetone-fixed smears of pure cultures of *B. abortus* strain 19, *B. abortus* field strain, *Staphylococcus aureus, Campylobacter fetus, Corynebacterium pseudotuberculosis* and *Chlamydia* spp.

Brucella abortus strain 19 as well as field strains of *Brucella* organisms stained red with AEC substrate when the IP technique was used with rabbit anti-*Brucella* IgG as primary antibody. No staining was observed with *S. aureus, C. fetus, C. pseudotuberculosis* and *Chlamydia* smears. Peroxidase-stained, *Brucella*-infected lochia smears showed the presence of organisms either as red-stained round to oval organisms or large clumps of stained bacteria. These structures were visible in neither uninfected lochia smears nor infected samples stained with PBS, or with normal rabbit serum only.

RESULTS

Results obtained in a series of examinations of lochia samples by culture and IP staining are summarized in Table 1.

The probability that an IP-positive sample originated from a brucellosis-infected case, was 64% (9/14), resulting in 36% false positive cases (Table 1). From

TABLE 1 Comparison of the results of the indirect immunoperoxidase (IP) staining and bacteriologic culture in the identification of *B. abortus* in lochia samples

IP	Culture		Total
	Positive	Negative	
Positive	9	5	14
Negative	2	83	85
Total	11	88	99

Chi-square: 7,36; $P < 0,01$
Sensitivity: 9/11*100 = 82%
Specificity: 83/88*100 = 94%

the same data it is also evident that 2,3% (2/85) false negative readings were obtained when the IP test was employed. The test had a sensitivity of 82% and a specificity of 94%.

DISCUSSION

Isolation of living brucellae from tissues or fluid samples has traditionally provided the most accurate method for the detection of *Brucella* infection; theoretically, a single viable *Brucella* organism in a sample can be detected (Alton, Jones & Pietz 1975; Mayfield, Bantle, Ewalt, Meador & Tabatabai 1990). In contrast, the detection limit for immunohistochemical techniques appears to approximate 10^6 organisms per g of tissue (Meador *et al.* 1986). The main feature of immunoenzymetic staining techniques, however, is that the procedure can be used to successfully localize *Brucella* and other antigens in formalin-fixed tissues (Meador, Hagemoser & Deyoe 1988). For this technique, therefore, the organism does not need to be alive in order to be detected (Haines & Clark 1991). According to Table 1, the IP test appears to be a suitable method for the detection of *Brucella*-infected cows from lochia samples.

False positive IP reactions, as revealed in Table 1, could be a direct result of cross reactions between the *B. abortus* antibody employed and organisms such as *Salmonella urbana,* particular serotypes of *Escherichia coli, Pseudomonas maltophilia* or *Yersinia enterocolitica* (MacMillan 1991), or they could be due to brucellae which succumbed during transit. As none of the former bacteria have been isolated from lochia samples from cattle by the local veterinary laboratories in the past, the presence of dead bacterial cells in the relevant samples may indicate an important reason for false positive reactions in the present test. The fact that samples stored in Stuart's transport medium were not necessarily stored at 4°C, nor transported to the laboratory without delay, could explain the high prevalence of false positive reactions.

There are two possible explanations for false negative IP results. Uterine discharges are not homogeneous and the small quantity taken for each smear may have contained insufficient organisms, or because of the predilection of *B. abortus* for foetal tissue, the levels of organisms in uterine discharges may have fallen to below the detection limit of the test after the expulsion of the placenta (McCaughey & Hanna 1973).

Because of the occurrence of false positive and negative test results and because IP does not identify species, strains or biotypes, this technique cannot replace culture techniques. Immunofluorescent labelling can be employed only when fresh or fresh-frozen tissue is being dealt with (Mayfield *et al.* 1991). In cases where brucellae succumb during transit, IP can be employed to detect infected brucellosis cases.

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