QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES
DIVISION OF ANIMAL INDUSTRY BULLETIN No. 125

# MELIOIDOSIS IN ANIMALS IN NORTH QUEENSLAND. V. SEROLOGICAL METHODS OF DIAGNOSIS IN SHEEP

By L. Laws, M.V.Sc.

## SUMMARY

**A complement fixation (C.F.) test and a haemagglutination (H.A.) test were developed.**

**The C.F. test was done on 188 sera from 10 sheep inoculated with cultures of *Ps. pseudomallei*, 18 sera from 2 sheep inoculated with cultures of *Ps. aeruginosa*, 4,058 sera from 2,058 sheep in the endemic area, which included 882 samples from sheep obtained at slaughter at an abattoir, and 385 sera from sheep kept outside the endemic area.**

**Seventy-seven sheep from the endemic area were autopsied. *Ps. pseudomallei* was recovered from 13 of these and from 9 of the 882 sheep killed at the abattoir.**

**The C.F. test was of considerable value in the diagnosis of either acute or chronic melioidosis in sheep, a C.F. titre of 20 or more being indicative of active infection or recent recovery. In experimentally infected sheep detectable C.F. antibodies had not developed by day 3 or day 4 post inoculation although they were present on day 7.**

**The 2 sheep inoculated with cultures of *Ps. aeruginosa* did not develop C.F. antibody titres to *Ps. pseudomallei*.**

**Infection was not confirmed in 6 of the flock sheep that gave positive C.F. tests.**

**False negative reactions occurred in 4 naturally infected sheep. In 2 of these it appeared no antibodies had developed. Examination of the sera of the other 2 sheep and the sera of sheep experimentally infected with cultures of the strains of *Ps. pseudomallei* recovered from them demonstrated the existence of antigenically heterologous strains of *Ps. pseudomallei*.**

**The H.A. test was not used as extensively as the C.F. test. It was done on all 188 sera from the 10 sheep inoculated with cultures of *Ps. pseudomallei*, and on 191 sera from sheep in the endemic area which included sera from 18 infected sheep and sera from reactors to the C.F. test. A titre of 4 was indicative of infection or recent recovery and a titre of 2 suspicious of infection. The H.A. test was less sensitive than the C.F. test and haemagglutinins developed later than C.F. antibodies but persisted longer after recovery. The heterogeneity that existed with C.F. antigens did not exist with H.A. antigens.**

## I. INTRODUCTION

Since Whitmore and Krishnaswami (1912) first described melioidosis in man the natural disease has been diagnosed in horses, cattle, sheep, pigs, goats, dogs, cats, wild rats, laboratory animals, an orang-outang and a tree-climbing kangaroo. The disease was first reported in Queensland as an epizootic in sheep in 1949 (Cottew, Sutherland, and Meehan 1952), when 80 of a total flock of 4,000 died. By 1955 melioidosis had been diagnosed in five sheep flocks in tropical Queensland (Cottew 1955) and it seemed that melioidosis could become an important disease in sheep in tropical Queensland. As in this area approximately 6 million sheep are depastured, the necessity for a reliable diagnostic test was apparent.

Although Olds and Lewis (1954) showed that in goats the intradermal melioidin test was far more accurate than the agglutination test, a serological test was considered to be desirable because intradermal tests are difficult to perform in small ruminants, and there is usually a period following inoculation when retesting is impossible. Laws (1956) had shown that the agglutination test was unreliable in sheep. As limited work on a complement fixation (C.F.) test in man and laboratory animals (Cravitz and Miller 1950) and a haemagglutination (H.A.) test in man (Boyden 1950) suggested that these tests might be of value, these two techniques were examined for the diagnosis of melioidosis in sheep. The results of the studies are reported in this paper.

## II. MATERIALS AND METHODS

### (a) Source of Serum Samples

#### (1) Experimental Infection

(*a*) Ten Merino sheep (1-10) were each inoculated subcutaneously with 1 ml of a 24-hr broth culture of *Ps. pseudomallei* as described by Laws and Hall (1963). The following blood samples were collected from sheep 1 to 8: preinoculation, time of inoculation, day 3 or day 4 post inoculation, day 7 post inoculation, then generally at weekly intervals until the sheep died or were killed for autopsy (Laws 1964*a*). Samples were collected when the sheep were killed for autopsy. Blood samples were collected from sheep 9 and 10 at the time of inoculation and at autopsy on day 9 post inoculation. A total of 188 sera was examined from these 10 sheep.

(*b*) Two Merino sheep (11 and 12) were each inoculated subcutaneously with 1·5 ml of a 24-hr broth culture of *Pseudomonas aeruginosa.* A different strain was used for each sheep. Blood samples were collected at the time of inoculation, days 5, 12, 16, 19, 23 post inoculation, then weekly for 3 weeks.

#### (2) Natural Infection

(*a*) *Laboratory flocks.*—Small breeding flocks of Merino sheep were depastured at the Animal Health Station, Townsville, in the endemic area and at the Animal Research Institute, Brisbane, outside the endemic area.

