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IDENTIFICATION OF SPECIFIC ANTIGEN OF *PSEUDOMONAS PSEUDOMALLEI* AND MONOCLONAL ANTIBODY PRODUCTION FOR THE DIAGNOSIS OF MELIOIDOSIS

PACHARIN RUGDECH

อภินันทนาการ
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ชื่อวิทยานิพนธ์ แอนติเจนจำเพาะสำหรับเชื้อ *Pseudomonas pseudomallei* และการผลิตไมโนโคลนอลแอนติบอดี เพื่อใช้ในการวินิจฉัยโรคเมลิออยโดสีส

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บทคัดย่อ

ในปัจจุบันนี้ วิธีการทางอิมมูโนวิทยาที่ใช้ในการวินิจฉัยโรคเมลิออยโดสีสชนิดเฉียบพลัน ยังให้ผลไม่เป็นที่น่าพอใจนัก ทั้งนี้เนื่องจากยังไม่มีการนำแอนติเจนที่จำเพาะต่อเชื้อก่อโรคมาใช้ และทำให้วิธีการทางอิมมูโนวิทยาที่ใช้กันอยู่ทั่วไปต้องใช้แอนติเจนชนิดหยาบจากตัวเชื้อก่อโรคมาตรวจหาระดับของแอนติบอดีในน้ำเหลืองของผู้ป่วย จึงทำให้ได้ผลที่ยังไม่เป็นที่น่าพอใจ ดังนั้น การศึกษาในครั้งนี้ผู้ทำจึงได้เตรียมโปรตีนจำเพาะขนาด 19.5 กิโลดาลตัน (fractionated 19.5-kDa antigen) จากแอนติเจนชนิดหยาบที่ได้จากตัวเชื้อแบคทีเรียก่อโรค (unfractionated *P. pseudomallei* whole cell antigen) เพื่อใช้ในการพัฒนาการตรวจวินิจฉัยโรคเมลิออยโดสีสให้ได้ผลดีขึ้น แอนติเจนจำเพาะที่เตรียมขึ้นนี้ได้นำไปใช้ในการตรวจหาระดับแอนติบอดีในน้ำเหลืองของผู้ป่วยเมลิออยโดสีสชนิดเฉียบพลัน (acute septicemic melioidosis) โดยวิธี indirect ELISA โดยเปรียบเทียบกับผลที่ได้จากผู้ป่วยที่ติดเชื้อแบคทีเรียชนิดอื่น และใช้ผลที่ได้จากการตรวจหาระดับแอนติบอดีโดยวิธีเดียวกันนี้ทั้งในกลุ่มคนปกติที่อาศัยอยู่ในพื้นที่ที่มีเชื้อแพร่ระบาด และในกลุ่มคนปกติที่อาศัยอยู่นอกพื้นที่แพร่ระบาด เป็นค่าปกติ (baseline) ผลบวกจากการตรวจด้วยวิธีนี้จะต้องมีค่า (O.D. value) มากกว่าค่าเฉลี่ยของ O.D. ของกลุ่มคนปกติบวกด้วย 2 เท่าของค่าเบี่ยงเบนมาตรฐาน ($\bar{x}$ + 2 S.D.) และการตรวจโดยวิธีนี้ใช้น้ำเหลืองที่เจือจางเป็น 1:250 จากผลการทดลองพบว่าแอนติเจนจำเพาะให้ผลเป็นที่น่าพอใจคือมีความไว 92%, ความจำเพาะ 91%, ค่าของคนเป็นโรคที่ตรวจได้ผลบวก 81% และค่าของคนปกติที่ตรวจได้ผลลบ 96% เมื่อคำนวนจากค่าเฉลี่ยของกลุ่มคนปกติที่อาศัยอยู่นอกพื้นที่ระบาด หรือมีความไว 82%, ความจำเพาะ 96% ค่าของคนเป็นโรคที่ตรวจได้ผลบวก 94% และค่าของคนปกติที่ตรวจได้ผลลบ 87% เมื่อคำนวนจากค่าเฉลี่ยของกลุ่มคนปกติที่อาศัยอยู่ในพื้นที่ที่มีการระบาดของโรค

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ในการศึกษานี้ ยังได้มีการผลิตโมโนโคลนอลแอนติบอดีต่อแอนติเจนชนิดหยาบของเชื้อก่อโรคขึ้น เพื่อใช้ในการพัฒนาวิธีการตรวจหาแอนติเจนในผู้ป่วยเมลิออยโดสิส จากผลการศึกษาพบว่าโมโนโคลนอลแอนติบอดี 5F8 ที่ผลิตขึ้นมานั้นมีความจำเพาะต่อเชื้อ *P. pseudomallei* ทั้ง 56 สายพันธุ์ ที่ใช้ในการวิจัยครั้งนี้ 5F8 เป็นโมโนโคลนอลแอนติบอดีชนิด IgM และสามารถทำให้ตัวเชื้อก่อโรคเกาะกลุ่ม (agglutinating antibody) จากการทดสอบคุณสมบัติโดยวิธี immunofluorescence test พบว่า 5F8 สามารถจับอย่างจำเพาะที่ผิวของเชื้อก่อโรค จากการนำ 5F8 ไปใช้ในการตรวจหาแอนติเจนโดยวิธี B-SA ELISA (Biotin-Streptavidin ELISA) พบว่าวิธีนี้จะสามารถตรวจหาระดับของแอนติเจนได้ต่ำสุดที่ความเข้มข้น 12.5 นาโนกรัม/มิลลิลิตร และข้อมูลขั้นต้นที่ได้จากการตรวจหาระดับแอนติเจนในบัสสาวะของคนปกติที่ผสมแอนติเจนในปริมาณต่าง ๆ เชื่อว่าวิธีนี้สามารถใช้ตรวจหาระดับแอนติเจนในสิ่งส่งตรวจที่เป็นบัสสาวะได้ อย่างไรก็ตามวิธีนี้จะใช้ตรวจหาระดับแอนติเจนในสิ่งส่งตรวจของผู้ป่วยที่เป็นโรคเมลิออยโดสิสชนิดเฉียบพลันได้ ผลดีหรือไม่จะต้องทำการทดลองโดยใช้สิ่งส่งตรวจเหล่านั้นมาทดสอบ

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Thesis Title Identification of Specific Antigen of *Pseudomonas pseudomallei* and Monoclonal Antibody Production for the Diagnosis of Melioidosis.

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ABSTRACT

The currently used serodiagnostic methods for acute septicemic melioidosis are not yet reliable and satisfactory because of the unavailability of specific antigen. Most reports depend largely on the use of crude unfractionated bacterial extract for the detection of serum antibodies. In this study, fractionated 19.5-kDa antigen was prepared from unfractionated whole cell antigen of *P. pseudomallei* and evaluated for its potential usefulness in the diagnosis of acute septicemic melioidosis. The antigen was used for detecting antibody in sera from patients with acute septicemic melioidosis by indirect ELISA and the results were compared with those from patients with other bacterial infections, using normal human sera from either endemic or nonendemic areas of infection as a baseline. The results were considered positive if the O.D. value of a 1:250 serum dilution were higher than mean +2SD. The fractionated 19.5-kDa antigen exhibited

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satisfactory results with 92% sensitivity, 91% specificity, 81% positive predictive value and 96% negative predictive value based on a background obtained with normal sera from the nonendemic area. These value were 82% sensitivity, 96% specificity, 94% positive predictive value and 87% negative predictive value based on normal sera from the endemic area.

Monoclonal antibodies were produced by immunizing BALB/c mice using unfractionated whole cell antigens with a purpose of developing specific reagent for antigen detection. Several specific clones were established and the 5F8 was selected for detailed study because it was highly specific for *P. pseudomallei* and could detect all 56 isolates currently available. It was IgM agglutinating antibody and could bind to the surface envelope of *P. pseudomallei* (detected by immunofluorescence test). This MoAb could be used successfully for detecting antigen based on B-SA ELISA method at the antigen concentration as low as 12.5 ng/ml. The preliminary data using an artificial mixture of antigen with urine or serum from normal individuals showed it to be promising for detecting antigen in the urine. However, whether or not this method will be useful in the diagnosis of acute melioidosis depends on further investigation when clinical specimens are available for analysis.

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CHAPTER I

INTRODUCTION

Melioidosis is a bacterial infection caused by *Pseudomonas pseudomallei*. The disease is endemic in Southeast Asia and Northern Australia (1). In Thailand, especially the northeastern part, melioidosis is one of the more important public health problems. Since, the majority of people are farmers they have higher risk of getting infection from *P. pseudomallei* which inhabits soil, stagnant water and rice paddies (2,3). The infection can occur by exposing broken skin or ulcers to contaminated soil or water or by inhaling dust particles. Because of its severity, acute septicemic form of melioidosis can cause death within 24-48 hours after the onset, with a mortality rate as high as 80-90% (4). Because melioidosis is a disease with broad clinical spectrum, it requires effective and specific diagnostic method for proper management. Such a method should not only be specific and sensitive, but also must be simple to perform and the result must be made available as soon as possible, e.g., within 24 hours. Until now, only bacteriological method has been used on a routine basis, but it is time consuming and requires experience in recovery and recognition. More recently, serological methods have been introduced to overcome the problems. However, the results are not reliable enough and the bacteriological method is still needed as a confirmatory test. A number of serological tests that have been developed for the detection of antibody to *P. pseudomallei* include indirect hemagglutination test (IHA), complement fixation test (CF), indirect immunofluorescent antibody test (IFA) and enzyme-linked immunosorbent assay (ELISA). The

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problems encountered with all of the methods are sensitivity or specificity. Moreover, some of the tests require elaborated special instruments, such as, fluorescent microscope. The unreliability of the currently used serological methods is most likely related to the use of crude antigens, many of which cross-react extensively with sera from patients with other bacterial infections or even sera from healthy persons resided in endemic areas of infection. Theoretically, such a problem can be minimized by using more refined and specific antigens. A number of investigators (5,6) have tried to characterize *P. pseudomallei* antigens in attempting to identify and fractionate specific antigens. The results are promising but need further investigation.

