

STUDIES ON CERTAIN BIOLOGICAL CHARACTERISTICS OF
MALLEOMYCES MALLEI AND MALLEOMYCES
PSEUDOMALLEI

I. MORPHOLOGY, CULTIVATION, VIABILITY, AND ISOLATION FROM
CONTAMINATED SPECIMENS

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The etiological agent of glanders, or farcy, was first isolated in pure culture by Loeffler and Schutz in 1892. The genus *Malleomyces* includes only two species—*Malleomyces mallei* and *Malleomyces pseudomallei* (Bergey *et al.*, 1939). Very few studies have been conducted with *M. mallei* in the last 25 years since, by the destruction of infected animals detected through mallein skin tests and serum complement fixation tests, the disease has been almost eradicated in most civilized countries from its natural equine hosts.

M. pseudomallei (*B. whitmori*) was identified by Whitmore (1912) as the etiological agent of melioidosis, a disease of man similar to glanders. The wild rat of southeastern Asia is reported to be the natural host, but natural infection has been found in rats, rabbits, cats, and dogs (Stanton and Fletcher, 1932).

The purpose of this paper is to present studies on morphology, cultivation, viability, disinfection, and isolation of the organisms from contaminated specimens. Studies on virulence and animal susceptibility are reported in the succeeding paper. Reports of studies on comparative serological reactions, chemotherapy, chemical fractions, pathological changes in infected animals, and six human cases of glanders will be reported elsewhere.

Eight strains of *M. mallei* and two strains of *M. pseudomallei* were used in the studies reported. The source, date of isolation, and reported virulence of each strain are given in table 1.

MORPHOLOGY

The strains of *M. mallei* and *M. pseudomallei* used in this work were indistinguishable morphologically. Pleomorphism was not marked even in old cultures. Apparent filamentous forms on careful study appeared to be long chains of bacilli that were closely united. When stained with common granule stains, the organisms showed scattered areas of increased density, giving a definite granular appearance. This was especially marked in direct films from infected tissues stained with Wright or Giemsa stains. Study of such preparations gave

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the impression that the organisms were encapsulated, but this could not be proved by the use of any of the common capsule stains. Flagellar stains showed that *M. pseudomallei* possessed lophotrichate flagella, whereas *M. mallei* was atrichous. Broth cultures of *M. pseudomallei* continued to show active motility after storage for 5 months.

When photographed in the electron microscope,² the organisms of both species showed marked variations in the internal structure of the cell. In some organisms there appeared to be only a bipolar increase in protoplasmic density. In

TABLE 1
Data on the strains of *M. mallei* and *M. pseudomallei* studied

ORGANISM	DESIGNATION	SOURCE	DATE OF ISOLATION	REPORTED VIRULENCE	DESIGNATION IN THIS PAPER
<i>M. mallei</i>	2024-2MP*	Unknown	Unknown— very old	Low	2MP
<i>M. mallei</i>	2024-3PP*	Unknown	Unknown— very old	Low	3PP
<i>M. mallei</i>	2024-3MP*	Unknown	Unknown— very old	Low	3MP
<i>M. mallei</i>	3†	Kweiyang, China	1942	Straus- positive	C3
<i>M. mallei</i>	4†	Lung of horse	1942	Straus- positive	C4
<i>M. mallei</i>	5†	Lung of horse	1942	Straus- positive	C5
<i>M. mallei</i>	K†	Unknown	Unknown	Unknown	C6
<i>M. mallei</i>	3873*	Fatal human case in China	1944	Very high	C7
<i>M. pseudomallei</i>	294‡	Unknown	Unknown	Avirulent	W294
<i>M. pseudomallei</i>	295‡	Unknown	Unknown	Avirulent	W295

* Cultures from U. S. Army Veterinary Laboratories used in the preparation of mallein. The authors are indebted to Colonel R. Randall, VC, USA, for supplying these strains.

† Type cultures from the China Epidemic Prevention Bureau (Dr. T'angs' laboratory). The authors are indebted to General R. A. Kelsner, VC, USA, for procuring these strains.

