MELIOIDOSIS IN ANIMALS IN NORTH QUEENSLAND. 3. BACTERIOLOGY

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SUMMARY

The bacteriology of 74 strains of *Pseudomonas pseudomallei* was studied. All strains had a characteristic earthy odour, showed marked bipolar staining and were motile with a single polar flagellum. When incubated at 37°C all strains grew well on MacConkey agar, nutrient agar, and 10 per cent. sheep blood agar. On incubation at 21°C on nutrient agar and 10 per cent. sheep blood agar and at 42°C on nutrient agar growth occurred but was slower.

Colonial type was variable. Rough and smooth forms and forms intermediate between these two were present.

All strains produced acid in litmus milk with slow digestion of the clot, liquified gelatin, reduced methylene blue, produced ammonia, formed catalase and fermented glucose with the production of acid, but without gas. Indole was not formed by any strains nor was hydrogen sulphide produced. In addition, the methyl red and Voges-Proskauer tests were negative. Growth occurred in the presence of potassium cyanide, in Koser's citrate and Levine's medium. Agglutination did not occur in 1/1000 trypaflavine or 0·4 per cent. or 0·85 per cent. saline. No pigmented colonies were produced.

The strains varied in their ability to ferment lactose, sucrose, maltose and mannite, to reduce nitrate, to liquify Leoffler's medium, to form urease and to grow on SS agar.

There was no demonstrable toxin in 24-hr broth cultures. The growth of *Ps. pseudomallei* was not inhibited by increased concentrations of neutral red, crystal violet or bile salts in MacConkey agar.

***In vitro, Ps. pseudomallei* was most sensitive to chloramphenicol, Aureomycin and tetracycline and partially sensitive to Terramycin and sulphafurazole.**

Forty-three guinea-pigs died from melioidosis following intraperitoneal inoculation with emulsions of pus or tissue, two after inoculation with cultures from muddy water and six after inoculation with broth cultures of *Ps. pseudomallei*. Invariably these showed abscesses of the liver, spleen and omentum and a peritonitis. Sometimes, also, there were subperitoneal abscesses and abscesses of the lungs and kidneys.

I. INTRODUCTION

The causative organism of melioidosis, originally described as *Bacillus pseudomallei* by Whitmore (1913) and since named *Bacillus whitmori* by Stanton and Fletcher (1921), *Pfeifferella whitmori* (Wilson and Miles 1955),

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Malleomyces pseudomallei (Anon. 1948), *Leofflerella pseudomallei* (Brindle and Cowan 1951), has been classified by Bergey (Anon. 1957) as *Pseudomonas pseudomallei.* Wetmore and Gochenour (1956) comment on the *in vitro* similarity between *Ps. pseudomallei* and the achromogenic forms of *Ps. aeruginosa.*

During investigation at Oonoonba Animal Health Station of the incidence and epidemiology of melioidosis in domestic animals in North Queensland, 194 strains of *Ps. pseudomallei* were recovered from 116 naturally infected animals and two strains were recovered from muddy water. This paper reports the examination of 67 of these, including two from water, two National Collection Type Culture strains (N.C.T.C.), three strains isolated by Lewis and Olds at the Animal Health Station (Oonoonba) and two strains from humans in Townsville.

II. MATERIALS AND METHODS

The method of examination of abscesses and central nervous system (C.N.S.) tissue by culture onto 10 per cent. sheep blood agar and MacConkey agar and by intraperitoneal inoculation of guinea pigs was described by Laws and Hall (1963).

Of the 65 strains recently isolated from animals, 40 were from pigs, 16 from sheep, 5 from goats, 3 from cattle and 1 from a horse. Both strains from muddy water were recovered from a swamp near Townsville. The two N.C.T.C. strains were No. 1688 from a rat and No. 8018 from a sheep. The origin of two of the strains of Lewis and Olds is not known. The third was Oonoonba ("O") strain recovered from a lamb (Case 1, Lewis and Olds 1952). It was used for the preparation of antiserum in rabbits and the preparation of antigens for serological tests. The two human cases were Rimington's Cases V and VI (1962).