Therefore, the purpose of the present study is to identify and purify specific antigen(s) of *P. pseudomallei* from crude unfractionated whole cell extract and to evaluate its potential for the diagnosis of melioidosis. Monoclonal antibodies specific for *P. pseudomallei* antigen will also be produced and used for the quantitation of antigens in the serum or urine of patients with acute septicemic melioidosis.

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CHAPTER II

BACKGROUND

1. Melioidosis

Human melioidosis was first encountered in 1912 in Rangoon by Whitmore and Kisnaswami (7). In 1913, Whitmore demonstrated that this disease was caused by bacteria and he named it *Bacillus pseudomallei* (8). The name *Pseudomonas (P.) pseudomallei* was introduced by Breed et al (9) in 1957. Subsequently, Stanton and Fletcher (10) observed a similar human infection in Malaysia and called the disease "Melioidosis". Some of the more obvious clinical manifestations include chronic pneumonitis with fever, wasting and haemoptysis which is difficult to distinguish from pulmonary tuberculosis (11). The causative agents were found at post mortem in the lungs, liver, spleen and other internal organs. More recently, melioidosis has emerged as an important health problem in many parts of Thailand. It increased from 3 cases in 1963 to over 800 cases in 1986 (12) with a mortality rate in severe and untreated cases as high as 80-90%.

1.1 The pathogen "*P.pseudomallei*"

Based on nucleic acid study, *P. pseudomallei* is very close to *P. mallei, P. cepacia, P. gladioli* and *P. caryophylli* (13)(Fig.1). *P. pseudomallei* can be recovered from soil and water in a limited geographical area, i.e., Southeast Asia, Northern Australia, parts of Africa and South and Central America (1,14). Damp climate and a terrain of flooded low-lying plains appear to favor the existing of the

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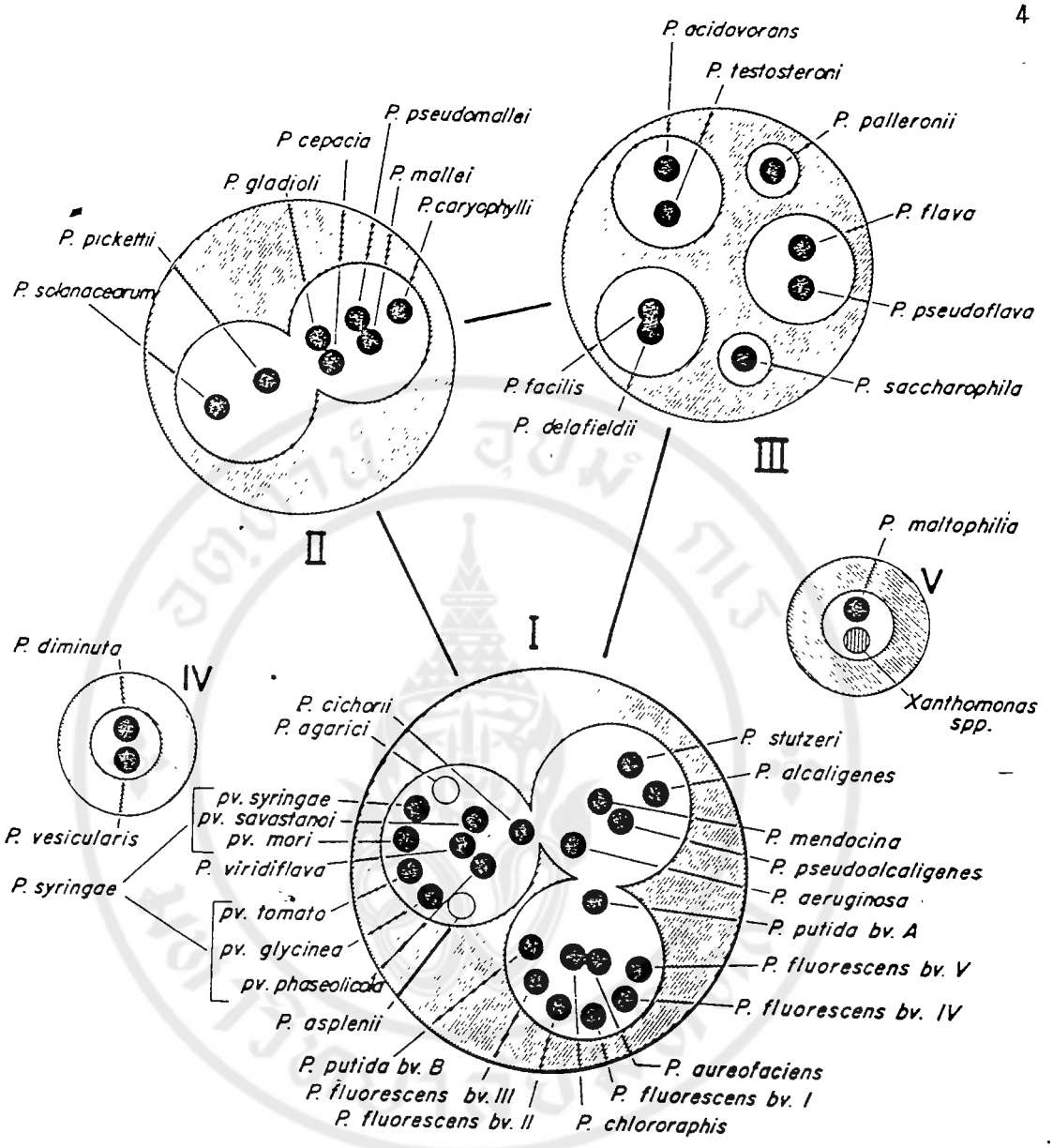

Figure 1 *Pseudomonas* species, biovars and pathovars arranged according to rRNA and DNA homologies. The *large circles* represent rRNA homology groups, within which DNA homology group are indicated by *white circles*. The diminuta group (group IV) and the *P. maltophilia-Xanthomonas* group (group V) are included for reference, but appear unconnected to the *Pseudomonas* group. Assignment of *P. agarici* and *P. asplenii* to the "fluorescent" DNA homology group within RNA group I is only tentative (13).

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organisms. Its morphology is slender rod, 0.3-0.5 μm in width, 1-2 μm in length and often appears as long parallel bundles of filaments. Gram stain is negative with a bipolar safety pin staining. When stained with Sudan black, the organism shows dark granules of poly-β-hydroxybutyrate (13).

P. pseudomallei grows well on simple media, including nutrient, blood and Mac Conkey's agar. After an overnight incubation at 37^0c under aerobic condition, the colonies are 1-2 mm in diameter. The growth may be smooth and mucoid or rough, with a dull wrinkled, corrugated or honeycombed surface, grayish-yellow color. After several days of incubation, they become opaque, yellowish-brown and have a characteristic earthy or musty odor (15). Young cultures usually give α -haemolysis on sheep blood agar but older cultures usually give β -haemolysis (13).

1.2 Epidemiology

The disease is almost exclusively associated with warm climate, and most of the reports of infection in man have been from Southeast Asia, notably Burma, Malaysia, Thailand, Vietnam and Hong Kong (16). A few cases have been reported from the Papua New Guinea (17). Another focus of human infection is in Queensland and the Northern Territories of Australia (1). Occasional infections have been reported from Central America and West Africa (18). In Thailand, melioidosis patients are commonly seen in the Northeast. Khon Kaen province is considered a highly endemic area; 8 cases(in 1978-1980) and 245 cases (in 1982-1985) were reported from Khon Kaen Provincial Hospital (19). Many investigators (4,20,21,22) have noted the high

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frequency of the disease in patients with diabetes or alcoholics. However, it may also occur in healthy persons. The disease was also often observed in soldiers on active service in the French (23) and American army (24) in Vietnam. The high frequency of respiratory infections in American soldiers in Vietnam has been attributed to the inhalation of contaminated dust floating by helicopter rotors. The farmers in the Northeastern Thailand (25) were frequently affected because they immersed in contaminated muddy water. Transmission between human subjects is very rare. There is also suggestive evidence for the occasional association of melioidosis with repeated injections and other invasive procedures (26).

In animals, the infection with *P. pseudomallei* is distributed irregularly in tropical areas and sometimes gives rise to outbreaks of clinical disease. Many animals have been reported to be infected with *P. pseudomallei* including laboratory animals in Malaysia (10), wild rats and sheep in Queensland (27), pigs in Malaysia (28) and Netherlands (29), but there is very little evidence of human infection from an animal source. Therefore, the disease in man appears not to be a zoonosis.

1.3 The pathogenesis

P. pseudomallei is most often transmitted to human through broken skin, respiratory tract and oral mucosa but transmission by a sexual route has also been suspected (30). The pathogenicity of this organism is reported to be associated with endotoxin, exotoxin or other biological products, e.g., enzymes (31-33). Any organ can be infected but the most common ones are lungs and liver (34). The disease may

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occur as : acute infection, chronic or subclinical infection and recurrent infection depending on the immune status of the host, routes of infection and inoculum size (35-37). Recrudescence may occur following chronic or subclinical infections. These patients have poor prognosis and may die suddenly following antimicrobial therapy (35). With the exception of endotoxin of *P. pseudomallei* (38), very little is known about the role of other biological products. More recently, a number of investigators have focused their attention on glycocalyx, an exopolysaccharide elaborated by the organism either in the liquid culture media or in the host tissues (39). This substance can help the organisms to build a biofilm growth which may be a factor which make *P. pseudomallei* able to withstand the host defence and antibiotics, thus enabling them to remain cryptic for a long period after an apparently successful treatment.