‡ Old laboratory stock cultures from the Calcutta School of Tropical Medicine. The authors are indebted to General R. A. Kelsner, USA, for procuring these strains.

others, the cell contained scattered, clear, refractile bodies resembling lipoidal globules and opaque bodies resembling dense protoplasmic accumulations (figures 1, 2, and 3). A definite cell wall was visible, and flagella were scattered through the field surrounding the cells of *M. pseudomallei* (figure 3). Electron micrographs of *M. pseudomallei* taken after a suspension was subjected to sonic vibration for 1 hour showed numerous small circumscribed bodies in the cellular debris, resembling extruded lipid globules (figure 4). Fat bodies within the cells of *M. mallei* have been demonstrated previously by special fat stains (Wor-

² We are indebted to Dr. L. A. Chambers for the preparation of the electron micrographs.

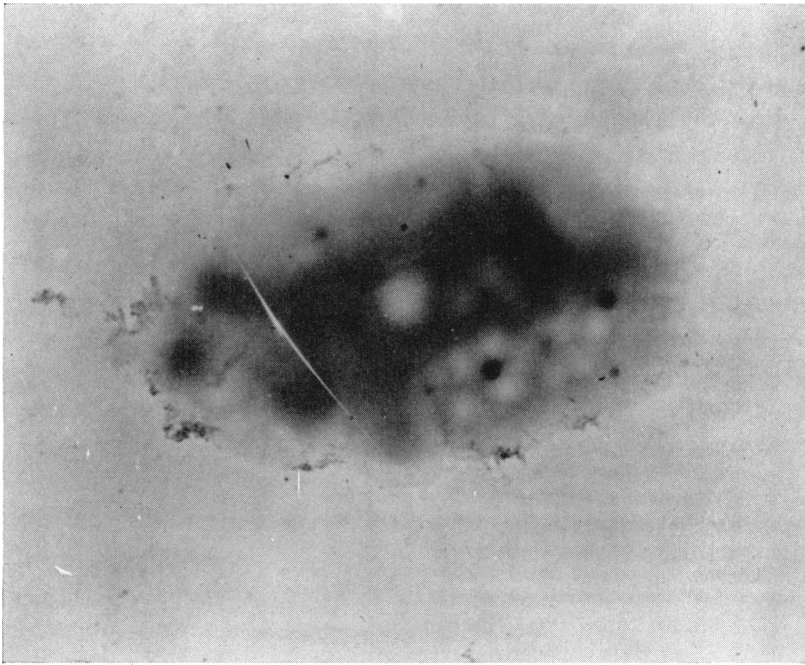


Fig. 1. ELECTRON MICROGRAPH OF *M. MALLEI*

Note areas of increased protoplasmic density, light areas resembling vacuoles, and refractile cell membrane. Magnification approximately 27,000 \times .

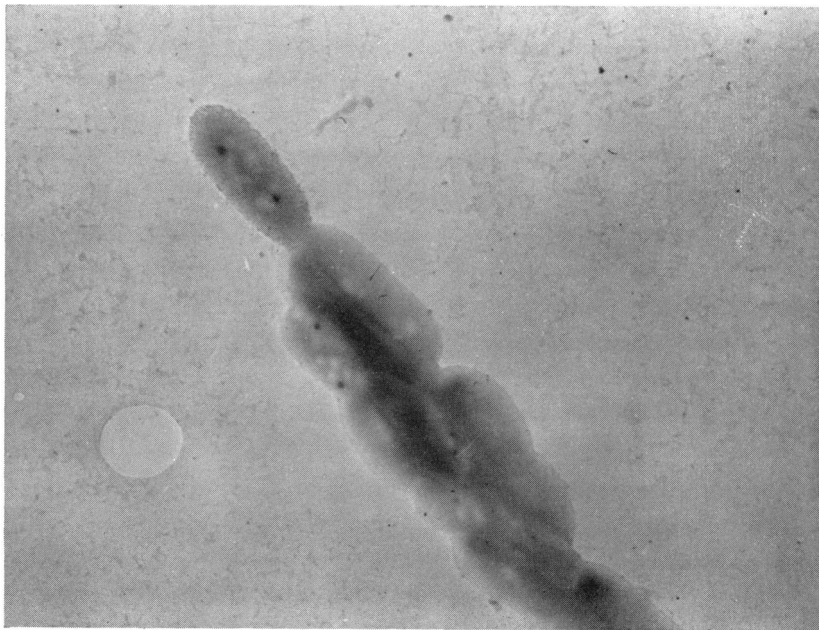


FIG. 2. ELECTRON MICROGRAPH OF *M. MALLEI* SHOWING DENSE GRANULES AND CLEAR REFRACTILE AREAS WITHIN THE CELLS
Magnification approximately 13,500 \times .



