Experimental infection of normal and immunosuppressed pigs with *Pseudomonas pseudomallei*

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SUMMARY: A single dose of $5x10^8$ bacilli of *Pseudomonas pseudomallei* by intratracheal injection resulted in acute (21 cases) or chronic (19 cases) melioidosis in 40 of 48 pigs. Fifteen (10 acute and 5 chronic) had been immunosuppressed by cyclophosphamide before inoculation. The major clinical signs were initial fever, marked neutrophilia and, in the acute cases, respiratory distress. There were no signs of the nasal and ocular discharge, paresis or diarrhoea seen in acute cases in south-east Asia. The cyclophosphamide treatment caused a significant decrease in the neutrophil count by 7 d after inoculation in all 15 immunosuppressed pigs, and all were culture positive at necropsy. Eight of the 33 non-treated pigs were culture negative at necropsy. Pigs overcoming the initial phase of infection had more abscess-like nodules that were bacteriologically sterile at necropsy than the pigs with acute cases of melioidosis. *P. pseudomallei* was isolated predominantly from the spleen, lungs and the injection site. Although only one strain was used in this study, it is likely that Australian strains of *P. pseudomallei* are not as virulent as the south-east Asian isolates.

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Introduction

Two forms of porcine melioidosis have been reported. One is the epizootic, mainly acute, form of the disease from which deaths occur mainly in young pigs (Nguyen-Ba-Luong 1956; Thonn *et al* 1960; Omar *et al* 1962). The other is the sporadic, mainly chronic, form which is diagnosed only at slaughter (Laws and Hall 1963; Ferry 1973; Little 1979; Thomas 1981). It is the chronic, form of melioidosis that is most common in Queensland (Thomas 1981; Webster *et al* 1984), although an abortion (Rogers and Anderson 1970) and an acute case of porcine melioidosis (Olds and Lewis 1955) have been recorded. A recent report of melioidosis in intensive piggeries in south-east Queensland (Ketterer *et al* 1986) where 159 culture positive pigs were condemned at slaughter has emphasised the need to learn more about the pathogenesis of the disease in pigs.

Pigs are relatively resistant to infection with *P. pseudomallei* (Ketterer *et al* 1986). Production of porcine melioidosis using a single inoculation by oral, nasal, scarification, subcutaneous or intramuscular routes has been ineffective (Stanton and Fletcher 1932; Nguyen-Ba-Luong 1961). The results of 3 oral doses at intervals of 2 d have been equivocal (Nguyen-Ba-Luong 1961; Thomas and Spinks 1983). As the intratracheal route proved successful in preliminary work at this laboratory (Thomas *et al* unpublished data) using $3x10^8$ to $3x10^{12}$ *P. pseudomallei* organisms, this single dose method was adopted for this trial. Weaner pigs were used because of their greater susceptibility to melioidosis than older pigs (Nguyen-Ba-Luong 1961; Omar *et al* 1962). A number of these trial pigs were immunosuppressed before inoculation.

The main objectives of the study were to assess the susceptibility of pigs to a standard dose of *P. pseudomallei,* and to assess the effect of immunosuppression on the susceptibility.

Materials and Methods

Pigs

Fifty pigs were obtained from a piggery with no history of melioidosis. The pigs were 7 to 9 weeks old and weighed between 12 and 15kg. Serums from these pigs were negative in the modified cold complement fixation and indirect haemagglutination tests for melioidosis (Thomas and Spinks 1983).

The pigs were housed in an isolation facility. Care was taken to prevent cross-infection.

Inoculum

The inoculums were prepared from a recently isolated, local porcine strain of *P. pseudomallei,* serotype I, grown in trypticase soya broth for 24 h at 37°C. The broth was shaken during incubation to avoid pellicle production. Suspensions of organisms in sterile saline were standardised optically and a final count determined using a Neubauer cytometer. The inoculums were used within 2 h of preparation; 0.5ml of each inoculum was also injected intraperitoneally into guinea pigs to test for virulence.

Experiment

Forty-eight weaner pigs were injected by the intratracheal route with a single dose (1ml) of 5×10^8 *P. pseudomallei,* followed by a 1ml rinse of the needles with sterile saline. Two d before inoculation, 500mg doses of cyclophosphamide* were prepared as recommended by the manufacturer and injected intravenously into 15 of the trial pigs. Two control pigs were given 1ml doses of sterile saline. The trial lasted 84 d. Two to 6 pigs per week were killed for necropsy. These were chosen at random except when obvious sickness warranted early intervention.