1.4 Clinical manifestations

Melioidosis varies greatly in its clinical manifestation. It may strike as an overwhelming swiftly fatal illness or as a chronic suppurative process or even an asymptomatic state detectable only by positive serology (40,41). More commonly, it occurs as an opportunistic infection against an array of debilitating diseases, particularly in protein-calorie malnutrition, diabetes mellitus and other immunosuppressive conditions. Patients with dysgammaglobulinemia and with acquired immunodeficiency syndrome are also at risk (42). The most common site of infection is the lungs and followed by the liver, spleen, kidneys, seminal vesicle, prostate gland and urinary bladder (41). The clinical features of the disease may depend on the mode of infection (for example, cellulitis by skin inoculation, pulmonary

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disease by inhalation, pharyngitis and cervical lymphadenitis by oral mucosa). However, the common features found in a majority of melioidosis patients are septicemia and pyogenic abscess (31). Antibiotic treatment may result in 80 percent cure rate for localized form and only 20 in septicemic form (43,44).

1.5 Immunity

The normal host defense mechanisms against *P. pseudomallei* are only partially understood. Because of its properties somewhat similar to those of other intracellular bacteria, cellular immunity is expected to play an important role in host defenses. Evidence for a protective role of cellular immunity is from a demonstration of mononuclear cell with organisms in phagocytic vacuoles by electron microscopy (45).

Antibody to *P. pseudomallei* can be detected in the second week after the patients are infected. The titres usually rise to a maximum level in the 4^{th} to 5^{th} month and may persist for as long as 9 months (46). However, there is no evidence to suggest that the antibody plays a role in defense against *P. pseudomallei*. It may however facilitate the ingestion and destruction of bacteria by immune macrophages (47).

1.6 Laboratory diagnosis

1.6.1 Bacteriological diagnosis

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Diagnosis of melioidosis is not always easy. *P. pseudomallei* may be grown from blood or pus of the patients but its presence is sometimes unexpectedly scanty. Although it can grow on plain agar, the growth is better on glycerol agar (48). Useful selective media include

40% glycerol agar containing 5 mg/L crystal (48) and Mac Conkey's agar containing colistin (49). Colony morphology is helpful for identification if the typical appearance, i.e., wrinkle, beta-hemolysis on blood agar or colony with musty odor, can be observed (50). Gram stain of clinical specimens or cultures showing bipolar staining can further help to identify the pathogen. Other characteristics, e.g., number of flagella, diffusible pigments and arginine dihydrolase are also quite useful to distinguish it from other closely related *Pseudomonas sp. (P. mallei, P. caryopylli, P. cepacia, P. gladiali, P. pickettii and P. solanaccarum)* (13).

1.6.2 Serological diagnosis

Bacteriological diagnosis is time consuming and may provide results too late for effective therapy. Therefore, serological tests have been developed. The latter should be more valuable for acute septicemia which requires rapid diagnosis and treatment.

IHA was first introduced by Ileri (51) and was found to be useful by many investigators (46,52). The method is based on the presence of antibody against polysaccharide or lipopolysaccharide of *P. pseudomallei* antigen in the serum of suspected individuals. The test is simple to perform, highly sensitive and moderately specific. Although the IHA is a useful indicator of previous infection, its main disadvantage is the high background value, particularly in those resided in the endemic area of infection.

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Ashdown et al (53,54) have introduced methods for the detection of specific IgM antibody against *P. pseudomallei* protein antigen by immunofluorescence (IgM-IFA) and ELISA (IgM-ELISA). They

found the level of specific IgM antibody to be a much more accurate diagnostic indicator for active melioidosis. The use of these methods to overcome the shortcomings of the IHA test and the combined use of IHA and IgM-IFA or IgM-ELISA seem to be the key for early diagnosis of acute septicemic melioidosis. However IgM-IFA and IgM-ELISA could not completely eliminate non-specificity because of the use of crude antigens.

More recently, different ELISA techniques have been developed with a prime objective of obtaining a better specificity and sensitivity. For example, Ismail and colleagues (6) developed an ELISA to detect antibody to exotoxin of the pathogen based on the availability of monoclonal antibody specific for a 31 kDa toxic component. Furthermore, an avidin-biotin enzyme immunoassay based on a rabbit polyclonal antibody to *P. pseudomallei* protein antigen has also been described (55). Antigen detection using monoclonal antibody against other components may prove to be valuable for the diagnosis of acute infection. DNA probe or when used in conjunction with polymerase chain reaction (PCR) represent another alternative approach.

2. The potential diagnostic antigens of *Pseudomonas pseudomallei*

P. pseudomallei antigens can be arbitrarily classified into soluble, flagella, somatic O and envelope (K) antigens (1). Attempts to identify, fractionate and characterize these antigens have providedus with a number of more refined diagnostic antigens such as melioidin, complement fixing (CF) antigen, Barber protein, exotoxin and endotoxin.

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Melioidin antigen

Melioidin is a soluble polysaccharide antigen. Originally prepared for use in the indirect hemagglutination test by Alexander et al (46). The melioidin was prepared based on the method of Rice et al (56) and consisted of a supernatant fluid of heat-killed two-week old protein-free broth culture. The antigen was preserved by the addition of phenol (0.5% by volume). On the other hand, Hatsadee et al (57) used autoclaving instead of heating.

A similar antigen was prepared by Wongratanacheewin et al (58). They used 3 synthetic media: glycine broth medium (45), modified Proskaver & Beck medium (59) and Rice, Kohet & Duthie medium (56) for culturing *P. pseudomallei* at 37 ^{0}c for 3 weeks. The supernatant culture fluids were collected by centrifugation at 12,800 g, 4 ^{0}c for 20 min and filtered through a 0.45 µm membrane. They found the 3 preparations to contain a specific component with a relative molecular weight of 40 kDa.

CF antigen

This antigen was first described by Nigg and Johnston (60) and used for the complement fixation test (46). The CF antigen was prepared by inactivating the bacterial suspension at 65 ^{0}c for 1 hr. The bacteria were then disrupted by oscillator and followed by a centrifugation at 18,000 x g for 30 min before subjecting to a millipore filtration. This antigen was found to be stable for over 3 years when kept at 4 ^{0}c. However, the CF antigen appeared to contain antigens cross reactive with some but not all types of *P. aeruginosa*.

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Barber protein (BP)

The BP or the partially purified cell extract (PCE) was prepared as originally described for *Salmonella typhi* by Barber et al (61). The BP protein is resistant to heat and it is immunogenic and toxic (62). It consists of both species specific and common antigens (63). Wongratanacheewin et al (55) prepared BP antigen from *P. pseudomallei* and used for the development of the assay for the detection of melioidosis. The preparation method used was based on that of Barber et al (61). Briefly, *P. pseudomallei* cells were extracted with veronol buffer and followed by trichloroacetic acid precipitation. The precipitate was dissolved and dialyzed against excess of distilled water prior to filtration through a 0.45 μm membrane.

Exotoxin

The role of toxic products of *P. pseudomallei* in the pathogenesis of melioidosis has been the focus of interest of many investigators included Ismail et al (6). They isolated the exotoxin of *P. pseudomallei* and used for the production of specific monoclonal anti-toxin. The organisms were cultured on Brain Heart Infusion Broth (BHIB) at 37^0c under static conditions for 7 days. The culture was harvested by centrifugation and the resulting supernatant fluid was recentrifuged, millipore filtered, dialyzed, concentrated and lyophilized. This toxin was used to detect mouse antibody to *P. pseudomallei* exotoxin.

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Endotoxin

Endotoxin or LPS had been prepared as a diagnostic antigen by

Petkanjanapong et al (64). It was used successfully for detecting specific IgG antibody in melioidosis patients based on indirect ELISA method. The preparation of endotoxin was done by culturing *P. pseudomallei* on Muller Hinton medium. After heating at 120^0c for 30 min, the bacterial mass was harvested, centrifuged and sonicated to make a lysate. Then endotoxin was extracted from the lysate by the phenol-water method (64).

3. Hybridoma technology

Serum contains many different types of antibodies that are specific for many different antigens. The use of mixed population of antibodies creates a variety of problems in immunochemical technique. Therefore, the preparation of homogeneous antibodies with defined specificity was a long-standing goal of immunochemical research. This goal was achieved with the development of the technology for hybridoma production. In 1975, Kohler and Milstein (65) showed that antibody-secreting hybrid cell line could be established routinely and maintained *in vitro* by somatic cell hybridization (fusion). The two cells commonly used as fusion partners are antibody-secreting cells isolated from immunized animals and non-secreting myeloma cells. The myeloma cells provide the correct genes for continued cell division in tissue culture, and the antibody-secreting cells provide the functional immunoglobulin genes. The fusion gives rise to hybrid cells that can produce and secrete antibody with a single specified reactivity (monoclonal antibody).

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3.1 Principle of hybridomas

The fusion can be mediated by Sendai virus or polyethyleneglycol (PEG) (66). PEG fuses the plasma membranes of adjacent myeloma and/or antibody secreting cells, forming a single cell with two or more nuclei. This heterokaryote retains these nuclei until the nuclear membranes dissolve prior to mitosis. During mitosis and further rounds of cell division, the individual chromosomes are segregated into daughter cells. Because of the abnormal number of chromosomes, segregation does not always deliver identical sets of chromosomes to daughter cells, and chromosomes may be lost. If the chromosomes that are lost contain genes for drug selection, then the growth of the hybridoma will be unstable and cell will die during selection (by drug selection to be described below). In practice, the selection for stable segregation of the drug selection marker is so strong that within a short time, the hybridoma is either lost completely or a variant is isolated that stably retains the selectable marker. Unfused cells from the immunized animal soon die. However, the unfused myeloma cells are well adapted to the *in vitro* tissue culture and must be eliminated by drug selection. The selection are commonly done with aminopterin, methotrexate or azaserine. The principle of drug selection is that the myeloma partner has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis [first reported by Littlefield (67)], namely hypoxanthine-guanine phosphoribosyl transferase (HPRT). The addition of a compound that blocks the *de novo* nucleotide synthesis would force the cells to use this salvage pathway. The myeloma cells which are defective in this salvage pathway cannot survive. Only the hybrids between myelomas with

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a nonfunctional HPRT and cells with a functional HPRT would be able to grow continuously.

