Evaluation of four serological tests for the diagnosis of caprine melioidosis

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SUMMARY: A complement fixation (CF) test, 2 indirect haemagglutination (IHA-A; IHA-L) tests which differed in antigen preparation and technique, and a microtitre agglutination (MA) test were compared in the serodiagnosis of melioidosis in goats. One hundred and eighteen experimental serums and 3143 field serums from goats in endemic and non-endemic areas of north Queensland were used in the evaluation. Culture of samples for *Pseudomonas pseudomallei* from 112 goats provided substantiating evidence of infection. The IHA-A test was the most sensitive, and the CF test the most specific. We advocate the use of the IHA-A as a screening test followed by the CF test for confirmation of active melioidosis. The IHA-A test is the better indicator of past infection.
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Introduction

Pseudomonas pseudomallei, the causative agent of melioidosis, is a soil saprophyte and opportunistic pathogen of man and animals (Chambon 1955; Howe *et al* 1971; Thomas *et al* 1979). In northern Queensland, there has been an increase in the human consumption of unpasteurised goats' milk. As *P. pseudomallei* has been isolated from both normal and mastitic goats' milk (Lewis and Olds 1952; Omar 1963; Thomas and Forbes-Faulkner, unpublished data), there is a potential public health risk.

Registration of dairy goat herds for freedom from melioidosis requires a reliable, quick and economic serological diagnosis. The serum agglutination test has been frequently used in the past, but its reliability has been questioned (Olds and Lewis 1954; Fournier and Chambon 1958; Ileri 1965; Malizia *et al* 1969). Ileri (1965) developed an indirect haemagglutination (IHA) test for goats, and Laws (1967) developed complement fixation (CF) and IHA tests for melioidosis in animals, including goats. These tests gave reliable results in naturally infected animals, but were not extensively evaluated.

This work was undertaken to evaluate 4 serological tests for melioidosis, using experimentally and naturally infected goats, and to examine the serological response of goats to experimental infection with *P. pseudomallei.*

Materials and Methods

For the purposes of this paper, goats from which *P. pseudomallei* has been isolated will be described as "culture positive" and goats from which *P. pseudomallei* was not isolated as "culture negative".

Goats

Experimental — Thirty-seven adult Saanen goats were used in a trial conducted at the Oonoonba Veterinary Laboratory to observe clinical and bacteriological reactions to subcutaneous infection with varying doses of *P. pseudomallei* (Thomas *et al* 1988). Eleven control goats were given injections of sterile saline. Twenty-six goats were inoculated with doses of *P. pseudomallei* ranging from 90 bacilli to $5x10^5$ bacilli. Of these, 15 goats (Table 1) developed clinical signs of disease and *P. pseudomallei* was isolated from 13 of these when killed for necropsy, 28 to 156 days after inoculation. The other 2 goats developing clinical signs of disease were culture negative at necropsy, 96 and 156 days after inoculation, although sterile lesions were found in the spleen and right prescapular lymph nodes (Thomas *et al* 1988). The remaining 11 inoculated goats and the 11 control goats showed no clinical signs of melioidosis and were culture negative at necropsy.

Field — Goats from domestic herds were distributed throughout areas of northern Queensland endemic or non-endemic for melioidosis (Figure 1). Feral goat serums were sent from the Prince of Wales Island. A domestic herd from Gayndah in southern Queensland was also tested.

Bacteriology — Methods for culture of clinical samples and identification of isolates have been previously described (Thomas *et al* 1988).

Serology

Tests — Serological tests evaluated were: the indirect haemagglutination (IHA-A) and complement fixation (CF) tests of Alexander *et al* (1970); the indirect haemagglutination (IHA-L) test of Laws (1967); and the microtitre agglutination (MA) test of Eurell *et al* (1979). All serums were tested by the 4 tests using doubling dilutions. The arbitrary minimum positive values used were 1/8 for the CF test, 1/40 for the IHA-A test, 1/10 for the IHA-L test, and 1/160 for the MA test. Titres were graded 0, or 1 to 4, depending upon the amount of haemolysis and/or buttoning at the bottom of the microtitre well in the CF, IHA-A and MA tests. In comparing the tests, only positive and negative results were used. Suspicious values were recorded as negative for this study, although values for suspicious readings are reported in routine field testing.

