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Application of Indirect Immunofluorescence Microscopy to Colony Identification of *Pseudomonas pseudomallei*

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SUMMARY **Indirect immunofluorescence microscopy was used as a colony identification method of *Pseudomonas pseudomallei* isolates. The antisera against lipopolysaccharide and protein fractions of *P. pseudomallei* were prepared in guinea pigs and rabbits. With these antisera and fluorescence-labelled anti-guinea pig IgG and anti-rabbit IgG prepared in sheep (goat), indirect immunofluorescence microscopy was conducted on the colonies of *P. pseudomallei* and other species of bacteria.**

The overall results indicated that this method is efficient, rapid and specific for identification of *P. pseudomallei* colonies from clinical specimens.

Pseudomonas pseudomallei is the causative organism of melioidosis, an endemic disease prevalent mainly in Southeast Asia and the northern area of Autralia. The disease takes various clinical courses; acute, subacute, chronic, and inapparent. Clinical manifestations are so varied resembling to many other infectious diseases that it is often called "the great imitator[1]". The lesions can be produced in various organs and tissues in melioidosis, and the specimens are obtained from blood, urine, pus, sputum, inflammatory fluid, etc. The definitive diagnosis of melioidosis is possible only by positive culture of *P. pseudomallei,* though serology is available for detection of the infection and for the purpose of epidemiological surveillance. However, a lack of familiarity with the cultural characteristics of the organisms in most clinical laboratories results in the misleading recognition or in the delay of diagnosis and treatment. This is particularly true in non-endemic areas. The isolation of the organisms is often accompanied by contaminating colonies. Colony morphology of *P. pseudomallei* is variable, and the wrinkled appearance of older colonies is misread as contaminants. Though there is a general scheme of colony identification on the basis of growth and biochemical characteristics and with commercial kits such as API 20NE, Microbact 24E, and Titertek NF, the identification of *P. pseudomallei* requires certain amount of laboratory experience. In this regard, screening of *P. pseudomallei* colonies from the clinical specimens by taking advantage of immunological specificity would be practical as a routine procedure. The objective of this communication is to develop an immunological method for the laboratory diagnosis of melioidosis.

MATERIALS AND METHODS

P. pseudomallei strains

1. Culture for antigen preparation : Five local strains (UB12, UB16, UB18, UB19, UB20) were employed, to cover strain differences if any, in the collection of *P. pseudomallei,* which were fresh isolates from melioidosis patients hospitalized in Sappasitthiprasong Ubon Hospital, Ubon Ratchathani during 1990 to 1991. The identification of these strains was described elsewhere.[2,3]

2. Test samples : One hundred eighty-seven isolates of *P. pseudo-*

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mallei and 77 isolates of the other species were subjected to immunofluorescence microscopy with the antisera (see below) for species identification. These strains were donated by Sappasitthiprasong Ubon Hospital, Ubon Ratchanthani by courtesy of Dr Vipada Chaowagul. They were the isolates from blood, pus, sputum, throat swab, urine, and pleural fluid. For hemoculture, the blood taken after careful skin cleaning was inoculated into brain heart infusion broth. The growth in 3 to 7 days was transferred to horse blood agar (HBA) and eosine methylene blue agar (EMBA). Other specimens were plated directly on to HBA, and EMBA, and the developed colonies were then subcultured in thioglycolate medium for 18 to 24 hours. The growth was transferred back to HBA. For urine culture, HBA and EMBA were inoculated with a calibrated loop of 0.01 ml. In the case of urine, only when viable counts of *P. pseudomallei* were more than 10^5 CFU per ml, the culture was employed for the study regarding it as non-contaminating and infection-derived *P. pseudomallei*. These first isolates were sent to our laboratory after having been confirmed by routine identification test[2,3] in Ubon Hospital, and then reconfirmed by fatty-acid profile analysis with gas-liquid chromatography[4], and by pH-activity patterns of acid phosphatase[4] in our laboratory.