3.2 Myeloma cell lines

Myeloma cell line, a partner cell for fusion, should have 3 characteristics as follow; lack of production of undesired heavy and light chains, the presence of drug resistance marker for selection against unfused myelomas and stability of the hybrids formed by cell fusion. Myelomas can be induced in a few strains of mice by injecting mineral oil into the peritonium cavity. Potter (68) had isolated myeloma cells from BALB/c mice, and was able to establish this cell line from these myelomas [mineral oil plasmacytomas (MOPC)]. Derivatives of MOPC most commonly used for fusion include X63Ag8.653, Sp2/0-Ag14 or NSI/1-Ag4-1.

3.3 *In vivo* immunization

The desired antibody producing lymphocytes, a partner for fusion, can be obtained by immunizing animals (mostly mice) with antigen of interest by a number of different protocols. The result of the immunization depends on three factors; firstly, dose and form of the antigen, secondly, route of inoculation and lastly, immunization schedule. These factors are interrelated and can result in a production of antigen-specific antibody producing lymphocytes at the stage when the cells are capable of undergoing fusion with myeloma cells.

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3.4 Screening method

Approximately one week after cell fusion, colonies of hybrid cells would be ready for screening. During the screening, samples of

the culture medium are removed from each well with growing hybridomas and tested for the presence of antibodies. A good screening procedure must: (1) reduce the number of cultures that must be maintained to a reasonable level, (2) identify potential positives in time and (3) be easy enough to perform. Currently, most screenings are done by antibody capture assays which are often the easiest and most convenient. The antibody capture assays can be done by either ELISA or immunoblot.

3.5 Cloning

The original positive wells often arise from more than one hybridoma cells and many hybrid cells have an unstable assortment of chromosomes, these problems may lead to an overgrowth of undesirable cells that are not producing antibody of interest. Therefore, the antibody producing cells must be recloned as soon as possible. Single cell cloning can ensure that antibody producing cells are stably maintained. This method is performed by plating a suspension of hybridoma cells that are diluted to the probable concentration of one cell per well, together with feeder cells (prepared from splenocytes, macrophages, thymocytes or fibroblasts from normal animal). In order to ensure that a hybridoma is stable and single cell cloned, the cloning should be repeated until every well tested is positive.

3.6 Producing and storing monoclonal antibody

Monoclonal antibodies are normally collected as either tissue culture supernatants or as ascitic fluids. For most immunochemical method, tissue culture supernatants will be the most useful source of monoclonal antibodies. In order to produce higher-titred supernatants, each culture is allowed to grow until the hybridomas die and after the

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debris is removed by centrifugation, the supernatant is collected. With this procedure, the supernatant is not contaminated with high levels of undesirable antibodies and the antibody concentration is high enough for most assays. For the production of monoclonal antibodies as an ascitic fluid, the peritoneal tumor is developed in mouse (same strain as used for fusion) by injecting hybridomas into the peritoneum, which serves as a growth chamber for cells. The hybridoma cells grow to high densities and continue to secrete the antibody of interest, thus creating a high-titred antibody solution for collection.

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CHAPTER III

MATERIALS AND METHODS

1. Serum specimens

The serum specimens were collected from four groups of people. Sera designated as "positive", were collected from 37 bacteriologically proven cases of acute septicemic melioidosis (26 sera were from endemic Northeast of Thailand, kindly provided by Wellcome Unit, Bangkok Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University and 11 sera were from the Department of Microbiology, Faculty of Medicine, Khon Kaen University) (each described in details in Table 1). Sera designated as "normal" from endemic area were collected from 25 healthy people who lived in Khon Kaen province and those from nonendemic area were collected from 60 blood donors of Vajira Hospital, Bangkok. Sera designated as "other infections" were collected from 25 patients (resided in the endemic northeast, provided by Faculty of Medicine, Khon Kaen University) who were infected with other bacterial agents (described in Table 2). The pooled melioidosis serum used as a positive control throughout this study was prepared by pooling the five melioidosis sera with high ELISA antibody titres against unfractionated antigen and the negative control serum was a pooled of normal sera from five blood donors of Vajira Hospital.

2. Preparation of antigens

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2.1 Bacteria

Fifty-six strains of *P. pseudomallei* were used in this study. They were isolated from the following clinical specimens: blood (31

Table 1. Details of individual septicemic sera from 37 patients with acute melioidosis

No.	Code	Date of blood collection for		IHA*
		Serology	Hemoculture	
1	Mel 142	16.06.88	15.06.88	640
2	A/C 76	31.10.88	31.10.88	1280
3	Mel 48	01.08.87	6.08.87	> 1280
4	Mel 93	29.10.87	27.10.87	1280
5	Mel 121	17.01.88	8.02.88	640
6	Mel 139	15.06.88	10.06.88	640
7	A/C 7	24.08.88	1.10.88	1280
8	Mel 60	27.08.87	22.08.87	> 1280
9	A/C 80	26.07.89	26.07.89	1280
10	Mel 103	12.12.87	18.11.89	160
11	Mel 154	29.06.88	30.06.88	640
12	A/C 12	3.09.88	3.09.88	1280
13	Mel 66	1.09.87	29.08.87	1280
14	Mel 67	3.09.87	3.09.87	640
15	Mel 87	15.10.87	15.10.87	1280
16	Mel 157	7.07.88	7.07.88	320
17	Chaivoot	NK	NK	320
18	Kreangsak	NK	NK	1280
19	A/C 193	28.06.90	19.06.90	> 1280
20	A/C 195	28.06.90	19.06.90	> 1280
21	A/C 213	8.08.90	8.08.90	160
22	A/C 220	23.08.90	20.08.90	1280
23	A/C 228	7.09.90	5.09.90	> 1280
24	A/C 240	2.10.90	27.09.90	1280
25	A/C 254	25.10.90	25.10.90	40
26	A/C 252	17.10.90	16.10.90	1280
27	Mel 301	12.03.35	NK	NK
28	Mel 302	21.02.34	After Serological	NK
29	Mel 303	21.06.34	Before Serological	NK
30	Mel 304	24.06.34	After Serological	NK
31	Mel 305	18.04.34	After Serological	NK
32	Mel 306	5.12.33	NK	NK
33	Mel 307	6.02.34	NK	NK
34	Mel 308	6.09.33	NK	NK
35	Mel 309	6.02.34	NK	NK
36	Mel 310	6.11.33	After Serological	NK
37	Mel 311	3.09.33	NK	NK

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NK : Data not provided.

* : Reciprocal melioidosis antibody titre by indirect heamagglutination test.

Table 2. Details of individual septicemic sera from 25 patients with other bacterial infections (all patients resided in endemic area)

Patient's name	Hemoculture	IHA*
1 Pimpa	*E. coli*	160
2 Say	*P. stuzeri*	320
3 Boonroong	*K. pneumoniae*	< 40
4 Dara	*K. pneumoniae*	160
5 Aree	*Salmonella* group B	< 40
6 Pimjai	coagulase - negative *Staphylococcus*	80
7 Tongsin	coagulase - negative *Staphylococcus*	160
8 Vitoon	*P. aeruginosa*	160
9 Son	*Enterobacter sp.*	80
10 Suai	*Enterobacter sp.*	80
11 Pimpa	*K. pneumoniae*	640
12 Pigoon	*Enterobacter sp.*	< 40
13 Tay	*K. pneumoniae*	80
14 Dongkamol	*K. pneumoniae*	160
15 Songjan	*α- streptococci*	160
16 Vimonrat	coagulase - negative *Staphylococcus*	80
17 Nuejan	coagulase - negative *Staphylococcus*	40
18 Pook	*Salmonella* group B	< 40
19 Montri	*α- streptococci*	160
20 Vason	coagulase - negative *Staphylococcus*	80
21 Son	*E. coli*	320
22 Shomshoon	*E. coli*	80
23 Sakar	coagulase - negative *Staphylococcus*	40
24 Noun	*E. coli*	160
25 Tongpang	coagulase - negative *Staphylococcus*	40

* : Reciprocal melioidosis antibody titre by indirect heamagglutination test.

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strains), pus (18 strains), sputum (4 strains) and ascitic or bronchial fluids (3 strains). All except one were isolated from patients resided in rural area, mostly from the northeastern part of the country. One isolate was from a septicemic patient admitted at Ramathibodi Hospital, Bangkok. Other bacteria used in this study including : *P. aeruginosa, P. cepacia* (1 strain from Khon Kaen and another from Ramathibodi Hospital), *P. putida, P. maltophila, P. alcaligenes, Klebsiella pneumoniae, Enterobacter cloaca, Escherichia coli, Salmonella typhi, S. enteritidis, S. krefeld, Proteus mirabilis* and *Staphylococcus aureus* were available from the stock cultures maintained at the Bacteriology Laboratory (Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok).

2.2 Unfractionated whole cell antigen preparation

All bacteria were cultured individually on blood agar and allowed to incubate at 37^0c under aerobic condition for 24 hours. The colonies were harvested by suspending in an appropriate volume of 0.15 M phosphate buffered saline (PBS), heating in boiling waterbath for 3 min and the protein content was determined by Folin-Ciocalteau method. When grown under this condition, a loopful (0.4 mm) of bacterial colonies suspended in 600 µl had protein content of approximately 22 mg/ml.

