STAINING BACTERIAL SMEARS WITH FLUORESCENT ANTIBODY

II. Rapid Detection of Varying Numbers of *Malleomyces pseudomallei* in Contaminated Materials and Infected Animals

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The identification of pure cultures of *Malleomyces pseudomallei* in dried smears by use of fluorescent antibody has been described in the previous paper (Moody *et al.*, 1956). The present report describes the application of the method in the detection of these organisms in the presence of other bacteria, in soil, and in animal tissues. The results obtained in this investigation illustrate the potentialities of the procedure for detecting certain bacteria in the environment.

EXPERIMENTAL METHODS

The methods used for preparing labeled antiglobulin, and for preparing, staining, and examining smears were the same as described previously (Moody *et al.*, 1956). All experiments were done with *M. pseudomallei* strain 53491 and its homologous antiglobulin. Cultures of *M. pseudomallei* were incubated on heart infusion agar (Difco) slants for 18 hr at 37 C. The cells were suspended in 0.85 per cent saline or in heart infusion broth (Difco) and tenfold dilutions were made in each menstruum. Plate counts of selected dilutions of *M. pseudomallei* showed that the original suspension contained approximately 2.2×10^9 viable cells per ml (hereafter referred to as a standard suspension). Formalin in a final concentration of 0.5 per cent was then added to all standard suspensions. Suspensions and dilutions of other bacteria were prepared in the same manner.

Smears approximately 15 mm in diameter were prepared on slides and allowed to dry. They were fixed by heat and exposed for 15 min to a 1:2 dilution of fluorescein-labeled anti-*M. pseudomallei* globulin.

Smears were examined by counting the number of fluorescing cells in each of 10 to 15 microscope fields using a 2 mm objective and a 10 × ocular. The results recorded were in terms of the average number of fluorescing cells per field. In smears in which the number of cells was very low, results were recorded as the total number of fluorescing cells observed in 2 or 3 trips across the smear.

Controls consisted of smears that were not exposed to any labeled globulin and of smears exposed to normal rabbit globulin.

RESULTS

Detection of individual cells in dilutions prepared from pure cultures of M. pseudomallei. Smears were prepared directly from broth and saline dilutions of standard suspensions of *M. pseudomallei*, and from sediments obtained by centrifuging 1 ml quantities of various dilutions of these suspensions for 15 minutes at 2500 rpm. Smears prepared from saline suspensions consistently showed more cells than the smears from comparable broth suspensions. In addition, more organisms were detected on smears prepared from centrifuged suspensions than on smears made directly from the various dilutions (table 1). Specifically-stained fluorescing cells were observed in smears prepared from saline suspensions containing as few as 220 organisms per ml.

Detection of M. pseudomallei in the presence of bacterial contaminants. Smears prepared from mixtures adjusted to contain varying numbers of *M. pseudomallei* and contaminants were exposed to homologous labeled globulin, and examinations were made of the original, and centrifuged, specimens.

M. pseudomallei was detected readily in the presence of other bacteria (figures 1 and 2). As before, saline suspensions consistently showed more fluorescing organisms than corresponding dilutions made in broth, and more cells were detected in the centrifuged suspensions. In all but one experiment, homologous organisms were