Preparation of antigens

1. Protein antigen (MH) from culture filtrate : One colony of each strain on blood agar was selected and transferred to tryptic soy broth. The overnight culture was plated onto tryptic soy agar by using cotton swab. The overnight growth of each culture was inoculated to 5 flasks containing 1.5 l of Mueller-Hinton liquid medium. These flasks were incubated at 37°C for 4 days. After autoclaving at 120°C for 10 minutes, the culture filtrates were harvested by centrifugation at 30,000 × g for 30 minutes. The pooled filtrate of 7.5 l was concentrated to 1,260 ml by a flash evaporator at 40°C. Ammonium sulfate was added to the concentrated filtrate to half saturation. The resulting precipitate was collected by centrifugation at 8,500 × g for 30 minutes, and then dissolved in 200 ml of 1/15 M phosphate buffer, to which were added 200 ml of saturated ammonium sulfate solution. The ammonium sulfate precipitate was dissolved in water and dialysed against water at 4°C for 4 days. After being condensed in volume with Ficoll Type 400 (Sigma Chemical Co., St. Louis, USA), the dialysed material was subjected to gel filtration with Sephadex G-75 column (Pharmacia, Uppsala, Sweden). The elution patterns according to protein determination by Lowry method[5] and sugar determination by anthrone test[6] are presented in Fig. 1. Three pooled eluted-fractions from tube Nos. 10 to 14, 15 to 19, and 20 to 25 were obtained and designated as MH-a, MH-b and MH-c, respectviely (Fig. 1). Their yields were 107, mg, 245 mg, and 74 mg in lyophilized weight, respectively. Their protein content was 17%, 21%, 22% and sugar content was 50%, 39%, 28%, respectively. MH-a was employed for antisera preparation since this fraction together with MH-b was considered to contain the antigens corresponding to those of UB 12 strain and UB 16 strain which reacted to the sera of melioidosis patients in gel-diffusion tests.[7]

2. Preparation of lipopolysaccharide (LPS) fraction : The preparation method was basically the same as that of Westphal and Jann.[8] Five grammes of cell pellet suspended in 50 ml of water was sonicated and then centrifuged at 1,500 × g for 10 minutes. The resulting supernatant was warmed up in a water bath at 68°C and 70 ml of 90% phenol prewarmed to 68°C were added. After being mixed vigorously, it was incubated at 68°C for 15 minutes. The mixture was then cooled to 10°C and centrifuged at 3,000 × g for 15 minutes at 10°C. The mixture was separated into two layers. The upper layer was set aside, and the lower was extracted with phenol as above. The upper layers were then combined and dialyzed against water for 4 days to remove phenol and low molecular weight materials. The dialyzed extract was concentrated to 5 ml with Ficoll, and centrifuged at 3,000 × g for 10 minutes. The supernatant was further centrifuged at 100,000 × g for one hour. The resulting sediment was resuspended in 5 ml of saline and washed 3 more times by centrifugation as above. The final sediment was suspended in water and lyophilized to be used as LPS fraction.

Preparation of antisera

The antisera each to MH-a and LPS were prepared by immunizing 5 guinea pigs and 3 rabbits. Guinea pigs weighing around 250 g were injected intramuscularly 3 times at one week intervals, each time employing 200 μg of MH-a or LPS emulsified with Freund complete adjuvant. Rabbits weighing aroung 2,000 g were immunized intramuscularly with 2 mg of the antigens in the same way as above. Three weeks after the final immunization, the animals were exsanguinated to separate the sera. The sera were pooled and inactivated, at 56°C for 30 minutes.

Immunofluorescence staining and microscopy

Colonies of *P. pseudomallei* on tryptic soy agar were suspended in PBS to a concentration of approximately 10^9 cells per ml. This suspension was made by turbidity comparison with McFarland standard tube. It was then treated with 0.35 per cent formalin to kill the cells. One drop amount of the

suspension was placed on a glass-slide and air-dried. The cells were fixed with acetone for 15 minutes. The fixed smears were covered with a 1:8 dilution of the pooled antisera and incubated in a moist chamber at 37°C for 45 minutes. The smears were washed 3 times with fresh PBS each-time, and air-dried. Finally, the smear was treated with 20 μl of a 1:20 dilution of fluorescein isothiocyanate-conjugated anti-IgG of homologous species (ICN Immunobiologicals, IL, USA) and incubated in a moist chamber at 37°C for 45 minutes. Fluorescence microscopy was conducted with a fluorescence microscope (Olympus BH).