3. Protein determination

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Protein concentration was determined by a Folin-Ciocalteau tyrosine method, using a microtechnique modified from the one originally described by Lowry et al (69). The reagents were prepared as described in the Appendix.

4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE for the analysis of *P. pseudomallei* and other bacterial proteins was performed according to a modified method of Laemmli (70) and the molecular weight (Mr.) of individual components were determined from a standard curve using Pre-stained protein molecular weight standards (high range)(BRL, Life Technologies, Inc., Gaithersburg, MD, USA).

4.1 Preparation of separation gel and stacking gel

The reagents were prepared as described in the Appendix. Separation gel was casted between 2 glass plates, using a 12% acrylamide solution in 1.875 M Tris-HCl pH 8.8 containing 0.1%(w/v) sodium dodecyl sulfate (SDS), 0.05%(v/v) TEMED and 0.05% (w/v) ammonium persulfate. The solution was allowed to polymerize at room temperature for at least 1 hr. Stacking gel prepared with 3% acrylamide solution in 1.875 M Tris-HCl pH 6.8 containing 0.1% (w/v) SDS, 0.025% (v/v) TEMED and 0.05% (w/v) ammonium persulfate was then added with plastic comb inserted to make sample slots, and the gel was allowed to polymerize for at least 30 min.

4.2 Preparation of samples

Both samples and standard protein markers were reduced in a boiling waterbath for 3 min in sample buffer containing 0.0625 M Tris-HCl pH 6.8, 2% (w/v) SDS, 10%(v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromphenol blue as an indicator dye and denatured by heating before application.

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4.3 Electrophoresis

Electrophoresis was carried out in a vertical direction (Hoefer Scientific Instruments, San Francesco, CA, USA) with a constant current of 25 mA/gel. The current was stopped when the blue dye marker reached the bottom of the gel which generally took about 4 hours. The gel was then carefully taken out for staining or Western blotting.

4.4 Staining of the gel

After the electrophoresis was terminated, the gel was stained and fixed with a protein stain consisting of 0.2% (w/v) Coomassie Brilliant blue R (Sigma, St. Louis, MO, USA) in 7 % (v/v) glacial acetic acid and 46.5% (v/v) methanol for 1 hour. The gel was then destained in a solution made up of 7% glacial acetic acid and 5% methanol until the background was clear.

4.5 Molecular weight determination

A procedure used for molecular weight determination was modified from the method described by Weber and Osborn (71) and by Davies and Stark (72). The molecular weight could be determined by comparing the relative electrophoretic mobility of component in question with standard protein markers of known molecular weight. A linear relationship was obtained when a relative mobility of the protein markers were plotted against a logarithmic value of their molecular weights. The relative mobility of the unknown was calculated according to the following formula:

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}} \times \frac{\text{Gel length before staining}}{\text{Gel length after destaining}}$$

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5. Immunoblotting

The method was performed as described by Towbin et al (73). In brief, proteins from electrophoresed gel was transferred to a nitrocellulose filter membrane of 0.45 µm pore size (Schleicher and Schuell BA85, Dassel, Germany) using a Transfer-Electroblotting unit (Hoefer Scientific Instruments) filled with Towbin's buffer containing 25mM Trizma-base, 192 mM glycine and 20% (v/v) methanol as an electrode buffer. A constant current of 0.6 A was applied overnight at 4^0c.

The proteins blotted onto the nitrocellulose filter membranes were probed with monoclonal or polyclonal antibodies of known specificity and stained by immunoenzyme reaction. The nitrocellulose was immediately immersed into a large volume of 0.15 M phosphate buffered saline containing 1% (v/v) Tween 20 (PBST) at 37^0c for 30 min. Unoccupied protein binding sites on the membrane were blocked with 3% (w/v) bovine serum albumin in PBST (3% BSA-PBST) at room temperature for 1 hour on a rotating platform. After blocking, the membrane was washed with PBST 3 times, each time for 3 min and was allowed to react with continuous gentle shaking in an appropriate volume and dilution of specific antisera in 1% (w/v) bovine serum albumin in PBST (1% BSA-PBST) at room temperature for 2 hours. The membrane was then washed in the same manner as previously described before a peroxidase-conjugated antibody (diluted 1:1000 in 1% BSA-PBST) (Dakopatts, Copenhagen, Denmark) was added and the reaction was allowed to proceed at room temperature for 1 hour on a rotating platform. The membrane was once again washed 3 times before the substrate (see the Appendix) was added. The enzymatic reaction was allowed to proceed for 20 min, and then the reaction was stopped by rinsing with tap water. Positive staining

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appeared as a dark brown band on the membrane.

6. Purification of unfractionated whole cell antigens

The individual protein components of *P. pseudomallei* was purified by eluting desirable components from a preparative SDS-PAGE and followed by a removal of SDS by ion-retardation. In brief, the unfractionated whole cell antigen from a *P. pseudomallei* extract as well as the pre-stained standard protein markers (BRL) were load onto SDS-PAGE and electrophoresis was carried out until the dye marker reached the bottom of the gel. Then desirable protein bands were located and desirable gel strips were cut. Each strip was sliced into smaller pieces prior to protein elution. The elution system was performed with Electro-eluter model 422 (Bio-Rad Laboratories, Richmond, CA, USA). The elution procedure was as described by the manufacture. In brief, the gels were put into the Electro-eluter tubes containing appropriate elution buffer (see the Appendix). The current of 10 mA/tube was applied for 2 hr at 4^0c. The eluent was dialyzed against distilled water at 4^0c overnight prior to lyophilization.

7. Removal of sodium dodecyl sulfate from protein

Protein eluted possessed a large amount of SDS that could interfere with immunological reaction. The SDS was therefore removed according to the procedure described by Kapp & Vinograd or (74). In brief, the calculated quantity of ion-retardation resin AG11A8 (Bio-Rad Laboratories) with the SDS-binding capacity of 1.7-2.2 mg of SDS per g of wet resin was weighted, allowed to swell in deionized distilled water and then packed into a column. The resin was washed with 5 volumes of 1 M NH_4Cl and followed by 20 volumes of deionized distilled

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water. The eluent was loaded to the column and eluted with deionized water at a flow rate of 15 ml per hour. Protein in the eluted fractions were measured by absorbance value of 280 nm. The high absorbance fractions were pooled, lyophilized and the protein content determined by the Lowry method.

8. Production of monoclonal antibodies

8.1 Immunization

Six weeks old female BALB/c mice (The National Institute of Health, Ministry of Public Health, Nondhaburi, Thailand) were immunized with either fractionated 19.5-kDa antigen or unfractionated whole cell antigen. One group of mice was immunized with fractionated antigen and another group received unfractionated whole cell antigen. Each mouse was first given an intraperitoneal injection of 100 µg antigen in 200 µl sterile normal saline mixed with equal volume of complete Freund's adjuvant (CEA)(Sigma) and boosted one month later with a similar injection of antigen in incomplete Freund's adjuvant (IFA)(Sigma). Three weeks later, all mice were bled from the tails. The antibody titers against unfractionated whole cell and fractionated antigens were determined by ELISA and immunoblot (described in Sections 1.7 and 1.4). The second booster was done one day later again with the antigen in IFA. Trial bleeding was done 20 days later and the mouse with highest antibody titre was chosen for fusion. This animal was given a prefusion booster with 100 µg of unfractionated whole cell antigen or 25 µg of fractionated antigen in aqueous solution again by intraperitoneal injection 3 days before fusion.

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8.2 Fusion procedure

Myeloma cells [P3-X63-Ag8-U1 (75) in special complete medium A (see the Appendix) or X63-Ag 8.653 (76) in special complete medium B (see the Appendix)] were maintained at the log phase about one week before the fusion date. The immune spleen cells were removed aseptically and made into a single cell suspension with a wire sieve. The cells were washed with cold incomplete medium (see the Appendix). Approximately 10^8 immune spleen cells were mixed with myeloma cells (spleen : myeloma at a ratio of 10:1 for P3-X63-Ag8-U1 or 2:1 for X63-Ag 8.653) and the mixture was centrifuged at 1,000 rpm for 5 min at room temperature. The supernatant was removed and the cell pellet was washed by suspending in 20 ml of warm incomplete medium and then centrifuged at 1,000 rpm for 5 min. The pellet was gently loosened and 1 ml of warm 37% polyethylene glycol solution [PEG-3000 (Sigma, No. P-3640, Lot 127F-0264) for fusion with P3-X63-Ag8-U1, or PEG-4000 (Accurate Chemical & Scientific Corp, 33163, Westbury, NY, USA) for fusion with Ag 8.653] was added dropwise over a one minute period. The reaction was allowed to proceed for one minute at 37^0c before one ml of warm incomplete medium was slowly added to stop the reaction within a one-minute interval. This step was followed by diluting the mixture to 20 ml with the same medium within 4 min. The cells were centrifuged at 1,000 rpm for 5 min at room temperature. After the supernatant was completely discarded, the pellet was resuspended in 100 ml of HAT medium (see the Appendix) and plated in 96-well, flat-bottom, microtitre plates (Nunc Immunoplate II; Intermed, Kamstrup, Roskilde, Denmark) in 0.1 ml aliquots. Two wells per microtitre plate were left as controls containing $2x10^5$ P3-X63-Ag8-U1 cells. All plates were

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incubated in 5% CO_2 humidified incubator at 37^0c for 4-5 days. After all of the control myeloma cells had died, one-half of the total volume of culture supernatant in each well containing growing hybridoma was removed and replaced with an equal volume of fresh HT medium (see the Appendix). The growth of hybridoma was observed daily whereas the medium was changed every other days. Whenever the size of the hybrid colony was one third the area of the well, the culture supernatants were collected for screening.