Clinical Observations and Samples

Morning temperatures were recorded daily. Injection sites were carefully examined for lesions, and any exudate was sampled for culture. Blood samples were collected for culture twice weekly for the first 2 weeks after inoculation and weekly thereafter. Total and differential white cell counts were done on blood collected into EDTA. Faeces and urine were collected weekly for culture, but not all pigs were sampled each week. Any nasal discharge was cultured.

Pathology

A complete necropsy was performed on each pig. Samples of heart blood, lung, liver, spleen, kidney, brain, cerebrospinal fluid, retropharyngeal lymph nodes, mediastinal lymph nodes, bronchial lymph nodes, mesenteric lymph nodes, tonsils, urine, faeces, injection site (trachea) were collected using aseptic techniques for bacteriological examination. Lesions were selected where present, otherwise pieces of tissue were removed for culture. Lesions present at any other site were also collected for culture.

The number, size and placement of lesions in the tissues were recorded and consistency of the lesions noted.

For histopathology, tissues were fixed in 10% buffered neutral formalin, embedded in paraffin, cut at 3 to 5 μm and stained with haematoxylin and eosin.

* Endoxanasta, Asta-Werke A-G Chem. Fabrik Germany

Culture

Samples were cultured on sheep blood agar, MacConkey agar and 3% glycerol agar with added antibiotics and crystal violet (Thomas *et al* 1979). The plates were incubated for 4 d at 37°C. Isolates were identified as *P. pseudomallei* by conventional biochemical tests and the Microbact 24E system† (Thomas 1983). Identity of the isolates was confirmed by agglutination with rabbit antiserum to *P. pseudomallei*.

The isolation of *P. pseudomallei* from infected tissue or pigs will be described as "culture positive", and failure to isolate the organisms as "culture negative".

Results

All guinea pigs injected with the *P. pseudomallei* inoculums died from melioidosis within 1 to 2 d.

The 2 control pigs were healthy throughout the trials.

Clinical Signs

Forty-seven of the experimentally inoculated pigs developed fevers between 40.2°C and 41.8°C (average 40.9°C) within 24 h of inoculation. The temperatures returned to normal within 2 to 7 d in 36 of the pigs and within 8 to 13 d in 7 of the pigs. Four pigs had undulating fevers. One pig had no marked temperature rise. There were no marked differences between pigs treated or not treated with cyclophosphamide.

All pigs ate well. Eighteen pigs (8 treated) had wheezing respiration within 2 to 28 d of inoculation. The 4 pigs killed 3 d after inoculation had respiratory distress and one of these had a vomiting attack. The remainder of the pigs appeared healthy throughout the trial.

Gross Pathology

The 2 control pigs had no gross lesions at necropsy.

Acute melioidosis was diagnosed in pigs on the basis of the presence of areas of consolidation in the lungs where exudative changes, with no proliferation or fibroblastic containment, were observed.

Chronic melioidosis was diagnosed in pigs when nodules had formed in the viscera which were characterised by a proliferative (fibroblastic) response. These nodules contained a collection of mononuclear and epithelioid cells which had undergone caseation necrosis. The spleen was the major organ involved.

Both the exudative and proliferative response could occur in the lung at the same time.

All 21 pigs (both treated and non-treated with cyclophosphamide) with the acute form of the disease were necropsied by 28 d after inoculation.

Acute cases showed more signs of respiratory distress (66.7% to 21.1%), more confluence of lung lesions (42.9% to 21%), more lung and thoracic lymph node involvement (85.7% to 21.1%) and more tissues infected per pig (3.8 to 2.0) than the chronic cases (Table 1). Spleen and head lymph node involvement was less (66.7% to 89.5%).