TABLE 1

Detection of Malleomyces pseudomallei cells in various bacterial suspensions

Number of *M. pseudomallei* Cells per ml of Mixture	Number of Cells of Contaminant Per ml of Mixture	Ratio of *M. pseudomallei* to Contaminant	Average Number *Malleomyces pseudomallei* Cells Seen*			
			Heart infusion broth diluent		NaCl diluent	
			Direct	Centrifuged	Direct	Centrifuged
2.2×10^8			13	—	TNTC‡	—
2.2×10^7			5	—	96	—
2.2×10^6			35†	1	14	55
2.2×10^5			10†	18†	3	14
2.2×10^4			0	5†	40†	2
2.2×10^3			0	3†	8†	80†
2.2×10^2			0	0	1†	6†
2.2×10^1			0	0	0	0
2.2×10^7	*Pseudomonas aeruginosa*, 2.3×10^9	1:100	5	—	—	—
2.2×10^6		1:1,000	0.1	—	—	—
2.2×10^5		1:10,000	0	50†	2	8
2.2×10^4		1:100,000	0	12†	13†	1
2.2×10^3		1:1,000,000	—	—	3†	10†
2.2×10^2		1:10,000,000	—	—	0	1†
2.2×10^7	*Serratia marcescens*, 1.7×10^9	1:77	6	—	—	—
2.2×10^6		1:770	3	—	—	—
2.2×10^5		1:7,700	2†	40†	3	9
2.2×10^4		1:77,000	0	12†	18†	1
2.2×10^3		1:770,000	—	—	3†	9†
2.2×10^2		1:7,700,000	—	—	0	1†
2.2×10^7	*Bacillus globigii*, 6.3×10^8	1:28	6	—	—	—
2.2×10^6		1:280	0.5	—	—	—
2.2×10^5		1:2,800	0	57†	2.5	10
2.2×10^4		1:28,000	0	15†	15†	2
2.2×10^3		1:280,000	—	—	2†	9†
2.2×10^2		1:2,800,000	—	—	0	2†
2.2×10^7	*Bacillus cereus*, 9.0×10^7	1:4	7	—	—	—
2.2×10^6		1:40	1	—	—	—
2.2×10^5		1:400	0	52†	1.5	6
2.2×10^4		1:4,000	0	13†	12†	1
2.2×10^3		1:40,000	—	—	3†	12†
2.2×10^2		1:400,000	—	—	0	0

* Average number of fluorescent cells per field observed in 10 to 15 oil immersion fields.

† Number of fluorescent cells observed in two trips across the smear.

‡ Too numerous to count.

detected in the samples containing as few as 220 cells of *M. pseudomallei* per ml regardless of the ratio of homologous to heterologous cells in the suspension. In the case of the mixture containing *M. pseudomallei* and *Pseudomonas aeruginosa* this ratio was 1:10,000,000.

Reactions with labeled normal globulin were negative. Controls in which the contaminants alone were tested with labeled anti-*M. pseudomallei* globulin also were negative.

Detection of M. pseudomallei in raw soil. In order to investigate the possibility of detecting *M. pseudomallei* in the presence of gross amounts of environmental debris, one ml each of tenfold (10^{-1} through 10^{-6}) dilutions of a standard saline suspension of *M. pseudomallei* were added

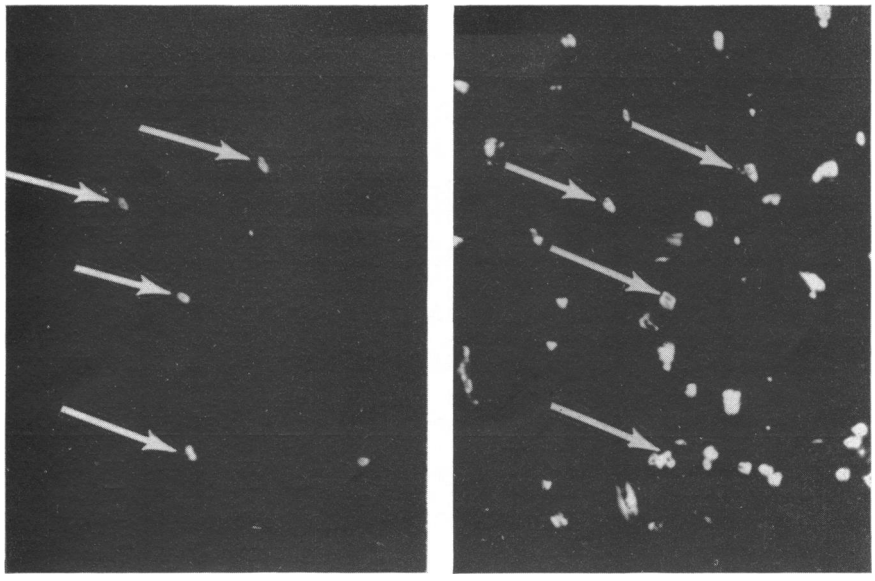

Figure 1. Malleomyces pseudomallei mixed with *Pseudomonas aeruginosa.* Photographs were 35 mm Super XX film. In figures 1, 2, and 3 the photograph on the left was made with fluorescence filters in place; the one on the right is the same field photographed without fluorescence filters and using a dark-field condenser. In figure 4, the one on the left is a fluorescence photograph; the one on the right shows the same field after staining with crystal violet, using a dark-field condenser.