8.3 Screening for antibody production by indirect ELISA

Indirect ELISA was used in a preliminary test for the ability of the hybrids to secrete specific monoclonal antibodies. The assay was carried out as previously described (77) in polystyrene U-bottomed, 96 well microtitration plates (Nunc Immunoplate II). The well was coated with 50 µl of unfractionated whole cell antigen of *P. pseudomallei* strain from Khon Kaen (PP844) or fractionated 19.5-kDa antigen. Each antigen was made up to a final concentration of 5 µg/ml with 0.05M carbonate buffer (see checkerboard titration Fig.10 and Fig.11) pH 9.6. After an overnight incubation at 4^0c, the plate was washed 3 times for 3 min each with 0.05% Tween 20 in phosphate buffered saline (PBST). Unbinding sites were blocked with 150 µl of 2% bovine serum albumin in PBS (2% BSA-PBS) at 30^0c for 1 hour. After washing the supernatant was added. The plate was washed 3 times with PBST before 50 µl of horseradish peroxidase (HRP) conjugated rabbit anti-mouse immunoglobulin (Dakopatts), diluted to 1:1000 in 1% BSA in PBST (1% BSA-PBST), was added. The plate was incubated at 30^0c for 1 hour. After washing, freshly prepared substrate solution (see the Appendix) was added and the plate was kept in the dark at room temperature for 40

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min. The enzymatic reaction was stopped by adding 25 µl of 5N H_2SO_4 to each well and the optical density (O.D.) was read at 492 nm by ELISA-plate reader (Titertek Multiskan MCC/340 Flow Laboratories, Finland).

The criterion for a positive result was arbitrarily designated based on those obtained from positive control (immune mouse serum) and negative control (myeloma culture medium), that is the O.D. value of 0.20 or above.

8.4 Clone expansion and cryopreservation

The selected monoclonal hybridomas were expanded into 1 ml of HT medium in the 24-well plate (Costar, Cambridge, MA, USA) and further expanded by transferring into 5 ml of HT medium in plastic flask (Costar). During expansion, all supernatants were collected and assayed for specific antibody to unfractionated whole cell antigen of *P. pseudomallei* and cross reactivity was checked with a panel of unfractionated whole cell antigens prepared from other bacteria. For storage, the hybridomas were harvested and centrifuged. The pellet was suspended in 0.5 ml of 10% (v/v) dimethyl sulfoxide (DMSO)(Sigma) in complete medium containing 50% (v/v) fetal bovine serum (FBS)(Flow laboratories, North Ryde, Australia). The suspensions were transferred to cryopreservation vials (Nunc) and slowly frozen to -70^0c over a 24-hour period. The cryovials were then transferred to a liquid nitrogen storage tank for storage.

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8.5 Preparation of feeder cells

Six months old female BALB/c mouse was used as a source of feeder cells. Only the animal whose serum was free from antibody to *P.*

pseudomallei or other gram negative bacteria tested by ELISA and Immunoblot was used. Either spleen cells or peritoneal exudate cells were used for making single cell suspension. The feeder cells suspended in complete medium at a concentration of 10^5 cells/ml was plated into 96-well plate, 50 µl per well and was preincubated overnight in a 37^0c humidified incubator with 5% CO_2.

8.6 Single-cell cloning by limiting dilution

The hybridoma culture was diluted to 80 cells per ml, 20 cells per ml and 10 cells per ml in complete medium. Each dilution was transferred (50 µl/well) to 10 cultured plate containing 50 µl per well of feeder cells. All plates were incubated in a 37^0c incubator with 5% CO_2. Approximately 4-5 days later, the plates were observed under microscope. After 7 to 10 days, colony growth was examined for single clones and the supernatants of growing colonies were assayed for antibody secretion. The wells that gave positive reaction were recloned by second limiting dilution. After the second cloning, the selected hybridomas were transferred to 24-well plates, rechecked for antibody secretion, cultured for supernatant collection and preserved by cryopreservation.

8.7 Ascites production

Six weeks old BALB/c mice were injected intraperitoneally with 0.5 ml of 2,6,10,14-tetra-methyl pentadecane (Pristane)(Sigma) 7 days prior to the intraperitoneal injection of approximately $5x10^5$ to $5x10^6$ hybridoma cells. One or two weeks later, ascites fluid was carefully withdrawn from the mouse with an 18-gauge needle attached to a 5-ml syringe. The fluid was allowed to stand at 37^0c for 1 hour, transferred

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to 4^0c overnight and then centrifuged at 3,000 g for 10 min. The supernatant was stored at -20^0c.

9. Characterization of monoclonal antibody (MoAb)

9.1 Testing for specificity by indirect ELISA

Monoclonal antibodies were tested for specificity by indirect ELISA using a panel of unfractionated whole cell antigens from 56 strains of *P. pseudomallei*, 2 strains of *P. cepacia*, 1 strain of *P. aeruginosa*, *P. putida*, *P. maltophila*, *P. alcaligenes*, *K. pneumoniae*, *E. cloacae*, *E. coli*, *S. enteritidis*, *S. krefeld*, *S. typhi*, *S. aureus and P. mirabilis*. All antigens were coated on microtiter plates (Nunc Immunoplate II) at the optimal concentration of 5 µg/ml (predetermined by checkerboard titration as described in Fig. 10). Reactivity against fractionated antigen of *P. pseudomallei* was also tested, using plates coated with 5 µg/ml of fractionated antigen (checkerboard titration described in Fig. 11).

9.2 Identification of components reactive with monoclonal antibodies

Immunoblotting against a panel of unfractionated whole cell antigens using monoclonal antibodies was analyzed as described in Section 5.

9.3 Isotype analysis

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The heavy chain and light chain isotypes of monoclonal antibody was determined by the ImmunoSelect™ Isotyping System (Gibco, BRL). This system is based on an antibody capture ELISA using a monoclonal rat anti-mouse isotype antibody. The monoclonal was used

exactly as recommended by the manufacture.

9.4 Partial purification of monoclonal antibodies by ammonium sulfate precipitation

The culture supernatant containing desirable monoclonal antibody was collected until after a sufficient volume was obtained. The pooled supernatant fluid was precipitated with an equal volumes of ice-cold saturated ammonium sulfate (Sigma) as described elsewhere. The mixture was stirred overnight at 4^0c and then centrifuged at 10,000 rpm for 30 min at 4^0c. The pellet was resuspended in normal saline and dialysed exhaustively against a large volume normal saline and finally followed by dialysing against PBS. Protein content of the dialysate was determined by measuring absorbance at 280 nm.

9.5 Radioimmunoprecipitation and autoradiography

9.5.1 Radioiodination of *P. pseudomallei* unfractionated whole cell antigen

The antigen was radioiodinated with I^{125} (Amersham Laboratories, England) by the chloramine-T technic originally described by Greenwood et al (78). One volume of a 20x concentration of 1 M sodium phosphate buffer pH 7.4 (see the Appendix) was added to 19 volumes of antigen prior to being labelled. Radioactive NaI was then added to the antigen at a ratio of 2 µCi/µg protein. Radioiodination was initiated by adding chloramine-T at a ratio of 0.2 µg/µg protein. After gentle shaking at 4^0c for 30 sec, the reaction was stopped by adding sodium metabisulfite (0.2 µg/µg chloramine-T) and followed by adding excess NaI (about 10 time the amount used above) and 1 µg of

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BSA/µg protein. All reagents were immediately prepared in distilled water before use.

9.5.2 Radioimmunoprecipitation

Immunoprecipitation was carried out by the technic modified from the one described by Ivarie and Jones (79). The I^{125}-labelled antigen was preabsorbed by the addition of normal mouse serum (0.2 µl/µg protein) and 2 volumes of a 50% (v/v) protein A-Sepharose CL-4B(Sigma). The solution was allowed to stand at 4^0c for 20 min and the supernatant was collected by centrifugation at 2,500 rpm at 4^0c for 5 min. The absorption step was repeated twice in order to minimize non-specific binding of labelled antigens to serum components and protein A-Sepharose. The preabsorbed antigen was then allowed to react with different concentration of (10x) monoclonal antibodies, using mouse polyclonal antibody against unfractionated whole cell antigen and normal mouse serum as positive and negative control respectively. The reaction mixture was incubated at 37^0c for 30 min, and then kept overnight at 4^0c. Immune complexes were separated from unreacted antigen by the addition of a precalibrated amount of protein A-Sepharose at 4^0c for 5 min. For the assay of IgM monoclonal antibodies, the immune complexes were coprecipitated with an excess of a predetermined quantity of specific rabbit anti-mouse IgM prior to the protein A-Sepharose step.

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The immune complexes absorbed to protein A-Sepharose were washed 3 times with cold PBS-Tris-HCl pH 8.1 (see the Appendix). The complexes were dissociated from protein A-Sepharose by heating in a boiling water bath for 2.5 min in the presence of sample buffer (see

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the Appendix). The suspension was then centrifuged at 2,500 rpm at 4^0c for 5 min and the supernatant was subjected to SDS-PAGE. For estimation of the optimal quantity of sample needed for electrophoresis and autoradiography, the radioreactivity of the supernatant fluid was determined in a γ-counter (1260 Multigamma II, LKB, Sweden). Typically, if the sample had 1,000 cpm, a 20 µl aliquot would be loaded and after electrophoresis the dried gel was exposed to an x-ray film for approximately 1 week.

9.5.3 Autoradiography

The technic used in the present study was slightly modified from that previously described by Laskey and Mills (80). The gel was first wrapped in a piece of cellophane and dried under a vacuum, using a slab gel dryer (LKB). The dried gel was then placed in contact with Fuji x-ray film (RX-100, Fuji Photo Film Co., Ltd., Japan) and kept at -70^0c in a film cassette made from two black plexiglass sheets with intensifying screen (Okamoto, Tokyo, Japan). The film was subsequently developed and fixed as recommended by the company.