Pigs treated with cyclophosphamide — visible lesions were seen in 12 of the 15 treated pigs at necropsy. Fibrous adhesions were present in the thoracic cavity of 3 of the pigs and the abdominal cavity of one pig. Ten pigs had acute melioidosis and 5 pigs had chronic forms of the disease. Abscess-like lesions were roughly spherical and averaged 4mm (range 1 to 5mm) in diameter by 14 d after inoculation. Thereafter, the nodules averaged 1cm (0.5 to 3cm). The number of nodules in organs varied from single to multiple lesions which tended to coalesce in the lungs covering areas up to 5cm by 8cm. In the 10 pigs with acute meliodiosis, the lungs showed consolidated pneumonic areas, firm to cut and grey to creamy/off white in colour. Three of these pigs had lesions too numerous to count in the lungs and were classed as having one confluent lesion. The 5 chronic cases of meliodiosis had lung nodules that varied from hyaline foci at 3 d to grey or creamy/off white foci with grey borders at 14 d after inoculation. In the spleen of all treated pigs, focal raised lesions of a similar colour to the spleen were seen by 7 d after inoculation. By 14 d, these nodules were raised, cream in colour and well encapsulated. Thirty-nine such nodules were recorded in one spleen. In the wall of the trachea, the lesions developed to form white fibrous capsules (2mm thick) containing cream to green-coloured, semi-gelatinous material. Lesions were rarely seen in the lymph nodes of pigs with acute forms of the disease. The bronchial, mediastinal and retropharyngeal lymph nodes were either normal in size or reddened and enlarged, often to twice their normal size. Single nodules, up to 3cm in size, were occasionally seen in chronic cases.

† Disposable Products, Adelaide, SA

TABLE 1

Differences observed between 40 experimentally infected pigs (treated or non-treated with cyclophosphamide) that were culture positive for *P. pseudomallei* at necropsy

Pigs	Number		Lung*	Spleen†	Tissues‡
CY§	A¶	10	10(100)#	7(70)	3.7
	C	5	1(20)	4(80)	1.4
non CY	A	11	8(72.7)	7(63.6)	3.8
	C	14	3(21.4)	13(92.9)	2.2
CY	A&C	15	11(73.3)	11(73.3)	2.9
non CY	A&C	25	11(44)	20(80)	2.9
CY & non CY	A	21	18(85.7)	14(66.7)	3.8
CY & non CY	C	19	4(21.1)	17(89.5)	2.0

* — lung and/or thoracic lymph nodes
† — spleen and/or head lymph nodes
‡ — average number of culture positive tissues per pig
§ — CY = cyclophosphamide
¶ — A = acute melioidosis
C = chronic melioidosis
\# — ( ) = percentage positive

Pigs not treated with cyclophosphamide — visible lesions were seen in 23 of the 33 non-treated pigs at necropsy. Fibrous adhesions were present in the thoracic cavity of one pig and the abdominal cavity of one pig. Eleven pigs had acute melioidosis and 14 pigs had chronic forms of the disease. The gross pathology of the lesions was similar to that seen in the treated pigs. Six of the 11 pigs with acute melioidosis had lesions too numerous to count in the lungs. In one pig, there was complete involvement of all lobes and multiple lesions. Eight non-treated pigs were free of disease at necropsy although 2 of these had sterile lung lesions.

Treatment with cyclophosphamide increased the number of pigs becoming infected (100% treated; 75.8% non-treated), the number of pigs developing acute forms of the disease (66.7% treated; 33.3% non-treated) and the number of pigs with lung and/or thoracic lymph node involvement (73.3% treated; 33.3% non-treated).

The most common sites for nodules in the lungs were the left dorsal diaphragmatic (13), the right dorsal diaphragmatic (11), the left cardiac (5), the right apical (4), the left apical (3) and the right cardiac (3) lobes.

Histopathology

There were two types of lesions seen in the lungs. One was an acute exudative bronchigenic reaction with associated consolidation while the other was a chronic granulomatous productive type lesion represented by nodules. The productive type either followed the exudative lesion or occurred independently. The exudative process showed focal exudation of leucocytes, mainly mononuclear cells and lymphocytes. With age, caseation foci and fibrous tissue appeared and then, a thin capsule of epithelioid cells along with fibrous tissue surrounded the caseated centre. The chronic productive type lesion was formed from initial focal exudation of leucocytes. These leucocytes could not be further differentiated because of intense karyorrhexis and finally complete caseous necrosis. An outer layer of fibrinous exudate was frequently present. With age, a thin zone of granulation tissue consisting of macrophages and fibroblasts replaced the fibrinous exudate and demarcated the lesions.