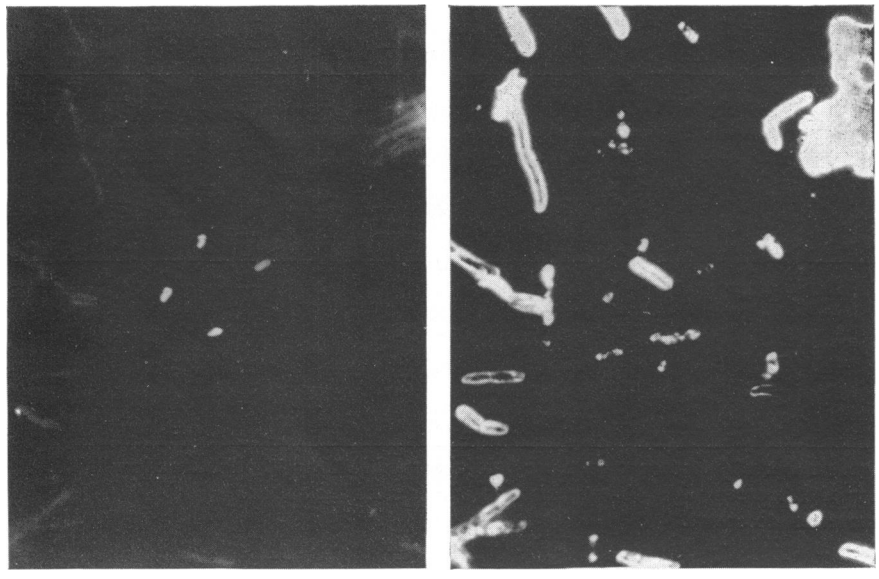

Figure 2. Malleomyces pseudomallei mixed with *Bacillus cereus.* See legend under Figure 1

to one-gram samples of raw soil collected near the laboratory. Smears of these mixtures were made on microscope slides. Each mixture then was diluted with 9 ml of saline, mixed thoroughly, and filtered through Whatman Number 4 filter paper to remove gross soil particles; smears were prepared directly from the filtrates. The filtrates then were centrifuged and additional smears prepared from the sediments. All smears were fixed and stained with labeled globulin as described previously.

Examination of the smears showed that *M.*

TABLE 2

Detection of Malleomyces pseudomallei in raw soil

Number of *M. pseudomallei* Organisms		Avg Number of Fluorescent *M. pseudomallei* Cells Seen*		
Dilution	Number cells	Direct smear of seeded soil	Smear of filtered soil suspension	Smear of sediment from filtrate
10^{-1}	2.2×10^{8}	9.0	7.5	15
10^{-2}	2.2×10^{7}	2.5	0.4	1.5
10^{-3}	2.2×10^{6}	3.0†	2†	16†
10^{-4}	2.2×10^{5}	0	0	6†
10^{-5}	2.2×10^{4}	0	0	1†
10^{-6}	2.2×10^{3}	0	0	0

* Average number of fluorescent cells observed per field in 10 to 15 oil immersion fields.

† Number of fluorescent cells observed in 3 trips across the smear.

pseudomallei could be detected when mixed with raw soil (table 2). Although certain particles in soil exhibited fluorescence, they were distinguished readily from the fluorescent bacteria on the basis of morphology. Other bacteria seen in the smears did not exhibit significant fluorescence (figure 3). Smears prepared from filtered soil suspensions were free of larger particles of debris and were easier to examine than smears prepared from the original seeded soil, but the number of organisms detected was essentially the same in both cases. As was expected, more organisms were found in smears from centrifuged filtrates than in the other types of smears. In such preparations *M. pseudomallei* was detected in mixtures containing as few as 2.2×10^{4} cells per g of soil. All controls stained with labeled normal globulin were negative. Controls in which smears were examined for fluorescence prior to staining with labeled globulin also were negative.