FIG. 3. ELECTRON MICROGRAPH OF *M. PSEUDOMALLEI*
Note intracellular refractile areas, areas of increased density, and flagellar strands scattered around the cells. Magnification approximately 12,500 X.

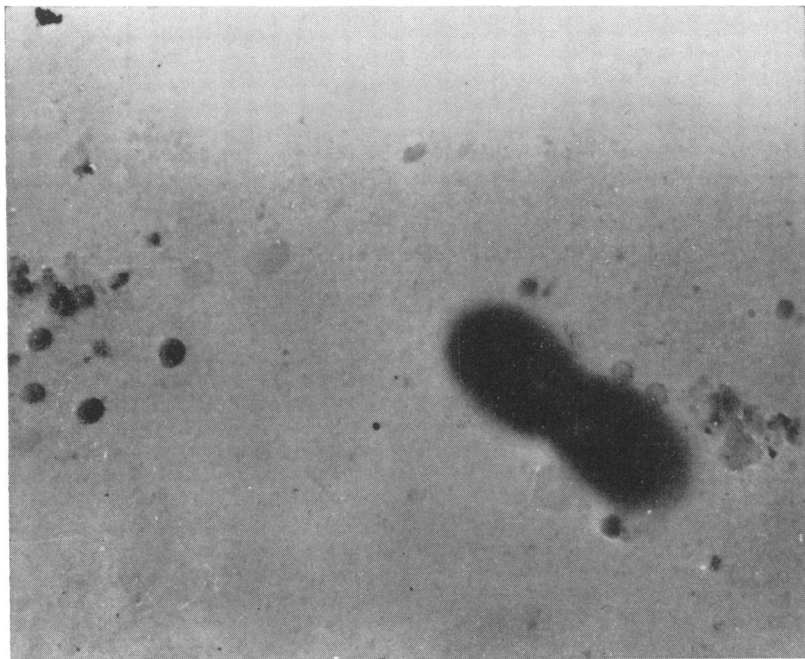


FIG. 4. ELECTRON MICROGRAPH OF *M. PSEUDOMALLEI* AFTER SONIC VIBRATION TREATMENT
The cell remaining intact appears to be in process of transverse fission. Numerous extruded granules believed to be of a lipoidal nature are seen. Magnification approximately 12,500 X.

ley and Young, 1945) and a complex lipoidal substance was extracted chemically from the organism in this laboratory (Hink, Miller, and Tanner, 1948).

CULTIVATION

The medium used routinely for *M. mallei* by former workers was a meat infusion base to which 4 per cent glycerol and 1 per cent peptone were added. *M. pseudomallei* was known to grow well on standard laboratory media. In an effort to find simple base media and synthetic media that were satisfactory for both organisms, the growth of both organisms on the test media was carefully compared with that on the glycerinated meat infusion peptone media. Comparison was made by plate counts (surface-streaking technique) and colonial study on solid media, and by turbidity measurement and viability counts of liquid media cultures. The optimum pH for both organisms was 6.8. The following media (all contained 4 per cent glycerol and were adjusted to pH 6.8) supported growth of both organisms, equal in every respect to the infusion base medium:

- (a) Beef extract (0.3 per cent) peptone (1 per cent) agar.
- (b) Potato infusion agar (no beef or peptone).
- (c) Cysteine (0.1 per cent) peptone (1 per cent) agar.
- (d) Asparagine (0.5 per cent) agar (no beef or peptone).
- (e) Beef extract (0.3 per cent) peptone (1 per cent) broth.

M. pseudomallei also grew well on all standard bacteriological media and did not require glycerol, although growth was somewhat enhanced by its presence. *M. mallei* required glycerol for optimum growth.

The colonial appearance and biochemical activity of the strains of *M. mallei* studied corresponded with those described by previous workers. The colonial appearance of both strains of *M. pseudomallei* was the same as that described by Stanton and Fletcher (1932), but our strains did not actively ferment any of the sugars tested. Colonies of *M. mallei* on agar were smooth, entire, convex, translucent, and similar to those of *Escherichia coli* except that the latter were somewhat larger. *M. pseudomallei* colonies were smooth after 24 hours' incubation and were identical in appearance with 48-hour *M. mallei* colonies. They became rough and flat and had finely crenated margins after 48 to 72 hours' incubation. Muroid and intermediate colonial types of *M. pseudomallei* (Stanton and Fletcher, 1932) and rough variants of *M. mallei* were observed from time to time, but serial colonial isolation gave only 75 to 90 per cent of the colonies in the variant phase, and on continued cultivation all reverted to the original type.