RESULTS

The results of immunofluorescence microscopy for colony identification of *P. pseudomallei* are summarized in Tables 1 and 2. Fluorescence reactions of the antisera raised in the guinea pigs and rabbits against LPS or MH-a to the isolates of *P. pseudomallei* from various clinical specimens are shown in Table 1.

Most of the test strains showed definite fluorescence of bacterial dimensions, whereas some strain gave faint fluorescence reaction. The faint reaction occurred in 5 and 9 strains out of the total 187 *P. pseudomallei* isolates when anti-LPS guinea pig and rabbit antisera were employed and 20 and 10 out of the 187 test samples when anti-MH-a guinea pig and rabbit antisera were used, respectively (Table 1).

The immunofluorescence reactions of the antisera against *P. pseudomallei* LPS and MH-a against various bacterial species other than *P. pseudomallei* are summarized in Table 2. Although most of the samples were negative in fluorescence, rabbit antisera against *P. pseudomallei* LPS and MH-a produced nonspecific reactions in 6 and 5 out of 19 *P. cepacia* isolates, respectively. The guinea pig antisera against *P. pseudomallei* LPS and MH-a also gave weak reactions to all 3 isolates of staphylococci.

DISCUSSION

Melioidosis is sometimes an acute septicemic disease of high mortality. The disease can also be the persistent infection with frequent opportunities to turn to the acute forms and relapse. Definitive diagnosis is provided only by culture-positivity of the clinical specimens. Therefore, the identification of causative organisms is of vital importance for the diagnosis of melioidiosis. We attempted to develop immunofluorescence microscopy for confirmation of the isolates of *P. pseudomallei.*

The immunofluorescence method has some advantages for this purpose. For immunofluorescence microscopy, the specimens can be stained within 4 hours, thus providing early laboratory information. The results obtained in this study indicated that our expectation was satisfied. The staining with anti-

Table 1. Identification of clinical isolates of *P. pseudomallei* by indirect immunofluorescence microscopy with antisera against lipopolysaccharide (LPS) and protein (MH-a) antigens.

Isolates from	No. of test strains	Fluorescence reactions with below-indicated antisera							
		Guinea pig antisera to				Rabbit antisera to			
		LPS		MH-a		LPS		MH-a	
		Clear	Faint	Clear	Faint	Clear	Faint	Clear	Faint
Blood	62	62	0	58	4	61	1	56	6
Pus	65	63	2	58	7	59	6	61	4
Sputum and Throat swab	39	38	1	35	4	38	1	39	0
Urine	16	14	2	12	4	15	1	16	0
Pleural fluid	3	3	0	2	1	3	0	3	0
Unknown	2	2	0	2	0	2	0	2	0
Total	187	182	5	167	20	178	9	177	10

Table 2. Immunofluorescence microscopy of clinical isolates of heterologous bacterial species with antisera against lipopolysaccharide (LPS) and fraction (MH-a) antigens of *P. pseudomallei*

Bacterial species	No. of test strains	Fluorescence reactions with below-indicated antisera											
		Guinea pig antisera to						Rabbit antisera to					
		LPS			MH-a			LPS			MH-a		
		C	F	N	C	F	N	C	F	N	C	F	N
P. cepacia	23	1	0	22	0	0	23	3	3	13*	1	4	14*
P. aeruginosa	24	0	0	24	0	0	24	0	0	24	0	0	24
Other *Pseudomonas* spp.	25	0	1	24	0	1	24	0	1	24	0	1	24
Staphylococci	3	0	3	0	0	3	0	0	1	2	0	1	2
E. coli	2	0	0	2	0	0	2	0	0	2	0	0	2
Total	77	1	4	72	0	4	73	3	5	65	1	6	66

*Not determined = 4 isolates; C = clear; F = faint; N = negative.