Detection of M. pseudomallei collected from exposed surfaces. Since, as described above, staining with fluorescent antibody permitted the identification of non-viable organisms of *M. pseudomallei*, the feasibility of using this procedure to detect this pathogen on experimentally exposed surfaces was investigated. Tenfold dilutions of standard suspensions of non-viable *M. pseudomallei* were prepared in heart infusion broth. Starting with the more dilute suspensions, 3 ml of dilutions 10^{-1} through 10^{-5} were sprayed with a small atomizer into a Blickman hood, approximately 40 cubic feet in volume, which contained a layer of tree leaves. The spraying was done while the exhaust fan was not in operation. Different batches of leaves were used for each dilution, and after they were sprayed they were allowed to stand (approximately 1 hr) until no more moisture could be seen on them. They were then transferred to sterile petri dishes and held overnight at room temperature. A cotton swab, moistened with 0.85 per cent saline, was rubbed over the surfaces of the leaves, and then rolled

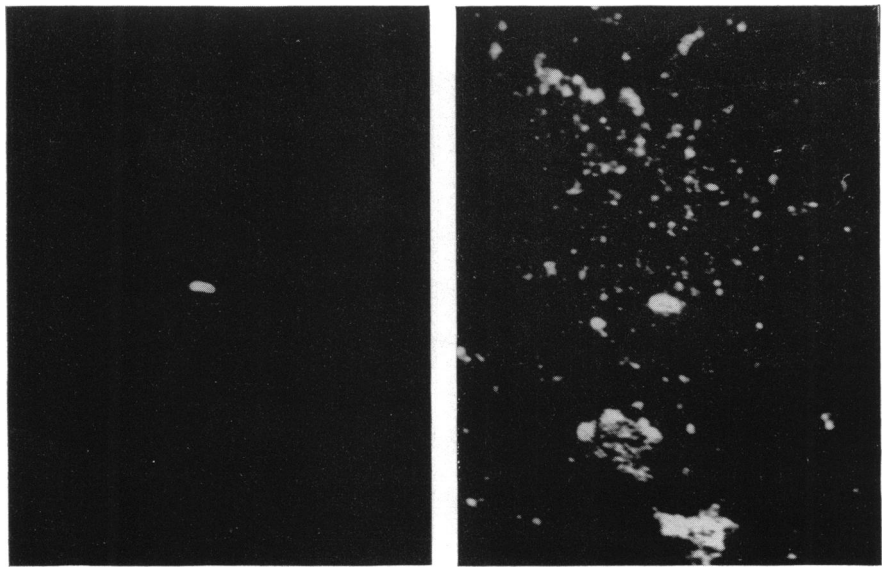

Figure 3. Malleomyces pseudomallei mixed with soil. See legend under Figure 1

TABLE 3

Detection of Malleomyces pseudomallei on surfaces exposed to aerosols

Dilution of Standard Suspension‡	Number Cells Sprayed Per Cubic Foot	Avg Number of *M. pseudomallei* Cells Seen*
10^{-1}	1.65×10^{7}	9.0
10^{-2}	1.65×10^{6}	1.5
10^{-3}	1.65×10^{5}	15.0†
10^{-4}	1.65×10^{4}	4.0†
10^{-5}	1.65×10^{3}	0

* Average number of fluorescent cells observed per field in 10 to 15 oil immersion fields.

† Total number of fluorescent organisms observed per entire smear.

‡ 3 ml of each dilution was sprayed.

onto the surfaces of two microscope slides. After air-drying and fixing with heat, one smear was stained in the usual manner with labeled anti-*M. pseudomallei* globulin, the other with labeled normal globulin.

The smears showed that *M. pseudomallei* could be detected on surfaces exposed to aerosols calculated to contain as few as 1.65×10^{4} cells per cubic foot (table 3). No fluorescent cells were observed in smears stained with labeled normal globulin.

Detection of M. pseudomallei organisms in impression smears from infected animals. M. pseudomallei can be recovered by cultural methods from lesions occurring in infected animals. In order to determine whether the organism could be identified by fluorescent antibody in smears made directly from infected tissue, the following experiment was performed. Eight male guinea pigs were injected intraperitoneally with approximately 5.5×10^{8} organisms of *M. pseudomallei* (53491). All of the animals died within 18 to 30 hr, presumably as a result of toxicity (Nigg *et al.*, 1955). They were autopsied and impression smears of the cut surfaces of the liver and spleen were prepared on microscope slides. The slides were exposed to labeled anti-*M. pseudomallei* globulin and labeled normal globulin in the usual manner. Impressions also were made on heart infusion agar plates which were incubated at 37 C overnight.

All plates showed pure cultures of *M. pseudomallei*. In all cases impression smears exposed to labeled anti-*M. pseudomallei* globulin revealed numerous bacteria which were brilliantly fluorescent (figure 4). No fluorescent bacteria were observed in smears exposed to labeled normal globulin.