The distinctive yellowish-brown pigmentation of *Malleomyces* organisms did not appear until after 4 to 7 days' incubation. *M. pseudomallei* showed rapid and complete proteolytic activity on all media containing colloidal or coagulated proteins.

Since it was observed that both organisms grew well on agar containing 0.5 per cent asparagine as the only source of nitrogen, synthetic media which contained asparagine as the source of nitrogen were chosen for study. Luhrs' (1927)

modification of Long's synthetic medium for *Mycobacterium tuberculosis* was found to support good growth of both organisms and the total bacterial counts were equal to those in the infusion base broth.

Aeration and oxygenation. Since *Malleomyces* organisms are obligate aerobes and produce heavy growth on the surface of media, studies were undertaken to determine the effect of increasing the oxygen tension in broth cultures. Aeration was effected by bubbling air under pressure through broth in sintered glass filter flasks or through Mandler filter candles in Erlenmeyer flasks of broth. Oxygenation was accomplished in like manner using pure oxygen under pressure. A thin layer of mineral oil or pure lard was placed on the surface of the medium to prevent foaming. The effluent from the sealed flasks was passed through a series of large cotton filters to prevent the liberation of infectious aerosols into the laboratory air. Cultures were incubated at 37 C until maximum growth had occurred (3 to 5 days), and aliquot portions were removed for plate counts.

Aerated cultures of *M. mallei* were homogeneous heavy suspensions without pellicle formation. Although cultures of *M. pseudomallei* showed moderate pellicle formation, the broth was cloudy throughout, and the amount of pellicle was much less than in nonaerated cultures. The maximum viability counts on aerated cultures of both organisms varied from 1×10^9 to 5×10^9 organisms per ml, compared with 8×10^7 to 2×10^8 organisms per ml in nonaerated cultures. Oxygenation produced maximum counts of 8×10^9 to 2×10^{10} organisms per ml.

DISINFECTION

Several of the strains (C3, C4, C5, C7, and W294) of *Malleomyces* studied were highly infectious for animals, and *M. mallei* proved to be infectious for man (Howe and Miller, 1947). It was of considerable importance, therefore, to determine the relative efficacy of common disinfectants against these organisms. Tests were made on sodium hypochlorite, phenol, "roccal" (benzalkonium chloride), tincture of iodine, lysol, mercuric chloride in alcohol, and potassium permanganate. One-tenth ml of a saline suspension containing 50 million organisms was added to 5 ml of each disinfectant solution. At intervals of 5, 10, 15, and 30 minutes, 0.2 ml of the organism-disinfectant mixture were withdrawn and inoculated into 10-ml broth tubes. In testing hypochlorite solutions, 0.01 per cent ammonium thiosulfate was added to the broth tubes to neutralize the hypochlorite carried over from the test mixture. The broth was observed for turbidity after 72 hours' incubation, and 0.1 ml of the broth was plated out as a final test for viability.

Table 2 gives the concentrations of the disinfectants tested and the minimum time necessary for sterilization of the suspension of organisms. Hypochlorite (500 ppm available chlorine), "roccal" (1:2,000), iodine, mercuric chloride in alcohol, and potassium permanganate were highly effective. Phenol was less effective and lysol was ineffective. "Roccal" (1:2,000) was chosen for routine use because of its high disinfectant action and its lack of corrosive and irritative action.

PRESERVATION OF VIABILITY AND VIRULENCE

The preservation of the viability and the virulence of the various strains was investigated, since the literature gave little information on this subject and no reference to the use of lyophilization was found.