(1) The sheep at the Animal Health Station, Townsville, were first examined in October 1956. By early July 1960 all the sheep in this flock (flock A) had been killed. Later in July 1960, flock B was introduced from southern Queensland. Between October 1956 and July 1960, flock A included a total of 65 sheep, and from these 1,465 serum samples collected at monthly intervals were examined. Flock B included a total of 48 sheep, and from these 525 monthly serum samples were examined between July 1960 and June 1961. Sixty-nine samples collected at autopsy of sheep in both flocks were also examined.

(2) The sheep kept at the Animal Research Institute, Brisbane, were bled in June 1957 (100 samples) and July 1960 (285 samples).

(*b*) *Commercial flocks, endemic area.*—(1) A small breeding flock of Merino sheep was maintained on farm P, 10 miles south of Townsville. The number of sheep in the flock fluctuated and a total of 80 sheep was examined. All sheep present were bled on three occasions and 68 were bled in December 1959, 66 in March 1960 (all bled previously) and 28 in April 1961 (16 bled on two previous occasions). Those positive to the C.F. test were killed for autopsy and further blood samples were collected.

(2) In January 1958 samples were examined from 119 Merino rams from a flock in the Julia Creek district.

(3) In August 1960 samples were examined from 64 Merino rams from a flock in the Hughenden district.

(4) In June 1961 a total of 800 samples was examined from Merino sheep on three widely separated properties in the Winton district. Three hundred of the samples were from a property where melioidosis had been diagnosed in sheep 5 years previously. The other 500 samples were from two properties where melioidosis had not been diagnosed previously in sheep. The two sheep on the first property that had reactions to the C.F. test were killed for autopsy and further samples were collected.

(*c*) *Flock sheep from the endemic area killed at abattoir.*—Between November 1959 and July 1960 a total of 882 samples was collected from sheep as they were slaughtered at the Townsville District Abattoir.

### (b) Testing of Sera

All sera were examined by the C.F. test. Generally the samples were examined within a week of collection and had been stored at 0–4°C.

Sera examined by the H.A. test were: (*a*) experimental infections, all 188 samples from sheep 1 to 10; (*b*) Animal Health Station flock, 36 sera with reactions to the C.F. test, 64 sera collected at autopsy of sheep and 44 sera collected from sheep in flock B in June 1961; (*c*) farm P, sera collected

at autopsy of 6 sheep with C.F. reactions; (*d*) Winton flocks, sera collected at autopsy of 2 sheep with C.F. reactions; (*e*) sheep killed at abattoir; 10 sera with reactions to the C.F. test, 4 sera with negative C.F. reactions but from sheep from which *Ps pseudomallei* was recovered, 25 sera from sheep with no gross lesions. The sera of sheep 1 to 8 and the sera of Animal Health Station flock sheep 142, 116, 189 and 136 were examined by the H.A. test after being stored at approximately—10°C for from 6 months to 3 years. When C.F. tests were done after the storage period, it was found the original C.F. titre had dropped. The remainder of the samples were examined by the H.A. test within a short period of being examined by the C.F. test.

### (c) Collection of Samples

Blood samples from living sheep were obtained by venipuncture of the jugular vein and were collected in sterile 1-oz pomade jars or 1-oz pill bottles.

Sheep were killed for autopsy by exsanguination. Samples of carotid blood were taken into 4-oz pomade jars and the required tissues were obtained for microscopic and bacteriological examinations.

At the abattoir sheep were also killed by exsanguination and blood was collected into labelled sterile 1-oz pomade jars. The carcass was identified immediately with a detachable label. Lesions detected at inspection were placed with the corresponding carcass labels in separate compartments of a disposable box.

### (d) Examination of Tissues

The techniques for bacteriological and histopathological examination of material from affected viscera, lymph nodes or central nervous system tissue, the identification of the *Ps. pseumonallei* strains recovered and the classification of the lesions have been described by Laws and Hall (1963) and by Laws (1964*b*).

### (e) Technique of the Complement Fixation (C.F.) Test

*Antigen preparation.*—The method was similar to that described by Van der Schaff (personal communication). A stock culture of *Ps. pseudomallei* grown overnight at 37°C in "Difco" nutrient broth was used to inoculate "Difco" nutrient agar plates. Five drops of the inoculum were spread over the entire surface of the medium with a sterile L-shaped glass rod. The plates were incubated at 37°C for 72 hr. Several smears were prepared from the growth on each plate and stained by Gram's method to determine whether any of the plates were contaminated. Then the growth was scraped off the medium with a sterile spatula and the pooled organisms from the plates suspended in 20 ml of 0·5% phenol in sterile 0·85% saline. The organisms were killed by heating at 65°C for 40 min and the suspension placed in the refrigerator overnight. Next day several

drops of the suspension were sown onto a 10% sheep blood agar plate and into nutrient broth and inoculated into a guinea pig to check for the presence of viable organisms. If the suspension was sterile the organisms were disintegrated by shaking. Initially this was done by shaking the suspension for 24 hr with large glass beads on a mechanical shaking device, but in all antigens prepared after July 1959 the cells were disintegrated by shaking the suspension for 30 min with Ballotini beads No. 12 on a Mickle shaking device. Following disintegration the preparation was centrifuged at 3,000 r.p.m. for 30 min and the supernatant withdrawn and stored in a refrigerator for use as antigen.