Antigen preparation and techniques — A local, serotype I isolate from a goat was used for inoculation of the experimental goats and in preparation of antigens. Local isolates, both serotype I, from a bird and a sheep, were also used to prepare antigens. Antigens and sensitised erythrocytes were prepared according to the respective authors listed in the previous paragraph. The 2 IHA tests were chosen to compare the difference in antigen preparations. The IHA-A antigen is prepared by growth of the isolate in a synthetic protein-free medium. The IHA-L antigen is grown on nutrient agar.

Techniques were used as recommended by the authors except for the CF test which was adapted to microtitre plates.

Serums

Serological response to melioidosis in goats — Serums (822) were collected from all 37 experimental goats. They were taken 3 times weekly for the first 3 weeks and then twice weekly until death or necropsy. Ninety serums from the 13 culture positive goats were used to determine the mean serological responses in goats to infection with *P. pseudomallei.* Mean titres were calculated on 13 serums on day 0 and at 7, 14, 21

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TABLE 1

Details of dose rate of *Pseudomonas pseudomallei* and number of trial days until necropsy for 15 goats showing clinical signs of melioidosis

Goat Number	Dose Rate	Necropsy (days after inoculation)	Number of tissues with Culture positive lesions	Culture negative lesions
1	$5x10^5$	60	3	0
2	$6x10^3$	51	4	0
3	500	104	3	4
4	500	34	12	0
5	500	34	5	0
6	500	34	7	0
7	500	96	0	2
8	500	28	4	0
9	225	31	14	0
11	225	156	3	2
13	225	31	9	0
14	225	31	5	1
17	225	31	13	0
18	90	156	0	2
19	90	71	3	2

and 28 days after inoculation; 5 serums at 35, 42 and 49 days after inoculation; 4 serums at 56 days after inoculation and 3 serums at 63 and 70 days after inoculation.

Evaluation of the 4 serological tests — Sensitivity and specificity values were calculated using the criteria of Fletcher *et al* (1982). The criteria for infected animals was culture positive lesions and not just clinical signs of melioidosis.

Sensitivity — Fifty-one necropsy serums (13 from experimentally infected goats and 38 from naturally infected goats) were used to determine diagnostic sensitivity values for the 4 tests. To determine the diagnostic sensitivity values of the 4 tests at the time of active infection, serums were collected from the 13 experimentally infected goats 28 days after inoculation.

Specificity — For the determination of diagnostic specificity, serums were collected at necropsy from 11 control goats inoculated with sterile saline and 48 field goats showing no signs of disease.

Comparison of the 4 tests — Five hundred and seventy-seven serums from goats with unknown melioidosis status, but from areas where melioidosis is endemic, were used to compare the 4 tests.

Choosing a serological regimen for melioidosis — Serums from 2,480 goats pastured in areas endemic or non-endemic for melioidosis were used to evaluate the most sensitive and the most specific of the 4 tests with a view to using them for routine screening and confirmation, respectively.

Comparison of goat, bird and sheep isolates of P. pseudomallei *as antigens* — Of the 237 positive serums collected from 13 experimentally infected goats, 130 were selected for retesting by all 4 tests using the antigens prepared from the sheep and bird isolates.

Results

Figure 2 shows the mean serological responses of the 13 goats experimentally infected with *P. pseudomallei.* Antibodies were detected by the CF, IHA-A and MA tests within 7 days of inoculation in goats receiving $\geqslant$500 bacilli and within 16 days of inoculation in goats receiving 90 to 225 bacilli. There was one exception. One goat that aborted 9 days after inoculation did not develop a positive titre in any test until 21 days after inoculation. Antibody detection by the IHA-L test was generally delayed until 16 to 25 days after inoculation. There was no IHA-L antibody response in 2 of the infected goats. Ten of the experimentally infected goats had an acute form of the disease and were all culture positive when killed for necropsy, 28 to 60 days after inoculation. CF and IHA-A titres were positive in all 10 goats; IHA-L and MA titres were positive in 8. The remaining 3 goats were killed in the chronic stage of the disease, 71 to 156 days after inoculation. *P. pseudomallei* was isolated from all. IHA-A, IHA-L and MA titres were positive in all 3 goats but the CF titre was positive in only 1.