DISCUSSION

Fuorescent antibody staining, when suitable controls are used, is of greater diagnostic value than other types of staining reactions used in

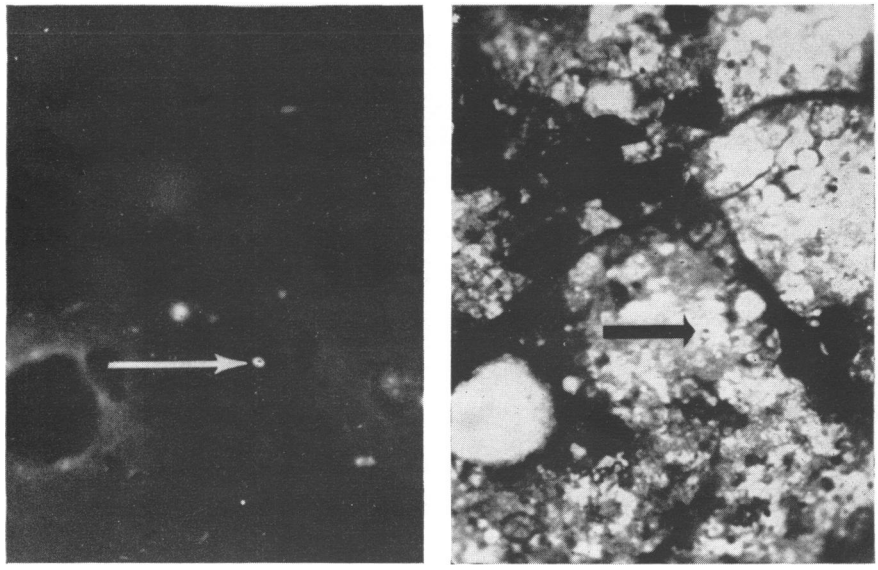

Figure 4. Malleomyces pseudomallei in impression smears from infected mouse liver. See legend under Figure 1.

bacteriology because it is dependent upon an antigen-antibody reaction. Also, the organisms, which occur as bright objects against a dark background, are generally easier to find.

The results presented here show that individual cells of *M. pseudomallei* were detected not only from pure cultures but also from soil, from tissues of infected animals, and from suspensions containing massive concentrations of other bacteria. In all cases the bright specific fluorescence of cells which reacted with homologous fluorescent antibody distinguished them readily from other materials present in the smears. These results suggest the possibility of using the fluorescent antibody technique to detect *M. pseudomallei* in clinical specimens as well as in other types of materials exposed to the organism.

Unpublished studies in this laboratory have shown that to detect *M. pseudomallei* by agglutination approximately 10^8 organisms per ml are required. The results presented here show that *M. pseudomallei* was identified in suspensions containing as few as 220 cells per ml when smears of such suspensions were stained with homologous fluorescent antibody. Similar results were obtained even in the presence of large numbers of other bacteria or soil particles.

The fact that killed cells of *M. pseudomallei* were detected from surfaces previously sprayed with the organisms suggests that staining with fluorescent antibody may be useful as a screening method for rapid identification of bacteria in areas suspected of being contaminated.

In impression smears of organs from infected animals the staining of bacteria with fluorescent antibody was of little value as a rapid diagnostic procedure since the number of organisms inoculated, 5.5×10^8, was sufficient to permit identification by the microscopic slide agglutination test. However, the results were useful in showing that the bacteria could be located so readily in tissues. It will be necessary to investigate further the relationship of dosage to speed of detection with fluorescent antibody before definite statements can be made about the value of animal inoculations.

The projection of these findings with *M. pseudomallei* to other bacteria of medical or public health importance must be made with caution. Before organisms can be identified with sufficient accuracy they must show some degree of characteristic morphology, and the specific staining reaction must be sufficiently strong to stand out against the low level, non-specific fluorescence which is found in practically all cells. In addition, it may be difficult or impossible to differentiate between closely related species because of cross reactions.

SUMMARY

Using fluorescein-labeled anti-*Malleomyces pseudomallei* globulin, it was possible to identify cells of the homologous species on smears prepared from: (a) Suspensions containing as few as 220 homologous cells per ml. This occurred when suspensions contained only *M. pseudomallei*, or *M. pseudomallei* plus other bacterial species in a ratio of as much as 1:10,000,000. (b) Experimentally seeded soil. (c) Material collected from surfaces previously sprayed with suspensions of the organism. (d) Tissues of experimentally infected animals.

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