*Titration of antigen.*—Before use, antigens were titrated to determine potency and were checked for anticomplementary and haemolytic properties and then a series of tests with known positive and negative sera were done in parallel with a known satisfactory antigen. The potency of the antigen was determined by the titration method of Campbell and Turner (1936). The Antigen Unit (A.U.) was the amount of antigen contained in unit volume of the highest dilution giving complete fixation with 2·5 minimal haemolytic doses (M.H.D.) of complement in the presence of a positive serum. It was found that antigen used at a concentration of 2 A.U. gave incomplete fixation but results were satisfactory with 4 A.U. In the test 4 A.U. of antigen were used. At this concentration all antigens used complied with the arbitrary rule of Kabat and Mayer (1961) "that a positive C.F. test is considered valid only if antigen and antiserum tested separately do not display anti-complementary action when used in twice the concentration as in the test proper."

*Diluent.*—Cohen's diluent (Cohen *et al.* 1953) was used.

*Haemolytic system.*—This consisted of a 3% suspension of washed sheep red blood cells sensitized at room temperature for 30 min with $6HD_{50}$ haemolysin. The haemolysin (glycerinated) was prepared by the Commonwealth Serum Laboratories. It was retitrated in the laboratory before use. Stored cells which had been obtained by aseptically collecting blood from three sheep into an equal volume of modified Alsever's solution (Chanock and Sabin 1953) were used. The 3% suspension of cells was standardized colorimetrically to contain 0·5 g of haemoglobin/100 ml.

*Complement.*—Serum from 4–12 adult male guinea pigs was collected either by exsanguination after stunning or by heart puncture under anaesthesia and was preserved by the method of Richardson (1941).

*Titration of complement.*—Each day on which the C.F. test was done the complement was titrated to determine the M.H.D. (A. W. Turner personal communication 1958). In the test, 2·5 M.H.D. were used.

*Inactivation of sera.*—One millilitre of a 1 in 10 dilution of serum in diluent was heated in tubes in a water-bath at 56°C for 30 min. Sera were inactivated on the day of the test.

*Test proper.*—A unit volume of 0·25 ml was used and the test carried out in rimless pyrex tubes 12 mm x 75 mm. One volume of a doubling series of dilutions commencing at 1 in 10 was placed in a series of tubes (routinely 3) followed by one volume of complement (2·5 M.H.D.) and one volume of antigen (4 A.U.). A serum control containing one volume of 1 in 10 serum, one volume of complement and one volume of diluent was included. After incubation in a water-bath at 37°C for 30 min (shaking every 10 min) one volume of sensitized red cells was added to all tubes. After a further incubation for 30 min (shaking every 10 min) the tests were read immediately. At least one negative, one weak positive and one strong positive serum tested to titre were included with each day's tests.

*Interpretation.*—Degrees of fixation were graded 4, 3, 2, 1, tr, neg (4, complete fixation; 2, 50% fixation; neg, complete lysis of the cells; other readings intermediary between these). All tubes in which there was fixation were centrifuged and the supernatant visually compared with a tube containing a standard corresponding to 50% lysis of cells. Titres were expressed as the reciprocal of the highest dilution of serum in which there was 4 (i.e. complete) fixation.

Laws (1964*a*) showed that the most satisfactory interpretation was: a titre of 20 or greater, positive; 3 or 4 fixation at 1 in 10 with 2 or 3 fixation at 1 in 20 serum dilution, suspicious; less than 50% fixation at 1 in 20 with any degree of fixation at 1 in 10 serum dilution, negative.

### (f) Technique of the Haemagglutination (H.A.) Test

*Antigen preparation and titration.*—A stock culture of *Ps. pseudomallei* grown overnight at 37°C in Difco nutrient broth was sown onto Difco nutrient agar "flats" in 4-oz McCartney bottles and was spread by tilting the bottle backwards and forwards. These were incubated for 72 hr at 37°C with the agar side of the bottle down. The growth was washed off with sterile 0·85% saline (10 ml per bottle) and smears stained with Gram's method to detect whether any of the cultures were contaminated. The suspensions were pooled in an air-tight bottle, heated in a steamer for 2 hr and checked when cool for viable organisms as described in the preparation of C.F. antigen. The suspension was centrifuged at 3,000 r.p.m. for 30 min and the supernatant without preservative stored in the refrigerator for use as antigen.

The antigen was not titrated quantitatively before use but was used in excess for adsorption. Generally with this method of preparation 10% antigen in a 3% suspension of red cells was satisfactory. Before any new batch of antigen was used routinely, parallel tests were done on known positive and negative sera with the current antigen and the freshly prepared antigen in various concentrations, to determine the minimum concentration of antigen necessary for adsorption.

*Diluent.*—Cohen's Diluent (Cohen *et al.* 1953) was used.