The serum of goat 4 showed a transient negative titre ($<1/40$) to the IHA-A test between 10 and 21 days after inoculation. Serums of goats 6 and 11 had transient negative

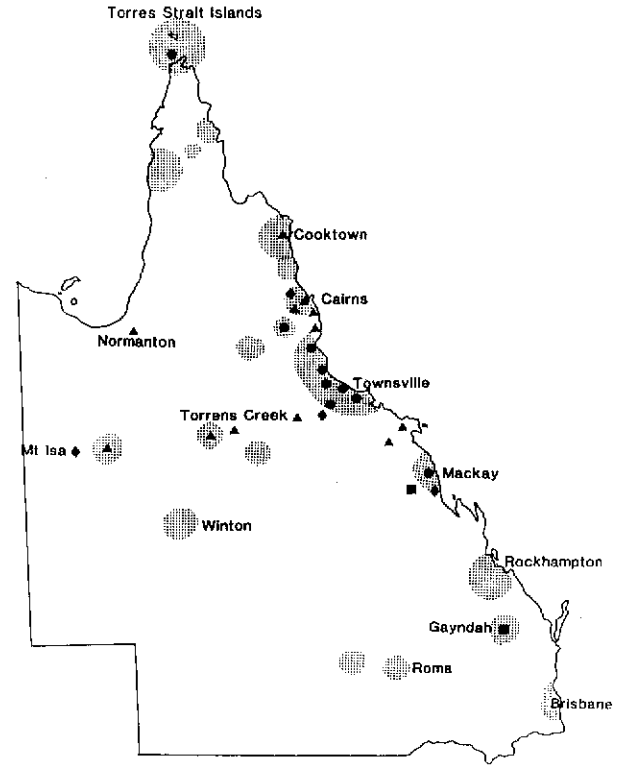

Figure 1: Distribution of melioidosis is Queensland.
▥ Serological or cultural positives recorded prior to this study — animals and man;
This study:
● Culture positive; IHA-A and CF tests positive.
■ Culture not done; IHA-A $\geqslant$ 1/160 and CF test negative.
♦ Culture not done; IHA-A 1/40-4/80 and CF test negative.
▲ Culture not done; IHA-A and CF tests negative.

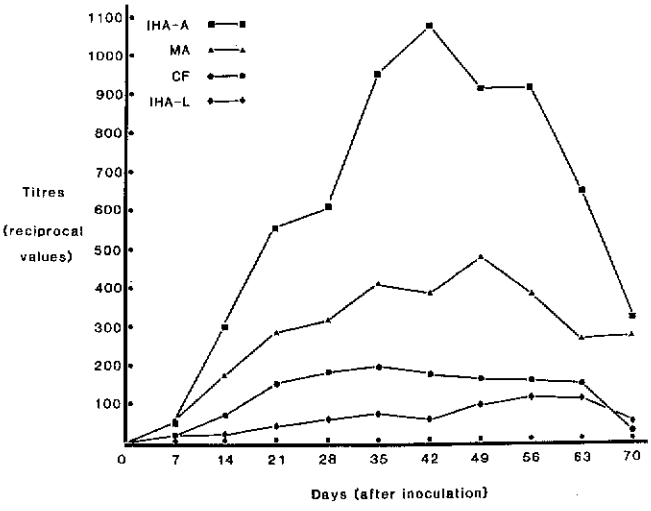

Figure 2: Mean serological responses to 2 indirect haemagglutination (IHA-A; IHA-L), complement fixation (CF) and microtitre agglutination (MA) tests for melioidosis in experimentally infected goats. As the goats were necropsied at various intervals during the trial, the mean response was calculated on 13 goats on day 0 and at 7, 14, 21 and 28 days after inoculation; 5 goats at 35, 42 and 49 days after inoculation; 4 goats at 56 days after inoculation and 3 goats at 63 and 70 days after inoculation.