9.6 Agglutination test

The specific MoAbs and polyclonal antibodies were tested for the capability to agglutinate homologous and heterologous bacteria. The growing colonies of bacteria were suspended in normal saline and the concentration was adjusted to $3x10^9$ bacterial suspension/ml by McFarland nephelometer (81), then one drop of each bacterial suspension was mixed with equal volume of undiluted MoAbs or polyclonal antibodies on a glass slide for 2 min at room temperature. The agglutination of bacterial cells were observed by naked eyes or under light microscope.

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9.7 Immunofluorescence staining

The technique was done to localize the binding site of specific antibodies on the surface of pathogenic cells (82). *P. pseudomallei* cell suspension was made by harvesting the growing colonies from culture plate and suspended in normal saline. A suitable concentration of cells for staining was selected from a 2-fold serial dilutions. For immunofluorescence, a selected cell suspension was smeared on a microscopic slide, fixed with acetone for 10 min and air-dried. The slide was washed by soaking in a solution of 1% BSA (Sigma) in PBS for 1 min. The washing step was repeated 3 times before overlaying the smear with one drop of appropriate concentration of monoclonal antibodies or hyperimmune serum. The slide was incubated in a moisture chamber at room temperature for 1 hr. The slide was washed and fluorescenated rabbit-anti-mouse immunoglobulin (Dakopatts) diluted 1:20 in 1% BSA-PBS was applied to the slide and left for 1 hr at room temperature in a moisture chamber. The slide was washed again prior to mounting with 90% glycerol in PBS. The slide was examined under a fluorescence microscope (Leitz Laborlux 12, Germany). The bacterial cell harbouring the antibody illuminated brilliant green fluorescence.

10. Biotin-streptavidin enzyme-linked immunosorbant assay (B-SA ELISA) for detection of *P. pseudomallei* antigen

The B-SA ELISA was developed for the assessment of its potential application in the diagnosis of melioidosis.

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10.1 Preparation of rabbit antisera against unfractionated whole cell antigen

Rabbits to be used for immunization were checked for the absence of antibodies against unfractionated *P. pseudomallei* antigen. Each animal was given the antigen in CFA (Sigma) by 4 subcutaneous injections over the back and was boosted 3 weeks later with the same antigen in ICF (Sigma) (83). Ten days later, all animals were bled and serum kept at -20^0c.

10.2 Purification of rabbit IgG by ion-exchange chromatography

The antiserum was precipitated with ammonium sulfate as described in Section 9.4. After exhaustive dialysis against several changes of normal saline to remove ammonium sulfate (as confirmed with 1% $BaCl_2$), the globulin fraction was then dialyzed against the buffer to be used in ion-exchange chromatography (84).

Approximately 1 g of pre-swollen DEAE-cellulose was used for the purification of IgG normally present in 1 ml of serum. Precycling, equilibration and packing of the DEAE-cellulose (DE-52, Whatman Ltd., Springfield Mill, Maidstone, Kent, England) column was carried out essentially as suggested by the manufacturer. The cellulose was finally equilibrated with 0.02 M phosphate buffer pH 8.0 prior to the application of sample (2.5 cm x 20 cm column for 10 ml serum). Under this condition, the IgG would be eluted with the starting buffer in the first peak at a rate of 0.5 ml/min. The protein content was estimated from the optical density (OD) value at 280 nm. Appropriate fractions were identified and pooled. The pooled IgG was dialyzed against 0.1 N NH_4HCO_3 pH 7.8, lyophilized and kept at 4^0c.

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10.3 Biotin conjugation of rabbit IgG

Chromatographically purified rabbit IgG was covalently conjugated with a biotin derivative by a method described by Neururkar et al (85). Briefly, the purified IgG at a concentration of 2 mg/ml was dialyzed overnight against 0.1 M $NaHCO_3$ pH 8.2 at 4^0c, clarified by centrifuged at 1,000 rpm, 4^0c for 10 min and the protein concentration was diluted to 1 mg/ml with 0.1 M $NaHCO_3$ pH 8.2. A 1 mg/ml of N-hydroxysuccinimidobiotin (Sigma) solution freshly prepared in dimethylsulfoxide (DMSO) was added immediately to the IgG at a ratio of 0.17/1 (W/W). The reaction mixture was allowed to take place at room temperature for 4 hr and was then exhaustively dialyzed for 48 hr against 0.15 M PBS at 4^0c to remove unconjugated biotin and DMSO. The supernatant was mixed with an equal volume of glycerol and stored at -20^0c.

10.4 B-SA ELISA method

The B-SA ELISA used for antigen quantitation was modified from the method described by Neururkar et al (85). The optimal concentration of rabbit anti-mouse Ig (Dakopatts), monoclonal antibodies and biotinylated-rabbit anti-unfractionated antigen were predetermined by a checkerboard titration as described by Voller et al (86) using varying concentrations of unfractionated whole cell antigen.

The titration was carried out in a U-bottomed, 96-well plate (Nunc Immunoplate II, Intermed). Each well was coated with 50 µg of rabbit anti-mouse Ig at a concentration of 10 µg/ml in 0.05 M carbonate buffer, pH 9.6 at 4^0c overnight. The plate was then washed 3 times for 3 min each with PBST and blocked with 150 µl per well of 2% BSA in PBST

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at 37^0c for 1 hr. After washing 50 µl of an appropriate concentration of monoclonal antibody was added. The plate was then incubated at 37^0c for 1 hr and washed again before the addition of 50 µl of antigens which was 2-fold diluted from 3.12 to 1,000 ng/ml with 1% BSA-PBST. The plate was incubated at 37^0c for 1 hr and washed 3 times, followed by the addition of 50 µl of biotinylated rabbit anti-unfractionated whole cell antigen (diluted 1:200) and the plates were reincubated at 37^0c for 1 hr. After washing, 50 µl of a 1:2000 diluted streptavidin peroxidase (Dakopatts) was added and the plate was incubated and washed once more. One hundred µl of freshly prepared substrate solution (same as 3.1) was added and allowed to react at room temperature in the dark for 40 min. The reaction was terminated by the addition of 25 µl of 5 N H_2SO_4. The optical density was determined at 492 nm by ELISA-plate reader. The result was considered positive if the optical density was more than 2 times the average optical density of the reagent control.

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CHAPTER IV

RESULTS

1. Specific antigen of *P. pseudomallei*

1.1 Characterization of unfractionated whole cell antigen by SDS-PAGE

The unfractionated whole cell antigen of *P. pseudomallei* used throughout this study was prepared from a strain originally isolated from a septicemic patient resided in Khon Kaen province [strain No. 18 in Table A (see Appendix) designated strain PP844 in this communication]. When the unfractionated whole cell antigen was subjected to SDS-PAGE under a standard condition, i.e., the sample was treated with a reducing agent (2-ME) and heated for 3 min before loading, there were more than 60 protein bands with molecular masses ranging between 11 and 210 kDa (Fig. 2 and Table 3). Of these, there were only 5-6 major components (judging from the intensity of the protein bands), i.e., 19.5, 33.7, 34, 54, 125 and 210 kDa. Similar protein patterns were observed with the extract from all 56 *P. pseudomallei* isolates. Essentially similar SDS-PAGE and immunoblotting patterns were obtained when the reducing agent was omitted and/or the sample was applied without heating (Fig. 3 and Table 4), suggesting that most of these components were present in a monomeric form. It should be mentioned that the interpretation of the above data should be taken with caution because the patterns, particularly the protein patterns, were highly complicated and it was very difficult to compare the patterns from different sets of conditions.

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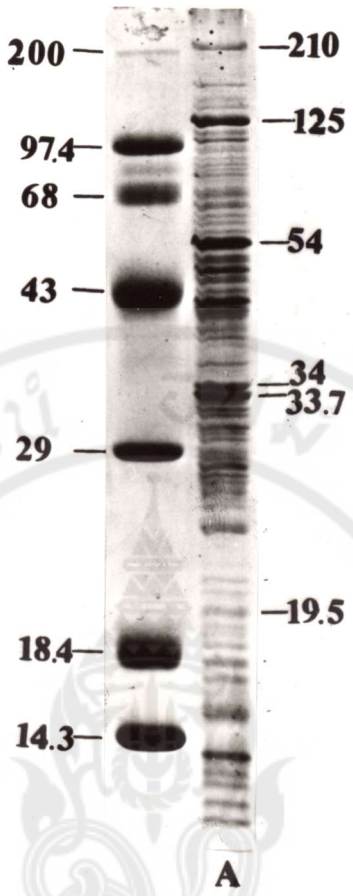

Figure 2. A representative of Coomassie blue stained SDS-PAGE profile of the unfractionated whole cell antigen of *P. pseudomallei* (PP 844, lane A). The molecular weights of major protein bands and the specific 19.5-kDa band are shown on the right. Standard protein markers were applied to the left lane and run simultaneously for the construction of a standard curve.

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Table 3. A summary of unfractionated and fractionated antigens detected by SDS-PAGE and immunoblot.