*Red cells.*—Sheep red cells were collected, stored, washed and a 6% suspension prepared as for the C.F. test. For adsorption of the antigen by the red cells, a volume of diluent containing excess antigen was added to an equal volume of 6% red cells. After thorough mixing, the resultant suspension of 3% cells was incubated in a hot-air incubator for 1 hr at 37°C. It was shaken every 10 min. The suspension was centrifuged, the supernatant was discarded and the red cells were resuspended in an equivalent volume of diluent.

*Inactivation and absorption of sera.*—Sera diluted 1 in 2 in diluent were inactivated at 56°C for 30 min in a water-bath. The tubes contained 1 ml of fluid. On cooling, the sera were absorbed with one-fifth their volume (0·2 ml) of washed packed sheep red cells for 1 hr at 37°C in a water-bath. The sera were centrifuged to pack the red cells. Beginning at the dilution of 1 in 2, serial doubling dilutions of sera in diluent were prepared in tubes. In routine tests six dilutions 1 in 2 to 1 in 64 were prepared.

*Agglutination of red cells.*—A rectangular piece of flat plate glass approximately 36 cm by 25 cm was divided by lines into squares approximately 2 cm square. One drop of each dilution of a serum was placed in a separate square in a row. A second drop of 1 in 2 dilution of serum was included in the row. The latter acted as a serum control and to it was added one drop of 3% unsensitized red cells. To the other drops of serum was added one drop of 3% sensitized red cells. The cells were suspended evenly throughout the fluid by gently backwards and forwards shaking of the plate for a few seconds then kept swirling by a rotary movement of the plate for 2 min and the reactions read at this time. After each batch of sera was tested the plate was washed in running tap water, rinsed in running distilled water and dried with a glass-cloth.

A negative serum, a weak positive and a strong positive serum were included as controls. In addition, the sensitized red cells were tested for auto agglutination.

The haemagglutinin titre of a serum was expressed as the reciprocal of the highest dilution of serum in which there was complete agglutination of red cells. The dilution was expressed as the dilution of a serum before the addition of an equal volume of red cells.

Agglutination was read as 4, 3, 2, 1 and negative (4, complete clumping of red cells; 2, clumping of 50% of the red cells; negative, no clumping of red cells; other readings between these).

*Strains of Ps. pseudomallei used for antigen preparation.*—Four strains of *Ps. pseudomallei* were used for antigen production, viz strain "O" (Case 1, Lewis and Olds 1952), strains J53 and G91 isolated from sheep killed at the abattoir (Laws and Hall 1963) and a National Collection Type Culture (N.C.T.C.) strain 8018 from a rat.

Unless otherwise stated, the antigen used in all tests was prepared from strain "O".

## III. RESULTS

### (a) Experimental Infection

#### (1) Sheep 1 to 10

The course of the disease, autopsy findings and bacteriological examination of these 10 sheep have been described by Laws and Hall (1963).

In both the C.F. and H.A. tests the sera of sheep 1 to 8 were tested with antigen "O" only, and the sera of sheep 9 and 10 were tested with antigens prepared from the four strains of *Ps. pseudomallei.*

*C.F. test.*—All the samples collected from sheep 1 to 8 up to day 3 or 4 post inoculation were negative to the C.F. test, but the samples collected on day 7 from each of these 8 sheep had a titre of 20 or higher. During the course of the disease these titres increased and the maximum titres for these sheep were respectively 160, 320, 160, 640, 80, 80, 320, 80. The sheep (3 to 8 inclusive) which had acute melioidosis maintained these maximum titres until they died or were killed for autopsy between days 17 and 29 post inoculation. Both sheep 1 and 2 developed chronic melioidosis and at autopsy *Ps. pseudomallei* was recovered from sheep 2 but not from sheep 1. Sheep 1 maintained a titre of 20 or higher to week 53 post inoculation, then it had a suspicious or negative reaction to week 98, when it was killed for autopsy. Sheep 2 had a titre of 40 or higher to week 28, a titre of 20 to week 44, then a titre of 40 to week 52, when it died.

The sera collected at the time of inoculation of sheep 9 and 10 gave negative reactions with all four antigens. The sera collected from both sheep at autopsy on day 9 post inoculatiton gave negative reactions with antigens "O" and N.C.T.C. 8018, but that from sheep 9 had a titre of 40 with antigen G91 (homologous) and a titre of 20 with antigen J53, and that from sheep 10 had a titre of 40 with antigens J53 (homologous) and G91. *Ps. pseudomallei* was recovered from lesions in sheep 9 and 10.