TABLE 2

Isolation sites of *P. pseudomallei* in 48 experimental pigs injected by the intratracheal route with $5x10^8$ organisms

Tissue Description	Pigs treated with cyclophosphamide (15)		Pigs not treated with cyclophosphamide (33)	
	Number of culture positive tissues	Number of culture positive tissues with visible lesions	Number of culture positive tissues	Number of culture positive tissues with visible lesions
Inoculation site/trachea	11	9	20	15
Lung	10	6	11	8
Spleen	9	8	17	15
Bronchial lymph node	5	0 (3)*	3	0 (1)
Mediastinal lymph node	3	0 (0)	3	0 (0)
Liver	2	2	3	0
Retropharyngeal lymph node	1	1 (1)	4	1 (2)
Mandibular lymph node	1	0 (0)	0	0
Kidney	1	0	1	0
Hoof	1	1	0	0
Tonsils	0	0	5	0
Iliac lymph node	0	0	2	0 (0)
Faeces	0	0	1	0
Tail	0	0	1	1

* — ( ) = number of lymph nodes reddened and enlarged

TABLE 3

Comparison of studies on natural cases of melioidosis in pigs with this experimental study

% of tissues from which *P. pseudomallei* was isolated				
Spleen	Lung	Thoracic lymph nodes	Disease	Reference
26.6	13.3	21.3	Chronic	Laws and Hall 1963
10.0	60.0	40.0	Acute	Omar 1963‡
40.7	40.7	51.9	Chronic	
26.4	4.5	0.9	Chronic	Thomas 1981
34.0	7.0	40.0	Chronic	Ketterer *et al* 1986
60.0	90.0	70.0	Acute (CY)*	This study 1990
60.0	20.0	0.0	Chronic (CY)	
63.6	81.8	45.5	Acute (non-CY)†	
71.4	14.3	0.0	Chronic (non-CY)	

* — CY = cyclophosphamide treated pigs
† — non-CY = non-cyclophosphamide treated pigs
‡ — south-east Asian study

The productive type nodules of the spleen and liver had well developed capsules. The thickness of this capsule was related to the age of the nodules.

Haematology

There was a marked neutrophilia in all infected pigs 3 d after inoculation. In the treated pigs, the neutrophil count returned to 'normal' levels by 7 d after inoculation, while in the non-treated pigs, the neutrophil count remained high until 21 d after inoculation. There was a slight drop in the lymphocyte count in both sets of pigs from 7 to 14 d after inoculation, but still within the 'normal' range.

Bacteriology

Table 1 shows the percentage of the 40 culture positive pigs that had *P. pseudomallei* isolated from the lung and/or associated lymph nodes and from the spleen and/or lymph nodes of the head. The average number of infected tissues per pig is also indicated.

Table 2 shows the isolation sites for *P. pseudomallei,* including the number of culture positive tissues showing visible lesions. Results for both treated and non-treated pigs are shown.

Pigs treated with cyclophosphamide — all 15 treated pigs were culture positive at necropsy. The average number of culture positive tissues was 2.9 (range 1 to 6). Five faecal and 18 urine samples collected during the trial were culture negative. One pig had a culture positive blood sample 21 d after inoculation, while another pig had a sterile lesion in the lung when necropsied 77 d after inoculation.

Pigs not treated with cyclophosphamide — P. pseudomallei was isolated from 25 of the 33 non-treated pigs at necropsy. The average number of culture positive tissues was 2.2 (range 1 to 7). Twenty-six faecal and 59 urine samples collected during the trial were culture negative. Two of the 8 culture negative pigs had sterile lesions in the lungs, and 2 culture positive pigs had sterile lesions in either the spleen or throat. All sterile lesions were in pigs necropsied 70 to 84 d after inoculation. There were 5 culture positive blood samples. These were from 3 pigs at 14 d after inoculation, one pig at 28 d after inoculation and one pig at 35 d after inoculation.

Discussion

This study confirmed preliminary work at this laboratory that $\geq 3 \times 1 \frac{1}{3} ?^8$ *P. pseudomallei* organisms injected by the intratracheal route would produce melioidosis in pigs. Forty of the 48 experimentally inoculated pigs developed the disease.

Natural infection by *P. pseudomallei* occurs through ingestion, inhalation or wound entry of soil or soil-contaminated food and water, *P. pseudomallei* being a soil saprophyte (Thomas *et al* 1979). Ingestion and wound entry generally lead to spleen and head lymph node involvement (Laws and Hall 1963; Thomas *et al* 1981) while aerosol inhalation (from high pressure sprayers and pressure nipple drinkers) leads to lung and thoracic lymph node involvement (Ketterer *et al* 1986). The chronic form of the disease is more common in Queensland (Laws and Hall 1963; Thomas 1981).

Twenty-one acute and 19 chronic cases of melioidosis developed during our trial. The larger numbers of acute cases seen was due to 2 major factors; the method of injection and the use of cyclophosphamide as an immunosuppressant.