Of the 67 strains isolated by the author, 65 were the original culture or the first, second or third subculture, while the other two, A234 and A709, were freeze-dried cultures. The N.C.T.C. strains were freeze-dried cultures also. The number of times the last four strains were subcultured is not known. Although "O" strain has been subcultured several times, the exact number is not known. The history of the other two strains of Lewis and Olds is not known. One had been stored for at least seven years at room temperature on a glycerol agar slope. The two human strains examined were the first and second subcultures respectively. Table 1 gives the subculture tested and the length of storage of the 74 strains.

All strains were inoculated into Difco nutrient broth, incubated for 24 hr and the resultant growth used to inoculate all test media. Nutrient agar, MacConkey agar and SS agar were prepared according to the Difco Manual (Anon. 1953). Blood agar plates were 10 per cent. sheep blood in Difco nutrient agar base. Glycerol agar plates were 5 per cent. glycerol in Difco nutrient agar base.

For growth of *Ps. pseudomallei* at different temperatures of incubation and on various media the following were tested:—

At 21°C—all cultures on nutrient agar plates and blood agar plates and 15 cultures on glycerol agar plates.

At 37°C—all cultures on nutrient agar plates, blood agar plates, MacConkey agar plates and SS agar plates.

At 42°C—all cultures on nutrient agar plates only.

For incubation at 21°C a thermostatically controlled refrigerated incubator was used. All plates were labelled and stored in the incubator for several hours before use. They were removed two at a time, sown and immediately returned to the incubator. Ten to 15 cultures were examined on any one day. The inoculum was at room temperature. Electric bacteriological incubators were used for incubation at 37°C and 42°C. A similar technique to the above was adopted.

To ensure that nutrient to support growth on SS agar was not being carried over with the inoculum of nutrient broth, the inoculation of many of the strains was done from either saline-washed organisms or the top of single colonies on nutrient agar.

Motility was determined by dark-ground microscopy on a drop of 24-hr broth culture incubated at 37°C. Flagella were stained by Kirkpatrick's method (Mackie and MacCartney 1938). If the examination was unsatisfactory on 24-hr cultures the organism was subcultured several times at 24-hr intervals and examined again. The fresh suspension of organisms in distilled water was examined. This was then incubated at 37°C overnight and the supernatant smeared again.

The tests for indole, hydrogen sulphide on triple sugar iron agar, methyl red, Voges-Proskauer and potassium cyanide were done by the methods given in the International Bulletin of Bacteriological Nomenclature and Taxonomy (1958). Where two methods are given the one used is indicated.

Methods given by Wilson and Miles (1955), using media prepared from Difco products, were used for methylene blue reduction, catalase production, the detection of hydrogen sulphide by the use of lead acetate paper and reduction of nitrate.

The methods given in the Difco Manual (Anon. 1953) were used for detecting urease, the liquefaction of gelatin and preparation of Koser's citrate medium. The test for liquefaction of gelatin was also done by Smith's Method (Smith 1946). Levine's medium was described by Levine *et al.* (1954). Litmus milk and Loeffler's medium were prepared according to Mackie and MacCartney (1938). Horse serum was used for the Loeffler's medium. Ability to ferment glucose, sucrose, maltose, mannite, and lactose in peptone water (Wilson and Miles 1955) was observed during 14 days' incubation at 37°C.

To test for agglutination in trypaflavine or 0·4 per cent. or 0·85 per cent. saline, the organisms were grown for 72 hr on nutrient agar at 37°C and suspended in each of the three solutions. These were incubated overnight at 37°C.

For observations on pigment production all strains were sown onto nutrient agar incubated at 37°C for 4 days then left at room temperature in the light for 7 days.

Growth of the organism on MacConkey agar containing either added crystal violet, neutral red or bile salts was tested. Growth was also tested in MacConkey broth containing added crystal violet and/or penicillin and streptomycin. The MacConkey agar was prepared according to Difco Manual (Anon. 1953) but with the following concentrations of the inhibiting substances: crystal violet 0·0002 per cent., 0·0003 per cent., 0·001 per cent., 0·01 per cent., 0·02 per cent., 0·1 per cent.; neutral red 0·006 per cent., 0·009 per cent., 0·03 per cent., 0·06 per cent.; bile salts 0·075 per cent., 0·15 per cent., 0·3 per cent. The MacConkey broth contained the constituents of MacConkey agar (Anon. 1953) without the agar, adjusted to pH 7·1 and with the following concentrations of the inhibiting substances: crystal violet 0·001 per cent., 0·02 per cent.; crystalline sodium penicillin 25 Units per ml and streptomycin sulphate 50 Units per ml; crystal violet 0·02 per cent. and penicillin and streptomycin as above.