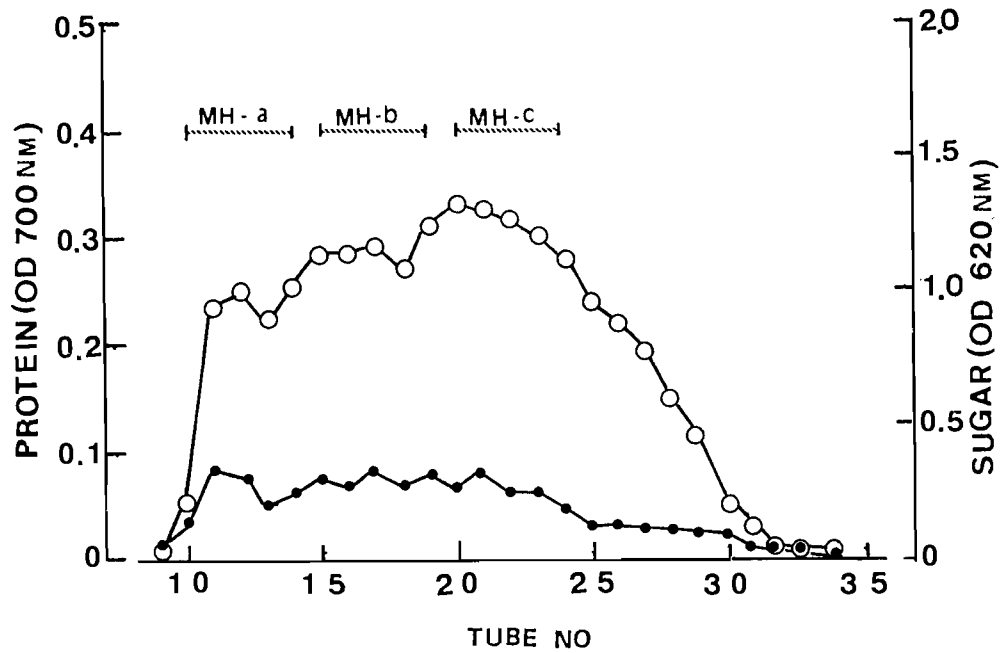

Fig. 1 Gel-filtration with Sephadex G-75 column of ammonium sulfate precipitate fraction obtained from the culture filtrate of *P. pseudomallei*.