Viability and virulence in stored cultures. Agar and broth cultures of *M. mallei* and *M. pseudomallei* remained viable for 2 to 3 months when stored at room temperature (22 to 28 C), but died more rapidly when stored in the 37 C incubator, in the refrigerator (3 to 5 C), or in the frozen state (minus 10 to 30 C). Although still viable, cultures of *M. mallei* showed a marked drop in viru-

TABLE 2

The germicidal activity of common disinfectants on M. mallei and M. pseudomallei

DISINFECTANT SOLUTION	TIME IN MINUTES NECESSARY TO KILL	
	<i>M. mallei</i>	<i>M. pseudomallei</i>
Sodium hypochlorite		
500 ppm chlorine	5	5
100 ppm chlorine	30	15
Phenol:		
1%	15	15
5%	15	10
"Roccal":		
1:2,000	5	5
1:5,000	>30	30
Tincture of iodine (7%)		
1:10	5	5
Lysol:		
1%	>30	>30
3%	>30	>30
Mercuric chloride:		
(1% in 35% ethyl alc.)	5	5
Potassium permanganate		
(1% in 1% HCl)	5	5

lence for experimental animals after storage for 4 to 6 weeks at any of the temperatures mentioned. When the cultures of *M. mallei* were transferred weekly or biweekly, virulence remained constant for 2 to 3 months. Virulence was kept at a constant level by transferring stock cultures weekly and passing each strain through hamsters once a month. *M. pseudomallei* (virulent strain W294), however, retained a constant degree of virulence for experimental animals without animal passage, even when stored in culture for 2 to 3 months.

Lyophilization preserved the viability and virulence of both organisms for relatively long periods. Heavy suspensions of organisms were prepared by emulsifying the growth scraped from agar plates into sterile inactivated rabbit serum. Horse serum could not be used, since it contained a thermostabile component that was lethal for *Malleomyces* organisms. Suspensions were lyophilized

with a portable lyophile apparatus in a refrigerated room held at 0 C. This was found to be essential since all organisms were killed when lyophilization was done with the apparatus in a room at 15 to 20 C. It was apparent that it was necessary to keep the temperature of the frozen suspensions considerably below 0 C to obtain successful lyophilization. The quantitative preservation of viability was determined by plate counts on suspensions before lyophilization and after various intervals of storage. The two species of *Malleomyces* showed about the same degree of preservation. The average of two separate tests with each species showed that with an original count of 6.9×10^{10} organisms per ml, 23 per cent of the cells were viable 24 hours after lyophilization and 8.3 per cent were viable 13 weeks after lyophilization.

In one additional test with *M. pseudomallei* the count after 6 months of storage was 6×10^7 compared with the original count of 6.8×10^8 organisms per ml. The virulence of *M. mallei* was lowered moderately by prolonged storage in lyophilized culture, but was rapidly restored by one animal passage through hamsters. The virulence of *M. pseudomallei* was not changed after 6 months' storage, as indicated by MLD titrations in hamsters.

TABLE 3
Viability of M. mallei and M. pseudomallei in tap water

TIME	M. MALLEI ORGANISMS PER ML	M. PSEUDOMALLEI ORGANISMS PER ML
At once	3.5×10^7	1.8×10^7
2 weeks	4.0×10^7	2.0×10^8
4 weeks	1.0×10^8	4.0×10^7
5 weeks	0	7.0×10^8
8 weeks	0	1.0×10^8

Viability in tap water. The transmission of glanders in horses has long been known to occur by contamination of water in drinking troughs (Hutyra and Marek, 1926). Several human cases of melioidosis are reported after ingestion or inhalation of lake or pond water (Huard and Long, 1937). The degree of survival of *Malleomyces* organisms in water was studied by making a dilute suspension of the 24-hour growth scraped from the surface of an agar slant culture into sterile tap water. The dilute suspension was stored at room temperature, and triplicate plate counts were made at once and after 2, 4, 5, and 8 weeks of storage. The results given in table 3 show that *M. mallei* survived well for 4 weeks and then decreased in numbers rapidly. *M. pseudomallei* apparently increased in numbers during the first 2 weeks, and a high percentage survived during the 8 weeks of storage. The degree of survival of these organisms in tap water indicates that water sources might remain infectious to animals or man for several weeks after pollution.

ISOLATION OF MALLEOMYCES FROM CONTAMINATED SPECIMENS

Because of the marked variation in the clinical signs and symptoms, the diagnosis of acute glanders or of melioidosis in man is largely dependent upon

isolation of the etiological agent. When bacteremia is present, isolation by blood culture offers no difficulties. However, isolation from contaminated biological materials such as exudates, sputum, feces, and urine and from possible natural sources such as food, water, or soil requires special techniques. Studies were undertaken to develop methods of differential cultivation and animal inoculation that would permit the isolation and recognition of these organisms when present in small numbers in contaminated specimens.