Both the solid and the liquid media were inoculated with a drop of a 48-hr nutrient broth culture of *Ps. pseudomallei,* then incubated at 37°C. After 48 hours' incubation, a drop of the liquid medium was plated on MacConkey agar.

In vitro sensitivity to chemotherapeutic agents was done with tablets and discs on 10 per cent. sheep blood agar. Strain A234 (bovine) was tested with Evans Sentest Tablets and "O" strain with Evans Sentest Tablets, Multo Discs (Oxoid) and tablets of Vancomycin and Ilosone (Eli Lilly).

To test the pathogenicity of four strains which were recovered by culture, and not by guinea-pig inoculations, and of two strains of Lewis and Olds, a 24-hr broth culture of each strain was inoculated intraperitoneally into a guinea pig.

To test for toxin production, bacteria-free filtrates of 24-hr broth cultures were prepared by filtration through Hormann-Ekwip D9 filters. These were inoculated subcutaneously into two sheep and intraperitoneally into two guinea pigs. Each sheep was given 1 ml and the guinea pigs 0·1 and 0·2 ml.

III. RESULTS

The results are summarized in Table 1.

All cultures of *Ps. pseudomallei,* whether primary isolations or subsequent subcultures, had the characteristic earthy aromatic odour, which was well developed after 24 hours' incubation.

TABLE 1

SOURCE, LENGTH OF STORAGE, AND SOME OF THE VARIABLE CHARACTERISTICS OF 74 STRAINS OF *Ps. pseudomallei*

Pigs

Strain No.	56/125	56/130	56/290	A 272	A 233	A 252	A 393	A 314	A 331	A 424	A 447	A 450	A 697	A 713	B 303	B 586	C 107	D 286	D 412	D 411
Subculture tested	1st	1st	3rd	1st	2nd	1st	3rd	2nd	1st	3rd	3rd	2nd	3rd	1st	1st	1st	1st	1st	1st	1st
Length of storage (months)	51	51	49	40	40	40	38	39	39	37	37	37	34	33	27	21	18	4	2	2
Lactose fermentation†	–	A5	–	A5	A3	A6	–	A5	A7	–	A7	A5	A13	A3	A3	A6	A7	A7	–	A5
Nitrate reduction	+	–	+	–	+	+	+	–	–	+	+	–	+	–	–	–	+	–	+	–
Liquefaction of Loefflers	+	+	+	–	+	+	+	+	+	–	+	+	+	–	+	+	–	+	+	+
Growth on SS agar	–	+	+	+	+	–	+	+	+	+	–	+	–	+	–	–	+	+	+	+

Strain No.	D 282	D 425	D 348	D 208	D 470	D 460	D 413	D 299	A 709*	A 714	E 218 (2)	E 218 (3)	E 218 (4)	E 222	E 230	E 273	E 286	E 288	E 298	E 300
Subculture tested	1st	1st	1st	1st	1st	1st	1st	1st	2nd	1st	orig.	1st	1st	orig.	orig.	orig.	orig.	orig.	orig.	orig.
Length of storage (months)	4	2	3	6	1	1	2	4	33	40	–	1	1	–	–	–	–	–	–	–
Lactose fermentation†	A3	A5	A2	–	A3	A3	A3	A1	A5	A7	A4	A4	A4	A14	A5	A5	A4	A4	A9	A5
Nitrate reduction	–	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	–	+
Liquefaction of Loefflers	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on SS agar	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–