*H.A. test.*—All samples collected pre-inoculation and at the time of inoculation of sheep 1 to 10 gave negative reactions. In sheep 1 to 7 a haemagglutinin titre of 4 or higher was first detected at the week post inoculation as indicated: sheep 1 (1), sheep 2 (4), sheep 3 (3), sheep 4 (2), sheep 5 (2), sheep 6 (1), sheep 7 (2). These titres increased as the disease progressed and the maximum titres were respectively 16, 64, 16, 64, 16, 8, 4. In the acutely affected sheep (3 to 7 inclusive) these titres were maintained until the animals died or were killed for autopsy. At the time of autopsy of sheep 1 and 2 the haemagglutinin titres

were 4 and 16 respectively. Both these sheep developed chronic melioidosis and at autopsy *Ps. pseudomallei* was recovered from sheep 2 but not from sheep 1. No haemagglutinins were detected in sera of sheep 8 even though *Ps. pseudomallei* was recovered from lesions examined at autopsy on day 28 post inoculation. No haemagglutinins to any of the four antigens were detected in the serum of sheep 9 collected at autopsy. The autopsy serum sample of sheep 10 had a haemagglutinin titre of 32 with antigens "O", G91, and J53 and a titre of 16 with antigen N.C.T.C. 8018. *Ps. pseudomallei* was recovered from lesions in sheep 9 and 10 detected at autopsy on day 9 post inoculation.

**(2) Sheep 11 and 12**

None of the serum samples from these sheep showed C.F. antibodies to *Ps. pseudomallei.* In both sheep an oedematous swelling developed at the site of inoculation of the cultures of *Ps. aeruginosa* but the swelling had regressed by day 10 after inoculation.

**(b) Natural Infection**

**(1) Laboratory Flocks, Animal Health Station, Townsville**

*C.F. test.*—Of the 1,465 monthly serum samples from the 65 sheep in flock A, 19 samples from 11 sheep had a positive reaction (Table 1), 20 samples from 15 sheep a suspicious reaction and the remaining 1,436 samples gave a negative reaction. Of the sera collected at autopsy of these 65 sheep, 9 had positive reactions (Table 1), 1 a suspicious reaction, and the other 53 negative reactions. *Ps. pseudomallei* was recovered from the 9 sheep with positive reactions. No *Ps. pseudomallei* was recovered from the other 56 sheep but 38 had multiple lesions due to *Oesophagostomum* sp. (4 of these sheep also had abscesses due to *Corynebacterium pyogenes*), 1 had an abscess due to *C. ovis,* 6 had bacteriologically sterile abscesses, and there were no gross lesions in the other 11 sheep.

Except in four instances the suspicious C.F. reactions occurred during the "wet" months of the year and at a time when melioidosis was present in other sheep in the flock.

Of the 525 monthly serum samples examined from the 48 sheep in flock B, only two from two sheep gave a positive result (Table 1). At autopsy these two sheep had positive reactions and *Ps. pseudomallei* was recovered. Only two other sheep in flock B were autopsied. Both had negative reactions. One had an epithelioma and the other a foot abscess due to *C. pyogenes.*

*H.A. test.*—Table 1 shows the H.A. reactions of the 12 sheep in flock A and the 2 sheep in flock B with positive C.F. reactions. One serum with a suspicious C.F. reaction had a haemagglutinin titre of 2, and one C.F. negative autopsy serum of a sheep in flock A had a titre of 4. The other 120 sera examined by the H.A. test had negative reactions.

**TABLE 1**

RESULTS OF THE EXAMINATION OF THE 14 SHEEP WITH POSITIVE C.F. TESTS: ANIMAL HEALTH STATION, FLOCKS A AND B

| Sheep No. | Serology Monthly Samples§ | | | Date Sheep Killed | Serology at Autopsy | | Abscesses Present at Autopsy | *Ps. pseudomallei* Recovered |
|---|---|---|---|---|---|---|---|---|
| | Date | C.F. | H.A. | | C.F. | H.A. | | |
| 109 | 14.xi.56 | 20 | N.D. | 20.xi.56 | 20 | N.D. | + | + |
| 141 | 7.xi.56 | 80 | N.D. | 7.xi.56 | 80 | N.D. | + | + |
| 108 | 8.x.56 | 160 | N.D. | 31.x.56 | 160 | N.D. | + | + |
| 145 | 21.i.57 | 40* | N.D. | 22.i.57 | 160 | N.D. | + | + |
| 142 | 17.ii.57<br>27.ii.57 | 40<br>40 | N.D.<br>4 | 28.ii.57 | 40 | N.D. | + | + |
| 116 | 24.ii.59<br>24.iii.59 | 20<br>20 | —<br>— | 29.iii.59 | 20 | — | + | + |
| 189 | 24.ii.59<br>24.iii.59 | 40*<br>40* | 4<br>4 | 29.iii.59 | 40* | 4 | + | + |
| 197 | 26.i.60 | S | 2 | 29.i.60 | 40 | — | + | + |
| 187 | 23.iii.60<br>26.iv.60<br>25.v.60<br>9.vi.60 | 40*<br>40*<br>40*<br>40* | 2<br>4<br>4<br>4 | 21.vi.60 | 160 | 4 | + | + |
| 207† | 23.iii.61 | 40 | — | 27.iii.61 | 40 | — | + | + |
| 243† | 23.iii.61 | 20 | — | 27.iii.61 | 20 | — | + | + |
| 136 | 21.iv.59 | 20 | — | 24.vi.59 | — | — | — | — |
| 201 | 23.iii.60<br>26.iv.60<br>28.v.60 | 40<br>40<br>20 | —<br>—<br>— | 21.vi.60 | — | — | + | — |
| 173 | 26.iv.60 | 20 | — | 21.vi.60 | — | — | + | — |

§ All other monthly samples from these 14 sheep gave negative C.F. reactions.
* Not tested at a higher dilution. N.D. = Not done.
S Suspicious reaction. — = Negative reaction.
† Nos. 207 and 243 were from flock B; the remaining 12 were from flock A.