TABLE 2

Sensitivity and specificity values for 2 indirect haemagglutination (IHA-A; IHA-L), microtitre agglutination (MA) and complement fixation (CF) tests foi caprine melioidosis

Test	28 days*		Necropsy†	
	SEN‡ (13)§	SPEC (11)	SEN (51)	SPEC (59)
IHA-A	100	100	98.0	92.5
IHA-L	84.6	100	88.2	98.8
MA	84.6	100	86.3	95.0
CF	100	100	82.4	100

* serums collected from 13 experimentally infected goats during active infection (sensitivity) or from 11 control goats at a similar period (specificity)

† serums collected from 13 experimentally and 38 naturally infected goats (sensitivity) and from 11 experimental and 48 field culture negative goats (specificity)

‡ SEN = Sensitivity; SPEC = specificity

§ number of serums

titres (<1/10) to the IHA-L test between 13 and 17 days and on day 77 after inoculation, respectively.

The diagnostic sensitivity and specificity values for the 4 tests are shown in Table 2. The most sensitive test based on sampling at necropsy of 51 goats was the IHA-A test. Table 3 shows the agreement between the IHA-A and the other 3 tests using 577 field serums.

To evaluate the IHA-A test as a sensitive screening test followed by the CF test as a specific confirmatory test, 2480 serums from domestic and feral goats were used. Of these, 2432 were screened as IHA-A negative. These were confirmed as negative by the CF test. Twenty of the 2432 goats were available for necropsy and all were culture negative. Forty-eight serums gave positive IHA-A titres of ⩾1/40. Fifteen of these were positive by the CF test. All 15 goats were available for necropsy and 14 of the goats were culture positive. IHA-A titres of the remaining 33 serums were 1/40 to 4/40 (27 serums), 1/80 (2) and ⩾2/320 (4). Two of the goats with IHA-A titres of ⩾2/320 were available for necropsy. Both were culture negative, although sterile abscesses were found in the spleen and/or prescapular lymph nodes.

The 130 serums repeat tested using the sheep and bird antigens consistently gave titres in all 4 tests within a 1 well variation of those titres obtained using the goat antigen.

The distribution of melioidosis titres and culture positive goats are shown on Figure 1.

Discussion

Both sensitivity and specificity are considered when selecting a testing regimen for the serodiagnosis of disease (Fletcher *et al* 1982). A rapid, sensitive screening test followed by a highly specific confirmatory test on any positive serums is a system often used (Dohoo *et al* 1986).

Alexander *et al* (1970) recommended the use of the CF and IHA-A tests for the serodiagnosis of human melioidosis. Our work indicated that the IHA-A test, with a sensitivity of 98.0% (Table 2), might be a useful screening test for caprine melioidosis with the CF test (specificity of 100%) as a confirmatory test. This was examined by testing 2480 field serums, of which 2432 (98.1%) were negative to both tests. Of the 15 (0.6%) serums reacting at positive titre in both tests, 14 were collected from goats that were cultured positive. Thirty-three (1.3%) serums were IHA-A positive, CF negative. Most of the IHA-A titres were low — 1/40 to 4/40 — and could have been due to cross-reactions with other pseudomonads or *Salmonella* spp (Nguyen-Ba-Luong 1961). Shields and Thomas (unpublished data) observed CF negative, IHA-A melioidosis titres from 1/40 to 1/160 in goats with confirmed cases of salmonellosis but culture negative for *P. pseudomallei*. This would explain the lower specificity value for the IHA-A test compared to the CF test (Table 2). One experimentally inoculated goat and 2 goats from domestic herds with CF negative, IHA-A ⩾2/320 titres were culture negative at necropsy, but had sterile lesions in the spleen and/or prescapular lymph nodes. Regression of melioidosis lesions with eventual sterility of abscesses is not uncommon in goats (Lewis and Olds 1952; Laws 1967; Thomas *et al* 1988). From our findings, IHA-A titres persist longer in the majority of surviving goats than do the CF titres, and the IHA-A test is a better indicator of past infection with *P. pseudomallei*.