Sheep

Strain No.	56/440	A 120	B 469	C 113	116	G 91	D 361	D 50	F 68	D 46	D 207	J 53	D 88	F 67	"O"	E 156 (1)	N.C.T.C. 8018*	E 156 (3)
Subculture tested	2nd	2nd	3rd	1st	3rd	1st	1st	1st	1st	1st	1st	1st	1st	1st	?	orig.	?	orig.
Length of storage (months)	46	42	24	18	18	6	3	8	8	8	6	5	7	8	104	–	?	–
Lactose fermentation†	A3	A5	A5	A3	A7	A7	–	A5	A3	A3	A3	A7	A5	–	A11	A4	A5	A4
Nitrate reduction	–	+	+	–	+	+	–	–	–	+	–	+	+	–	+	–	+	+
Liquefaction of Loefflers	+	+	+	–	+	+	+	+	+	–	+	–	+	+	+	+	+	+
Growth on SS agar	+	+	+	+	–	–	+	+	+	+	+	+	+	+	–	+	+	+

	Goats					Cattle			Horse	Human		Unknown (Lewis & Olds)		Rat	Soil	
Strain No.	D 136	D 196	T 878	D 131	D 212	A 234	A 234*	B 297	C 119	D 238	S	No. 6	No. 121	N.C.T.C. 1688*	5	10
Subculture tested	1st	1st	1st	1st	1st	1st	2nd	1st	1st	1st	1st	?	?	?	1st	1st
Length of storage (months)	6	6	3	6	6	40	40	27	18	5	4	>84	>84	?	1½	1
Lactose fermentation†	A3	A2	A5	A3	A4	–	A5	A4	A3	A3	A4	–	A5	A9	A14	A4
Nitrate reduction	–	+	–	+	+	–	+	+	–	–	+	+	+	+	–	+
Liquefaction of Loefflers	–	+	+	–	+	+	+	+	–	+	–	–	+	+	+	+
Growth on SS agar	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+

? Not known.
* Freeze dried.

† A followed by a numeral indicates acid production after this number of days' incubation.
orig. Original culture.

In all freshly isolated strains, the organisms were short Gram-negative rods with marked bipolarity. On subculture they became pleomorphic and bipolarity decreased. The large elements 15-20 μ in length mentioned by Stanton and Fletcher (1932) and not observed by Cottew (1950) were seen occasionally in subcultures on solid media. They were not observed in primary isolations.

After 24 hours' incubation in nutrient broth all strains produced turbidity with a thin surface pellicle. On further incubation the pellicle thickened and a ropy deposit developed at the bottom of the tube.

On primary isolation, most of the strains had the colonial form of Cottew's (1950) "type A." A high percentage of these colonies showed concentric rings of growth. After prolonged growth on both blood and MacConkey agar, radiate wrinkling sometimes appeared. This is described by Cottew as occurring on glycerol agar. All of these "type A" colonies were of glutinous consistency. They were opaque, frosty white on blood agar and pink on MacConkey agar. Several strains produced round, smooth, moist, mucoid colonies with an entire edge. They were translucent on blood agar and red on MacConkey agar. By successive subculturing all could be changed to "type A" colonial form. This change occurred after a variable number of subcultures. At times both "type A" and smooth forms or forms intermediate between these two appeared on the same plate. Regardless of colonial type, the ability to ferment lactose on MacConkey agar was sometimes lost. On three occasions, these lactose nonfermenting organisms were inoculated into guinea pigs. They fermented lactose normally on MacConkey agar on recovery from the guinea-pig tissues.

Broth subcultures inoculated from either "type A" or "smooth" colonies and incubated for 24 hr were pathogenic on intraperitoneal inoculation of guinea pigs.

Fifteen strains were tested for growth on glycerol agar at 21°C. These included the N.C.T.C. strains and all grew under these conditions.

The following characteristics were possessed by all 74 strains examined: Growth on nutrient agar occurred at 21°C, 37°C and 42°C. Growth occurred on 10 per cent. sheep blood agar at 21°C and 37°C. (No tests were done on blood agar at 42°C.) Growth occurred on MacConkey agar at 37°C. The bacteria were motile with a single flagellum at one pole. Acid was produced in litmus milk with clot formation and then slow digestion of the clot. Gelatin was liquefied when tested after seven days (Difco and Smith's gelatin). Methylene blue was reduced. The catalase test was positive. Ammonia was produced. Indole was not formed (Erlich-Boehme). Hydrogen sulphide was not produced in triple sugar iron agar or by the lead acetate paper method. Methyl red and Voges-Proskauer tests (Barritt) were negative. Growth occurred in the presence of potassium cyanide, in Koser's citrate (Difco) and Levine's medium. Glucose was fermented without gas formation. Agglutination did not occur in 1/1000 trypaflavine or 0·4 per cent. or 0·85 per cent. saline. No yellow colonies were seen.