## (2) Laboratory Flock, Animal Research Institute, Brisbane

All 385 sera were negative to the C.F. test. No H.A. tests were done. Several sheep from this flock were killed for autopsy in other experiments but none showed evidence of melioidosis.

### (3) Commercial Flocks, Endemic Area

The results of the examination of sheep in these flocks are shown in Table 2.

**TABLE 2**

SEROLOGICAL, PATHOLOGICAL AND BACTERIOLOGICAL EXAMINATION OF SHEEP IN THE COMMERCIAL FLOCKS, ENDEMIC AREA

| Flock Identification | Number of Sheep Tested | Identification Sheep Reactors C.F. Test | Reactions C.F. Test | Subsequent Examination of Sheep Reactors, C.F. Test | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Days till Autopsy | Serology at Autopsy | | Abscesses Present at Autopsy | *Ps. pseudomallei* Recovered |
| | | | | | C.F.* | H.A. | | |
| Farm P .. | 163 | T 916 | 40 | 15 | 20 | — | + | — |
| | | T 919 | 40 | No autopsy | | | | |
| | | T 920 | 40 | 15 | 40 | 4 | + | — |
| | | T 940 | 20 | 15 | S | — | + | — |
| | | T 948 | 20 | 15 | 40 | 4 | + | + |
| | | T 952 | 20 | 39 | 20 | — | + | — |
| | | T 965 | 20 | 39 | 20 | -- | + | + |
| Julia Creek | 119 | Nil | | | | | | |
| Hughenden | 64 | Nil | | | | | | |
| Winton .. | 800 | 305 | S | 28 | — | — | — | — |
| | | 666 | 20 | 28 | 20 | 4 | — | — |

* 1 in 40 highest serum dilution tested.

S Suspicious reaction.

### (4) Flock Sheep from the Endemic Area Killed at Abattoir

*Ps. pseudomallei* was recovered from 9 of the 882 sheep killed at the Townsville District Abattoir. Six other sheep had C.F. reactions to *Ps. pseudomallei*. The results of the serological ("O" antigen), pathological and bacteriological examinations of these 15 sheep are shown in Table 3. The remaining 867 sheep had negative C.F. reactions for melioidosis. The lesions detected in these sheep were: 158 abscesses from which *C. ovis* was recovered, 1 abscess from which *Pasteurella multocida* was recovered, 66 bacteriologically sterile abscesses, 125 small grossly calcified lesions in the viscera which were not examined bacteriologically, 18 hydatid cysts, 28 lesions due to *Oesophagostomum* sp. infestation, 8 *Cysticercus* spp. cysts, 1 neoplasm of the liver, and 1 pneumonia from which an unclassified Gram-negative bacillus was recovered.

**TABLE 3**

RESULTS OF THE SEROLOGICAL ("O" ANTIGEN), PATHOLOGICAL AND BACTERIOLOGICAL EXAMINATIONS OF THE 9 INFECTED SHEEP AND THE 6 OTHER SHEEP WITH C.F. REACTIONS ("O" ANTIGEN) KILLED AT THE TOWNSVILLE DISTRICT ABATTOIR

| Sheep No. | Owner | C.F.* | H.A. | Pathology and Bacteriology |
|---|---|---|---|---|
| F67 | R | 20 | — | *Ps. pseudomallei* recovered |
| F69 | R | 20 | 4 | *Ps. pseudomallei* recovered |
| G17 | A | 40 | — | *Ps. pseudomallei* recovered |
| D88 | A | 40 | 2 | *Ps. pseudomallei* recovered |
| DS46 | B | 40 | — | *Ps. pseudomallei* recovered |
| G12 | A | — | — | *Ps. pseudomallei* recovered |
| G91 | B | — | 2 | *Ps. pseudomallei* recovered |
| G92 | B | — | — | *Ps. pseudomallei* recovered |
| J53 | B | — | 4 | *Ps. pseudomallei* recovered |
| D56 | R | 40 | — | *C. ovis* recovered from abscesses |
| D46 | A | 20 | N.D. | No visible lesions |
| G33 | R | S | — | Abscess 0·2 mm in liver—nil bacteria |
| G50 | SW | S | — | Calcified lesion 0·2 mm in liver—not cultured |
| G51 | SW | S | 2 | No visible lesions |
| H29 | A | S | — | No visible lesions |

* 1 : 40 was the highest dilution of serum tested

N.D. = Not tested

S = Suspicious reaction

— = Negative reaction

The sera of infected sheep G12 and G92, which had negative C.F. reactions with antigen "O", also gave negative results when examined with antigens G91, J53 and N.C.T.C. 8018. The serum of infected sheep G91 was anticomplementary when tested with these three antigens. The serum of infected sheep J53, which had a negative C.F. reaction with antigen "O", had a C.F. titre of 40 with the homologous antigen J53.