Differential cultivation. Plate counts were performed on a suspension of one or more strains of each organism on agar plates containing serial dilutions of a number of common inhibitor dyes and on plain glycerinated agar plates. The interpretation of the results was based on the plate counts and the appearance of the growth. A list of suitable inhibitor dyes with the maximum concentration that gave no inhibition of growth of *M. mallei* and *M. pseudomallei* is as follows:

Crystal violet.....	1:200,000
Proflavine.....	1:500,000
Acriflavine.....	1:500,000
Acridine orange.....	1:500,000
Acridine yellow.....	1:500,000
Basic fuchsin.....	1:100,000
Acid fuchsin.....	1:100,000
Malachite green.....	1:1,000,000
Brilliant green.....	1:1,000,000

All of these dyes, in the concentration indicated, inhibited gram-positive organisms. Although any one of the dyes could be used successfully to inhibit gram-positive organisms, crystal violet was chosen for routine use. None of the dyes were suitable for differential inhibition of other gram-negative organisms since concentrations adequate for this purpose also inhibited *Malleomyces* organisms.

Eosin-methylene-blue medium was adapted for use with *Malleomyces* by adding 4 per cent glycerol. *Malleomyces* grew well on this medium and the colonies were clear or slightly bluish. Differentiation from coli-aerogenes colonies was made with ease. The limitations of this method for isolation of *Malleomyces* from fecal suspensions were: (1) coli-aerogenes colonies become comparatively large in the 48-hour incubation period necessary for the development of *M. mallei*, and (2) the lack of any significant inhibitory action of this medium allows overgrowth of gram-positive as well as gram-negative contaminants in all except the higher dilutions of the specimen.

Rapid colonial identification tests. Studies were undertaken to find methods of rapid identification of suspicious *Malleomyces* colonies on crystal violet agar. The oxidase test (Ellingworth *et al.*, 1929; Gordon and McLeod, 1928) was found to be positive on *Malleomyces* colonies. Since there was an interval of about 5 minutes between the development of a positive test and death of the organisms, the colonies giving a positive test could be subcultured or inoculated into hamsters.

The rapid slide agglutination technique was applied and gave dependable

results for confirming suspected *Malleomyces* colonies. The two species of the genus could not be differentiated by this test, however, because of the serological cross reactions (Cravitz and Miller, 1948).

Use of antibiotics. Since penicillin had been found to exert no effect on *Malleomyces in vitro* (Miller, Pannell, and Ingalls, 1948), this antibiotic was used to reduce the number of the other organisms in contaminated specimens. Specimens were incubated for 3 hours at 37 C in saline containing 1,000 units of penicillin per ml and were then plated out on crystal violet agar or injected into hamsters. Gram-positive organisms were killed or inhibited and isolation of *Malleomyces* organisms was greatly facilitated.

Animal inoculation. Saline extracts or suspensions of suspected materials were injected subcutaneously and intraperitoneally into adult male hamsters. For grossly contaminated material, the suspension was incubated with penicillin for 3 hours as described above before animals were inoculated. The development of a Straus reaction, the death of the animal with typical findings at autopsy of acute glanders or melioidosis, and cultivation of the organisms from infected tissues gave proof of the presence of *Malleomyces* organisms in the specimen.

Application of the methods. In order to test the efficiency of the methods, studies were conducted to determine the lowest proportion of *Malleomyces* to total contaminant organisms that would permit recovery of *Malleomyces*. Normal animal cage sweepings were suspended in isotonic saline solution as a source of contaminants and plate counts were made to determine the number of organisms present. Known numbers of *M. mallei* and of *M. pseudomallei* were added to aliquot portions of this suspension. The cultural isolation technique was tested by inoculating these mixed suspensions on crystal violet agar plates and identifying the colonies of *Malleomyces* by the oxidase reaction and slide agglutination tests. The animal inoculation technique was tested by treating the mixture with penicillin for 3 hours and inoculating aliquot portions subcutaneously and intraperitoneally into hamsters.

It was found that *Malleomyces* could be easily and uniformly recovered by the cultural method when the original proportion of these organisms to contaminants was as low as 1 to 100. Since hamsters were very susceptible to glanders and melioidosis (MLD 15 to 20 organisms), and not very susceptible to infection with the contaminants present, *M. mallei* and *M. pseudomallei* could be uniformly recovered by the animal inoculation method when the original proportion of *Malleomyces* to contaminants was as low as 1 to 12,500.