The strains varied in their ability to ferment lactose, reduce nitrate, liquefy Loeffler's medium, grow on SS agar. These results are given in Table 1.

The variable cultural characteristics were: Acid was produced from lactose by 64 strains, from sucrose by 44 strains, from maltose by 61 strains, from mannite by 66 strains. No gas was produced. Nitrate was reduced to nitrite by 45 of the strains. Loeffler's medium was liquefied by 60 strains. An indefinite, faint pink, urease reaction was seen with 10 strains (Kauffmann). Growth on Difco SS agar was demonstrated in 57 of the strains but it was variable, with only a few colonies developing from a loop of inoculum on some of the plates. Both lactose and non-lactose fermenting colonies were seen.

Ps. pseudomallei grew on MacConkey agar containing crystal violet in concentrations from 0·0002 per cent. to 0·02 per cent. inclusive. It did not grow on MacConkey agar containing 0·1 per cent. crystal violet. The colonies on agar with 0·02 per cent. crystal violet were umbonate and even after prolonged incubation did not develop greater than 1-mm dia. The colonies on the remaining plates had the same colonial form as those on MacConkey agar. They were purple instead of pink. At all concentrations of neutral red and bile salts, growth of the organism was comparable to that on MacConkey agar. The colonies on plates containing added neutral red were red. Growth occurred in the MacConkey broths containing added crystal violet, penicillin and streptomycin and crystal violet plus penicillin and streptomycin.

Strain A234 was sensitive to chloramphenicol, Aureomycin and tetracycline, partially sensitive to Terramycin and resistant to penicillin, streptomycin and erythromycin.

Strain Oonoonba was sensitive to chloramphenicol, tetracycline and Aureomycin, partially sensitive to sulphafurazole and Terramycin, and resistant to Vancomycin, Ilosone, bacitracin, neomycin, nitro-furantoin, penicillin, polymyxin B, streptomycin, erythromycin, novobiocin and oleandomycin.

One hundred-and-sixty-five samples of pus or tissue from animals were inoculated into guinea pigs to check for melioidosis. Of these, 43 died with lesions indicative of melioidosis and from which *Ps. pseudomallei* was recovered. The two strains recovered from muddy water were isolated from fatally infected guinea pigs. A further 6 guinea pigs inoculated with cultures of strains "O", G91, A234, D207, J53, and Lewis and Olds No. 6 died.

All of the infected guinea pigs had multiple abscesses of the liver and spleen, peritonitis and multiple abscess formation and folding of the omentum into a firm mass along the greater curvature of the stomach. The Strauss reaction was seen in male guinea pigs. On a few occasions abscesses were also detected in the lungs, kidneys and subperitoneally on the ventral abdominal wall.

Inoculation of the bacteria-free filtrate produced no ill-effects in the sheep or guinea pigs.

IV. DISCUSSION

The bacteriological findings agreed with those described by Bergey (Anon. 1957) and Cottew (1950) with the exception that not all of the strains reduced nitrates or liquefied Loeffler's medium. Some strains grew on SS agar. Glucose was regularly fermented with the production of acid but without the production of gas. The variation in fermentation of the other carbohydrate media tested, limits the use of these tests for identification. Fermentation of lactose when it occurs helps to distinguish *Ps. pseudomallei* from *Ps. aeruginosa.*

Kirkpatrick's method for staining flagella was most satisfactory on organisms which had been subcultured on nutrient agar several times at 24-hr intervals. The best results were obtained if the bacterial suspension in distilled water was left at 37°C overnight and the supernatant smeared.

The marked variation of colonial type reported by Nigg, Ruch, Scott, and Noble (1956) was seen. Some colonies resembled those of the coliform bacillus. Broth cultures prepared from either "type A" or smooth forms were pathogenic for guinea pigs on intraperitoneal inoculation.