Twenty-five sera from sheep with negative C.F. reactions and with no gross lesions were examined by the haemagglutination test. One had a titre of 2; the other 24 gave negative reactions. The sera of infected sheep G12, G91 and G92 were examined in the H.A. test with antigens G91, J53 and N.C.T.C. 8018, as well as with antigen "O". Serum G91 had a H.A. titre of 2 with all antigens, but G92 and G12 gave negative reactions with all antigens.

## IV. DISCUSSION

The results of the C.F. test on sera of sheep 1–8 experimentally infected indicated that this test was of considerable aid in the diagnosis of either acute or chronic melioidosis in sheep. In the acute disease there was a rapid rise in complement-fixing antibodies, antibodies being present at 7 days but not at 3 or 4 days after inoculation. That this was a satisfactory test was supported by the results of the regular monthly testing of sera from sheep in the laboratory flocks

which were kept in the endemic area. In both the experimentally infected sheep and naturally infected sheep in the laboratory flocks, *Ps. pseudomallei* had been recovered from animals with a C.F. titre of 20 or higher, and Laws (1964*a*) considered that a titre of this order in single samples at least was indicative of infection and was termed a positive reaction.

Six flock sheep in which infection was not confirmed had positive reactions to the C.F. test. Three of these had bacteriologically sterile abscesses in the viscera or lymph nodes, two had no visible lesions, and in the sixth sheep there were abscesses due to *C. ovis*. The three sheep with bacteriologically sterile abscesses and one of the sheep with no visible lesions were from flocks where there were active cases of melioidosis. It would seem that these four sheep had been exposed to *Ps. pseudomallei* and the titres were persisting after recovery from infection. The antigenicity of the strain of *C. ovis* recovered from the sheep with a positive C.F. reaction to *Ps. pseudomallei* was not studied further, but 157 other sheep killed at the abattoir, many from the same flock as the C.F. positive sheep, had *C. ovis* abscesses and all were negative to the C.F. test.

Delbove and Reynes (1942) and Wetmore and Gochenour (1956) have stressed the close relationship between *Ps. aeruginosa* and *Ps. pseudomallei*. The sera of sheep 11 and 12 inoculated with cultures of *Ps. aeruginosa* did not develop C.F. antibodies detectable by *Ps. pseudomallei* antigen. Cook (1962) reported cross reaction serologically with *Ps. pseudomallei* and *Ps. aeruginosa* and found false positive reactions to melioidosis in marsupial sera. He reported that several of these marsupial sera gave positive reactions when tested with a similar *Ps. aeruginosa* antigen prepared by me from the strain of *Ps. aeruginosa* used to inoculate sheep 12.

It was not known how long C.F. antibodies persisted following recovery from infection, but the results of the examination of sheep 201, 136, 173 and T940 suggested the period could be as short as 4 weeks.

Except in one sheep, all the suspicious reactions in the Animal Health Station flock occurred in one monthly sample only. In addition, most occurred during the "wet" months of the year when there were known infected sheep in the flock. Laws and Hall (1964) have described the recovery of *Ps. pseudomallei* from swamp water in Townsville during this period of the year and discussed the possibility of infection of animals from the soil reservoir during this time. These "fleeting" reactions in sheep might represent antibody response to abortive infections from *Ps. pseudomallei* in the soil.

From the results of the experimentally infected sheep the C.F. titre developed between the fourth and seventh days after inoculation. This indicated that, in natural infections, if samples were taken early in the infection false negative reactions could result. This can be seen in Animal Health Station sheep 197, in which there was a rise in titre from suspicious to 40 in 3 days. When no infected sheep were detected in the laboratory flock at slaughter, it was believed that false negative reactions were not occurring. Table 3 shows that four infected sheep killed at the abattoir had negative C.F. reactions. It was not possible to establish

the time of infection of these sheep, but in three of them (G12, G91 and G92) the lesions appeared of recent origin, and in the other (J53), in which there were multiple abscesses in the lungs and kidney, it seemed the animal had been infected for some time. Although sheep G12 and G92 were not tested with homologous antigen they gave negative C.F. reactions to all four antigens used and they were from flocks in which other infected sheep gave positive reactions to the C.F. test for melioidosis. This suggested that the negative results in these two sheep were false negatives because of no serum C.F. antibody development. When the serum of sheep G91 was tested with the homologous antigen the serum was anticomplementary, but the serum of sheep J53, negative with antigen "O", had a C.F. titre of 40 with the homologous antigen. This showed that in the C.F. test there were heterologous strains of *Ps. pseudomallei*. This was supported further by the examination of sera of sheep 9 and 10 inoculated with cultures of *Ps. pseudomallei*. The reports of both Alexander, Griffin, and Gochenour (1955) and Chambon and Fournier (1956*b*) showed that *Ps. pseudomallei* was not a serologically homogeneous species. Leclerc and Sureau (1956) isolated a bacteriophage which appeared to be specific for *Ps. pseudomallei* and which lysed all strains of *Ps. pseudomallei* isolated in North Vietnam but only some of those isolated in South Vietnam, thus demonstrating the heterogeneity of this organism.