Our intratracheal inoculation provided both aerosol (forcible injection by needle) and ingestion (initial swallow plus possible reinfection from tracheal lesions) routes of infection. Where aerosol infection played the major part, acute cases of bronchopneumonia ensued, with associated involvement of the thoracic lymph nodes. Where ingestion was the major route of entry, chronic cases were more common and the spleen was more often affected than the lung (Table 1).

Treatment with cyclophosphamide did not significantly affect the clinical signs, histopathology and bacteriology as seen in the non-treated pigs. However, it did increase the number of pigs affected and the number of pigs with acute forms of the disease. This is possibly due to the marked drop in the neutrophil count at 7 d after inoculation in the treated pigs. Cyclophosphamide, a cytotoxic drug, is known to act against

cells of the immune system, particularly polymorphonuclear cells and macrophages (Buhles and Shifrine 1979; Tatsukawa *et al* 1979; Muneer *et al* 1988) which are important in the primary stages of infection (Sawada *et al* 1987).

The signs of acute melioidosis seen in pigs in south-east Asia, such as nasal and ocular discharge, paresis of the hind legs and diarrhoea (Nguyen-Ba-Luong 1956; Thonn *et al* 1960; Omar *et al* 1962) were not seen in this trial. Table 3 compares results of spleen, lung and thoracic lymph node involvement in pigs with melioidosis in field and experimental cases in Queensland and south-east Asia. It would appear that strains of *P. pseudomallei* isolated in Queensland may not be as virulent as the Asian isolates.

The histopathology seen in our trial was similar to that described by Omar (1963) in field cases of porcine melioidosis. However, he found that the apical and cardiac lobes were the most commonly affected lung lobes in the natural cases he investigated. In contrast, the diaphragmatic lobes were the most commonly affected lobes in 13 of the 14 pigs with lung lesions in our experimental work. The forcible injection of *P. pseudomallei* into the trachea of pigs held vertically, head upwards, allowed direct entry into the diaphragmatic lobes. No changes in the thoracic lymph nodes, other than oedema and congestion, were evident in acute cases and this is in agreement with the observation on acute melioidosis made by Omar (1963).

The age of the lesions could be deduced from the size of the nodules, the degree of encapsulation and the consistency of the contents. Very few nodules were seen in pigs killed at 3 to 7 d after inoculation. At 7 to 14 d after inoculation, the roughly spherical nodules averaged 0.4cm in diameter. After 14 d, nodules were found to vary from 0.5 to 3.0cm. In the lungs, nodules were either large, discrete and well-encapsulated with semi-solid, tenacious contents, or small and tending to coalesce into areas up to 8x5cm which were filled with caseated material. The older lesions in the spleen were multiple, discrete, large well-encapsulated lesions that protruded above the surface of the organ. As the lesions aged, the contents turned from moist and caseous to dry and crumbling. Older lesions were often sterile. Five pigs had sterile lesions suspected of being melioidosis in regression. Sterile lesions are not uncommon in natural infections in older pigs (Omar *et al* 1962) and goats (Olds and Lewis 1954; Thomas *et al* 1988). In this trial, the sterile lesions were found in pigs killed between 70 and 84 d after inoculation.

P. pseudomallei was isolated from the faeces of only one pig, emphasising the fact that transmission of melioidosis by this means is rare (Nouvel *et al* 1976; Thomas *et al* 1988), even though faecal spread of *P. pseudomallei* in horses in France has been documented (Galimand and Dodin 1982).

Two pigs had culture positive lesions in unexpected sites. One pig had a nodule in the right hoof (other sites being lung, spleen and retropharyngeal lymph node) and another pig had a nodule near the tail (other sites being the injection site and the spleen). A thorough necropsy is recommended when melioidosis in pigs is suspected.

This trial has supplied relevant data towards the understanding of the pathogenesis of *P. pseudomallei* in pigs. However, it was not a true indication of what occurs in the field where the chronic form of melioidosis is more common. Further work, involving use of a lower dose of organisms more consistent with field concentrations (up to $5x10^6$/g — Thomas and Forbes-Faulkner 1981) and use of an aerosol or multiple dose oral inoculation technique to avoid reinfection from tracheal lesions, could be worthwhile.

The spleen will be the major organ involved in field cases where oral and wound entry are normal methods of ingress for *P. pseudomallei*. A high incidence of lung and thoracic (especially bronchial) lymph node involvement would indicate an aerosol infection. Any stress on the pigs, especially weaners, will increase the risk of infection.

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