Application of the methods to feces samples to which *Malleomyces* organisms were added was less successful. EMB agar gave satisfactory differentiation of colonies, but, because of the limitations of the method, the proportion of *Malleomyces* to *Escherichia* and *Aerobacter* organisms had to be larger than 1 to 100 to get uniform recovery. Injections of fecal samples into hamsters frequently produced fatal coli-aerogenes infections unless the specimen was highly diluted.

M. mallei and *M. pseudomallei* were isolated from blood, urine, and purulent exudates from infected hamsters and guinea pigs by the foregoing methods. *M. pseudomallei* was isolated from the purulent nasal discharge of infected rabbits. Attempts to isolate *Malleomyces* from feces of infected animals were un-

successful. Application of the methods in six human cases of acute pulmonary glanders failed to reveal the specific organisms in the blood or sputum (Howe and Miller, 1947).

DISCUSSION

The intracellular granulations prominent in stained preparations of the organisms and in electron micrographs appear to be lipoid globules and accumulations of increased protoplasmic density. This is in contrast to the apparently similar granulations in the genus *Corynebacterium*, which are thought to be protein materials within the cells.

The vigorous growth and the stability in respect to viability and virulence of *M. pseudomallei* were outstanding characteristics of this organism. The fact that both species of *Malleomyces* organisms remained viable for at least 4 weeks, when suspended in tap water, emphasized the importance of ingestion or inhalation of polluted water supplies as a means of transmission of glanders and melioidosis.

The two species of the genus *Malleomyces* are very closely related. They are indistinguishable morphologically, difficult to distinguish serologically (Cravitz and Miller, 1948), and they produce diseases in experimental animals that are practically indistinguishable clinically and pathologically (Miller, Smith, and Tanner, 1948). The two species differ in only a few respects. *M. pseudomallei* is motile, grows in 24 hours on ordinary media, produces predominately rough colonies on agar, has greater proteolytic activity, and tends to produce a more fulminating disease. *M. mallei* is nonmotile, requires 48 hours' incubation for growth, and requires glycerol in the culture medium.

Malleomyces organisms have a number of characteristics that suggest a relationship to *Mycobacterium tuberculosis*. The cells contain a complex lipoidal material (Worley and Young, 1945; Hink, Miller, and Tanner, 1948); the organisms grow in a synthetic medium developed for *Mycobacterium tuberculosis*; *M. mallei* requires glycerol in the medium and the growth of *M. pseudomallei* is enhanced by its presence; skin test substances (mallein and whitmorine) are produced that have many of the properties of tuberculin; and the lesions produced are granulomatous in character. Furthermore the gross pathological changes in experimental animals show a marked resemblance to those in acute miliary tuberculosis (Miller, Smith, and Tanner, 1948). The two genera differ in a number of other characteristics, but the similarities are worthy of note.

The lack of detailed knowledge of the mechanisms of natural transmission suggests the need for careful studies on the epidemiology of glanders and melioidosis. The methods described for isolation of the organisms by differential cultivation and animal inoculation would be of assistance in analyzing the role of animal carriers, insect vectors, and polluted food and water supplies in the transmission of these diseases.

SUMMARY

Electron micrographs of *Malleomyces* organisms show intracellular refractile bodies resembling lipoid globules and opaque areas of increased protoplasmic

density. *Malleomyces pseudomallei* possesses lophotrichate flagella, whereas *Malleomyces mallei* is atrichous.

Both organisms grew well on beef extract base media. *M. mallei* required the addition of glycerol. Both organisms grew well in Luhrs' modification of Long's synthetic medium for *Mycobacterium tuberculosis*. Aeration and oxygenation of broth cultures produced a heavy growth with an even turbidity and little or no pellicle.

Studies of the efficacy of common disinfectant solutions showed "roccal" (benzalkonium chloride), hypochlorite, iodine, and mercuric chloride to be highly effective. Phenol was less effective and lysol was ineffective.

Viability and virulence of cultures were well preserved over periods of at least 3 to 6 months by lyophilization.

Both organisms remained viable for at least 4 weeks when suspended in ordinary tap water, and *M. pseudomallei* apparently increased in numbers.

Effective methods of isolation of *Malleomyces* organisms from contaminated specimens by differential cultivation, rapid colonial identification, and animal inoculation are described.

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