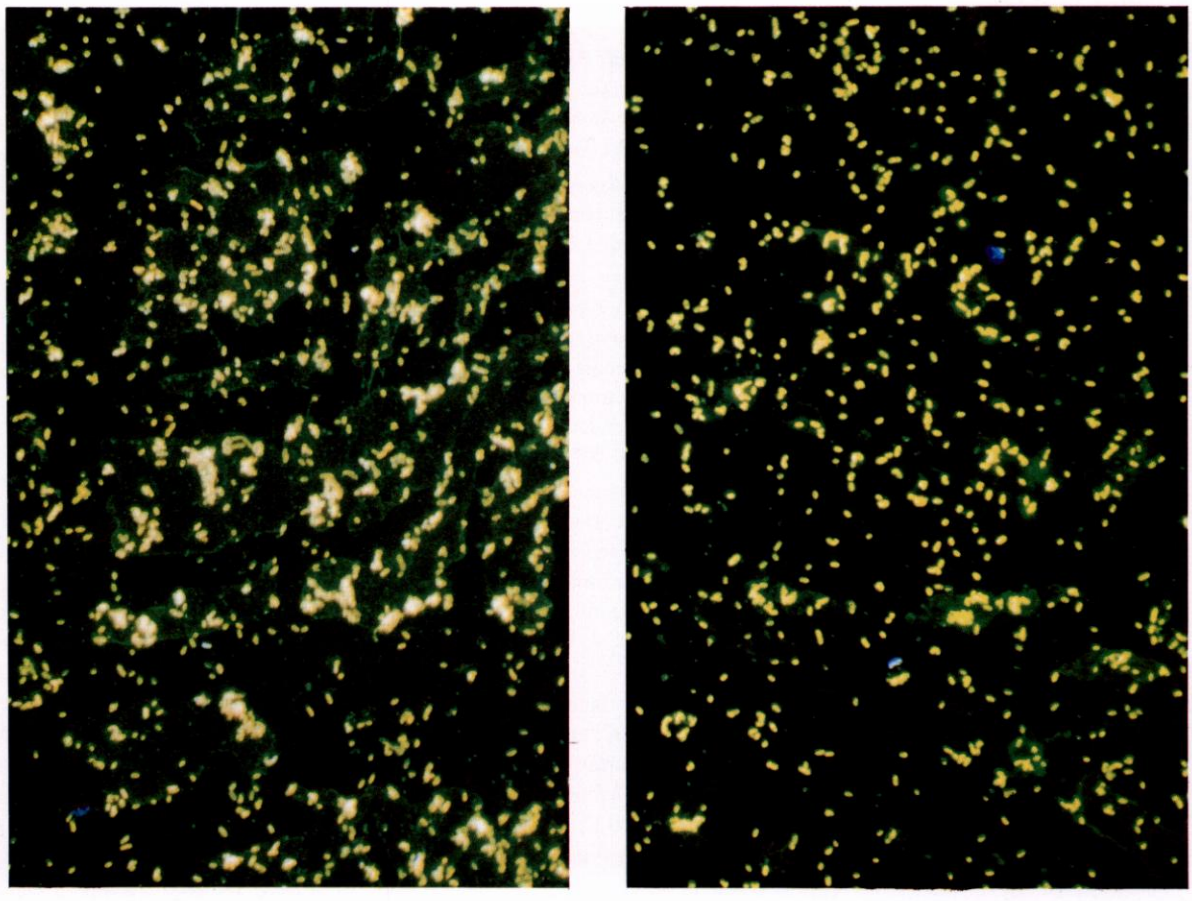

Fig. 2. Immunofluorescence-stained *P. pseudomallei* organisms from the colonies grown on horse blood agar (1,200X).

P. pseudomallei antigens was able to identify the isolates specifically, although weak cross-reaction may occur sometimes to *P. cepacia* and *Staphylococci.* The organisms exhibited clear fluorescence around the periphery, thus revealing a distinct bacterial shape and dimension. Sometimes, however, the stained forms were smaller and irregular suggesting that they were debris of the organisms.

It was also found that the guinea pig and rabbit antisera gave some false positivity of immunofluorescence reactions against the other species of bacteria. Among these, *P. cepacia* is the species very similar to *P. pseudomallei.*[9] Our previous paper reported that, though there are definite differences in metabolism,[10] endotoxicity[11] and growth behaviour[12] between *P. pseudomallei* and *P. cepacia,* cellular fatty acid patterns by gas-liquid chromatography and pH-activity patterns of acid phosphatase were the same in both species.[4] However, the immunofluorescence microscopy with guinea pig antisera to *P. pseudomallei* antigens did not give fluorescence reaction to all the 23 test strains of *P. cepacia,* excepting one case with the antiserum against LPS. Meanwhile, rabbit antisera gave a rather high rate of "false-positivity". Though the reason was not clear, one possibility would be that the immunized rabbits had been naturally contaminated with *P. cepacia.* We have no experience with *P. mallei.* Previous direct immunofluorescence microscopy study with anti-*P. pseudomallei* rabbit sera gave positive fluorescence to *P. mallei.*[13,14] Another "false-positivity" was also found with *Staphylococci.* This occurred in both rabbit and guinea pig antisera and to both of *P. pseudomallei* antigens. There would be some intrinsic questions in this misleading reaction. Since *Legionella* which often exhibits cross-reaction with *P. pseu-*

domallei in IFA staining of antibodies,[15] it must be examined also for our study in future.

An attempt to detect *P. pseudomallei* cells in the pathological specimens has been made in our laboratory. Our preliminary result is successful and indicates that the technique can be used as the rapid diagnosis of melioidosis.

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