Nigg (1963), Johnson (1967) and Laws (1967) reported that CF antibody did not persist for very long after melioidosis infection was resolved, and that persistence of a CF titre indicated persistence of infection (Nigg 1963). Howe *et al* (1971) suggested that the CF test was a highly sensitive and specific test for the diagnosis of active infection and subclinical disease in man. Our work has shown that the CF test indicates active infection. All 10 experimentally infected goats killed during the acute stage of the disease had positive CF titres. Only one of the 3 experimentally infected goats killed during the chronic stage of infection had a positive CF titre, although all 3 were CF positive at 28 days after inoculation. This result contributed to the difference between the sensitivity values for the CF test in Table 2. Melioidosis in goats in the field is most often a chronic disease (Laws and Hall 1963).

Agreement between the IHA-A test and the other 3 tests was high (Table 3) when evaluating 577 field serums. This was not unexpected as the majority of these serums were from clinically negative goats, even though the herds were from areas endemic for melioidosis. As seen in Table 2, the specificity values for all 4 tests were relatively high.

We would not recommend either the IHA-L or MA tests for routine diagnostic testing. Sensitivity values for both tests were low (Table 2) and this could be due to antigen preparation. Neither of these antigens were prepared in protein-free media and protein extracts can reduce sensitivity and/or specificity values (Malizia *et al* 1969; Alexander *et al* 1970). The IHA-L titres were delayed when compared to the other 3 tests, although they did tend to persist in the longer surviving goats as observed by Laws (1967). The IHA-L test is done on a glass slide with a rocking technique and can be time-consuming when dealing with large numbers of serums. The MA test is easy to perform and requires no addition of red cells. However, it is an overnight cold test which delays results and there are problems with subjectivity. Both sensitivity and specificity values are low (Table 2).

Alexander *et al* (1970) found transient titres to the CF and IHA-A tests in 19% of human cases, though rarely in the 2 tests simultaneously. Transient titres occurred in our study with the IHA tests. These were recorded in 3 of the 13 experimentally infected goats (23%). Delayed seroconversion in animals after abortion is not uncommon (Christie 1969; Worthington 1982).

Use of a *P. pseudomallei* isolate from goats is not neccesary for the diagnosis of caprine melioidosis. Antigens prepared from local sheep and bird isolates of *P. pseudomallei* gave similar results to the goat antigen.

None of the 4 tests gave both high sensitivity and specificity values at 28 days after inoculation and at necropsy. However, use of the sensitive IHA-A test for screening in conjunction

TABLE 3

Comparison of indirect haemagglutination (IHA-L), microtitre agglutination (MA) and complement fixation (CF) tests against an indirect haemagglutination (IHA-A) test for caprine melioidosis on 577 serums from goat herds in areas endemic for melioidosis

Tests			IHA-L		MA		CF	
			Pos	Neg	Pos	Neg	Pos	Neg
IHA-A	Pos	42	34	8	33	9	30	12
	Neg	535	2	533	0	535	1	534
			(98.3)†		(98.4)		(97.7)	

* Pos — positive; Neg — negative;

† ( ) — percentage agreement with the IHA-A test.

with the specific CF test on IHA-A positive serums provides a reliable procedure for the serodiagnosis of active melioidosis in goats. This is important in the accreditation of goat dairy herds. The lower sensitivity of the CF test in chronic infections must be recognised and goat serums with high IHA-A and negative CF titres should be regarded with suspicion and further testing implemented. Although this was not an epidemiological trial, plotting of our results on a distribution map of Queensland (Figure 1), showed that both tests, the IHA-A test in particular, could be valuable as epidemiological tools in the identification of areas endemic for *P. pseudomallei.*

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