In sheep 2 the C.F. titre showed an initial rise, then a fall, and a second rise for 7 weeks before it died, and *Ps. pseudomallei* was recovered from tissues. This rise, then fall, in C.F. antibody titre to a lower level at which it is maintained has been described in contagious bovine pleuropneumonia (Campbell and Turner 1936).

The haemagglutination test was not used in sheep as extensively as the complement fixation test, and as this test was developed later, sera from the experimentally infected sheep and some of the naturally infected sheep were examined after long periods of storage at approximately −10°C. With this test a titre of 4 was interpreted as positive and a titre of 2 was considered a suspicious reaction. The results indicated that haemagglutinins developed later in the disease than C.F. antibodies and persisted longer after recovery from infection. While there were few false positive reactions, there was a high percentage of false negative reactions. The heterogeneity that existed with C.F. antigens did not exist with haemagglutination antigens.

## V. ACKNOWLEDGEMENTS

I am indebted to Messrs. D. F. Mahoney, R. E. Dunham, K. C. Beaumont, P. Goener and abattoir staff for assistance at the Townsville District Abattoir and to the Abattoir Board, who made facilities available. Animals were donated or made available from various sources. These included Messrs. D. Scott and J. Maxwell, The Australian Estates Company Pty. Ltd. and Her Majesty's Prison, Stuart. Messrs. R. Swan, J. Aubrey and the late F. Ward submitted some of the blood samples from the field. Mr. R. D. Oakes assisted in bleeding the sheep at Winton. The project was financed in part by the Wool Research Trust Fund.

## REFERENCES

ALEXANDER, A. D., GRIFFIN, A. M., and GOCHENOUR, W. S. (1955).—*Res. Rep. Army Med. Grad. School Walter Reed Inst.* (Cited by Fournier and Chambon 1958).

BOYDEN, S. V. (1950).—*Proc. Soc. Exp. Biol.* 73:289.

CAMPBELL, A. D., and TURNER, A. W. (1936).—*Bull. Coun. Scient. Ind. Res., Melb.* No. 97.

CHAMBON, L., and FOURNIER, J. (1956*a*).—*Annls Inst. Pasteur, Paris,* 91:355.

CHAMBON, L., and FOURNIER, J. (1956*b*).—*Annls Inst. Pasteur, Paris,* 91:472.

CHANOCK, R. M., and SABIN, A. R. (1953).—*J. Immun.* 70:271.

COHEN, H. H., VAN LINGE, B., DE BOER, J. H., and DOLMAN, H. (1953).—*Leeuwenhoek Ned. Tijdschr.* 19:197.

COOK, I. (1962).—*Aust. Med. J.* 2:627.

COTTEW, G. S. (1955).—*Aust. Vet. J.* 31:155.

COTTEW, G. S., SUTHERLAND, A. K., and MEEHAN, J. F. (1952).—*Aust. Vet. J.* 28:113.

CRAVITZ, L., and MILLER, W. R. (1950).—*J. Infect. Dis.* 86:52.

DELBOVE, P., and REYNES, V. (1942).—"Acquisitions recentes sur quelques Maladies Infectieuses", A. Portail Saigon. (Cited by Cottew 1955).

FOURNIER, J., and CHAMBON, L. (1958).—"La Mélioidose et le Bacille de Whitmore". Collection de L'Institut Pasteur. (Ernest Flammarion: Paris).

KABAT, E. A., and MAYER, M. M. (1961).—"Experimental Immunochemistry". 2nd Ed. (Charles C. Thomas: Illinois.)

LAWS, L. (1964*a*).—"Melioidosis in Animals". M.V.Sc. Thesis, Univ. of Queensland.

LAWS, L. (1964*b*).—*Qd J. Agric. Sci.* 21:15.

LAWS, L., and HALL, W. T. K. (1963).—*Qd J. Agric. Sci.* 20:499.

LAWS, L., and HALL, W. T. K. (1964).—*Aust. Vet. J.* 40:309.

LECLERC, H, and SUREAU, P. (1956).—*Bull. Soc. Path. Exot.* 49:874.

LEWIS, F. A., and OLDS, R. J. (1952).—*Aust. Vet. J.* 28:145.

NIGG, CLARA, RUCH, J., SCOTT, EVA, and NOBLE, KATHRYN (1956).—*J. Bact.* 71:530.

NIGG, CLARA, and JOHNSTON, MARGARET M. (1961).—*J. Bact.* 82:159.

OLDS, R. J., and LEWIS, F. A. (1954).—*Aust. Vet. J.* 30:253.

RICHARDSON, G. M. (1941).—*Lancet* 2:696.

WETMORE, PSYCHE W. and GOCHENOUR, W. S. Jr. (1956).—*J. Bact.* 72:79.

WHITMORE, A., and KRISHNASWAMI, C. S. (1912).—*Indian Med. Gaz.* 47:262.

(Received for publication November 29, 1966)

The author is an officer of the Pathology Branch, Division of Animal Industry, Department of Primary Industries, and is stationed at the Animal Research Institute, Yeerongpilly, Brisbane.