Wetmore and Gochenour (1956) examined 15 strains of *Ps. pseudomallei* and 20 strains of *Pseudomonas aeruginosa.* They found that *Ps. aeruginosa* grew on glycerol agar at 21°C and on Difco SS agar at 37°C, while *Ps. pseudomallei* did not. Fifty-two of my strains and the N.C.T.C. strain 8018 grew on Difco SS agar at 37°C. All strains grew on blood agar at 21°C and all of the 15 strains tested, including the two N.C.T.C. strains, grew on 5 per cent. glycerol agar at 21°C.

Cottew (1950) and Brygoo and Richard (1952) described yellow pigmented colonies. No yellow colonies were seen in either primary isolations or subsequent subcultures on any of the media used. For storage, cultures were incubated for 48 hr at 37°C on nutrient agar slopes, in air-tight bottles, then placed in a dark cupboard at room temperature. One of these stored cultures developed a salmon pink colour.

Gallie (1942) obtained good inhibition of most lactose-fermenting coliform bacilli by modifying the constituents of MacConkey agar. Tests were done to determine the inhibition of *Ps. pseudomallei* by increase in concentration of the inhibitive substances in MacConkey agar. No tests were done on the inhibition of other Gram-negative bacilli or Gram-positive organisms. As an increase in concentration of bile salts or neutral red did not inhibit the growth of *Ps. pseudomallei,* and as it grew on MacConkey agar containing up to 0·02 per cent. crystal violet, media with increased concentration of these dyes were prepared for examination of soil and water samples for *Ps. pseudomallei.*

Chambon, De Lajudie, and Fournier (1954) stated that chloramphenicol was the only antibiotic effective against melioidosis and Aureomycin and Terramycin were useless. Moustardier, Dulong de Rosnay, and Salvat (1959) reported that a strain of *Ps. pseudomallei* from a man was sensitive *in vitro* to chloramphenicol, novobiocin and the tetracyclines. Hezebicks and Nigg (1958)

demonstrated the efficacy of chloramphenicol, sulphonamides and chlortetracycline in prolonged doses on melioidosis in mice. Novobiocin was effective in a low percentage of cases and the other tetracyclines had a suppressive action on this infection. Laws and Hall (1963) showed temporary clinical improvement in sheep experimentally infected with melioidosis following treatment with chloramphenicol, tetracycline or sulphadiazine, but their tests were not critical. *In vitro,* both strains A234 and Oonoonba were sensitive to chloramphenicol, tetracycline and Aureomycin and partially sensitive to Terramycin. Oonoonba strain (the only strain tested) was also partially sensitive to sulphafurazole but resistant to novobiocin.

The lesions in guinea pigs were similar to those described by Whitmore (1913) and Cottew (1950). Abscess formation, with folding of the omentum, was a constant feature of melioidosis produced by intraperitoneal inoculation. Cultures or tissue suspensions containing *Brucella suis, Corynebacterium ovis* or *Chromobacterium violaceum* produced visceral abscesses in guinea pigs on intraperitoneal inoculations. In these latter infections there were no abscesses in the omentum. Francis (1958), quoting Griffith (1911), stated that guinea pigs dying following intraperitoneal inoculations of 0·1 mg of avian *M. tuberculosis* showed discrete nodules filled with yellow pus in the omentum.

Colling, Nigg, and Heckly (1958) stated that their early work in which mice and hamsters died in 1 or 2 days with no gross lesions after inoculation of viable organisms suggested that death was due to a lethal toxin and subsequently they proved that *Ps. pseudomallei* produced a toxin. Heckly and Nigg (1958) demonstrated two thermolabile exotoxins and a thermostable endotoxin in bacteria-free filtrates of cultures of *Ps. pseudomallei* incubated for 7 days. The rapid rise in rectal temperature of experimentally infected sheep (Laws and Hall 1963) suggested that perhaps a toxin was produced even though cultures for inoculation had been incubated for 24 hr only. Bacteria-free filtrates of broth cultures similar to those used to infect the experimental sheep produced no ill-effect when inoculated into sheep or guinea pigs. These sheep did not produce detectable C.F. antibodies.

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