Methods	Molecular weights (kDa)	
	Unfractionated antigen	Fractionated antigen
SDS-PAGE	11.0, 11.6, 12.2, 12.7 13.5, 14.3, 15.0, 15.5 17.5, 17.8, 18.7, 19.0 19.5, 20.0, 21.0, 21.6 23.0, 24.5, 25.5, 26.6 27.1, 28.0, 33.7, 34.0 43.0, 54.0*, 105.0, 125.0* 160.0, 210.0*	17.5, 18.7, 19.5*, 21.0, 21.6, 23.0, 25.5
Immunoblot+	12.7, 18.7, 19.5*, 21.0 21.6, 24.5, 25.5, 33.7 34.0, 39.0, 43.0, 54.0* 66.0, 68.0, 125.0*	19.5*

+ : The immunoblot was developed with pooled melioidosis serum

* : Major components

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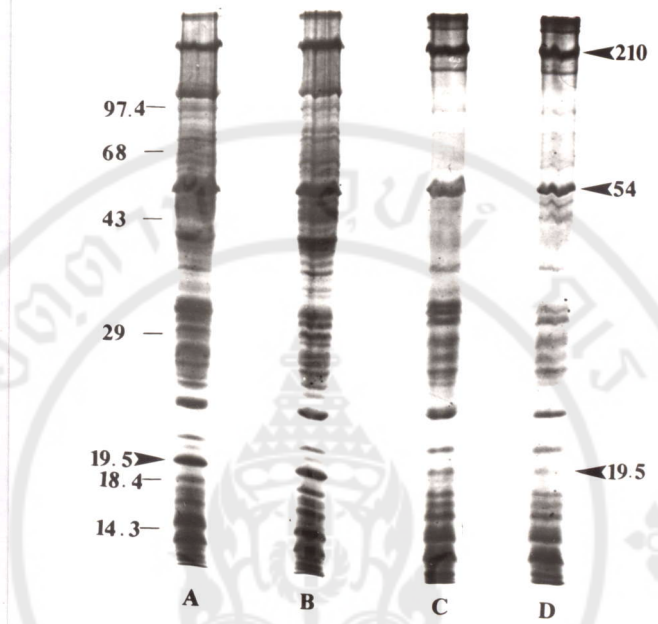

Figure 3. Effect of reduction and heating on the SDS-PAGE profiles. The unfractionated whole cell antigen of PP 844 was subjected to heating (lanes A and C) or reduction (lanes A and B) prior to being electrophoresed. The pattern of lane A represented that of a standard condition shown in Fig. 2. See legend to Fig. 2 for other explanation.

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Table 4. Effect of reduction and heating on SDS-PAGE profiles.

Relative molecular weights (kDa)	Relative intensity of staining			
	with 2-ME		without 2-ME	
	heated	unheated	heated	unheated
210.0	3*	3	3	3
160.0+	0	0	2	2
125.0+	3	3	1	1
54.0	4	4	4	4
50.0, 46.0, 43.0, 41.0+	1	1	0	0
34.0	3	3	3	0
33.7	3	3	3	2
28.0	3	3	3	2
26.5	1	1	1	1
25.5	1	1	1	1
24.5	3	3	3	3
23.0	2	2	2	2
20.5	1	1	1	1
19.5+	3	3	2	2
18.7	2	2	2	2
17.5	2	2	2	2
16.5	2	2	2	2
15.0	4	4	4	4
13.5	4	4	4	4

* The numbers 0 to 4 represent the relative intensity of staining.

+ The components markedly affected by reduction.

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1.2 Identification of specific components of *P. pseudomallei* by immunoblotting

The SDS-PAGE protein profiles of *P. pseudomallei* was rather complicated and could not be distinguished from those of other *Pseudomonas* species or other gram-negative bacteria (Fig. 4). It was not possible therefore to identify any protein component that was unique for *P. pseudomallei* by this simple analysis. However, from preliminary analysis using pooled melioidosis serum as a probe in the Western blot analysis, it appeared possible to demonstrate *P. pseudomallei* specific antigen. Therefore, in the present study detailed analysis was carried out to further elucidate and confirm this finding. Immunoblotting analysis of unfractionated whole cell antigen from PP844 was carried out using a 1:500 dilution of pooled melioidosis serum as a specific probe. The results showed that there were at least 28 immunoreactive components and the most immunogenic one appeared to be the one migrated to a 19.5-kDa position (Fig. 5, Table 3). Essentially the same immunoreactive pattern was obtained with 55 other strains of *P. pseudomallei*. Again, the 19.5-kDa component seemed to be the strongest and unique immunogenic antigen present in all *P. pseudomallei* isolates (Fig. 6). It is interesting to note that among a large number of other gram-negative bacteria tested, the 19.5-kDa immunoreactive component was present only in *P. cepacia* (Fig. 7). However, the presence of the immunoreactive component at this position does not mean that it is immunologically identical with the 19.5-kDa component of *P. pseudomallei*.

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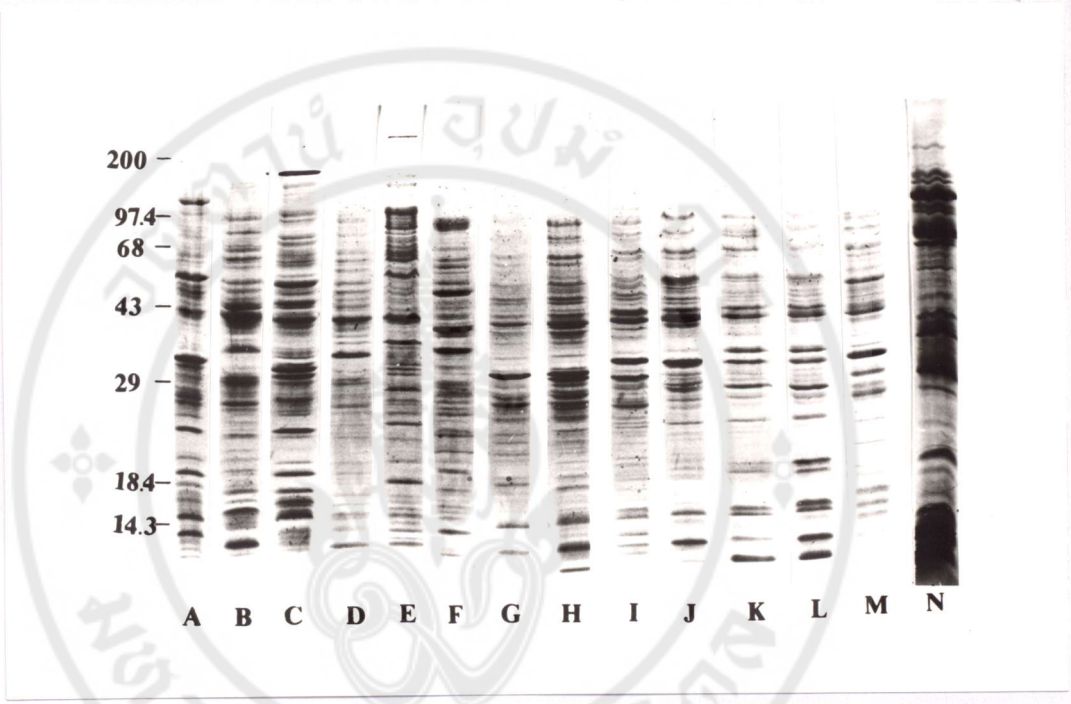

Figure 4. Coomassie blue stained SDS-PAGE profiles of the unfractionated whole cell antigens prepared from PP844 (A), *P. aeruginosa* (B), *P. cepacia* (C), *P. putida* (D), *P. maltophila* (E), *P. alcaligenes* (F), *K. pneumoniae* (G), *E. cloaca* (H), *E. coli* (I), *S. typhi* (J), *S. enteritidis* (K), *S. krefeld* (L), *P. mirabilis* (M) and *S. aureus* (N). Molecular weight markers are shown on the left.

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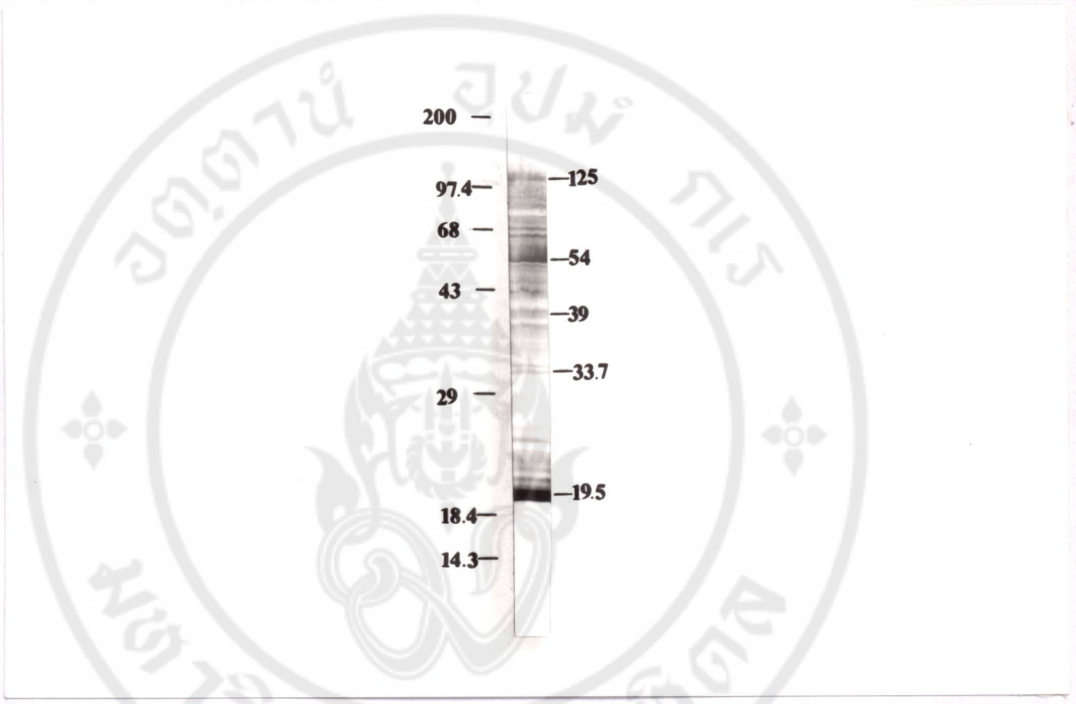

Figure 5. Immunoblotting pattern of unfractionated whole cell antigen PP844 probed with pooled melioidosis serum. The positions of major immunoactive components are shown on the right and molecular weight standard markers on the left.

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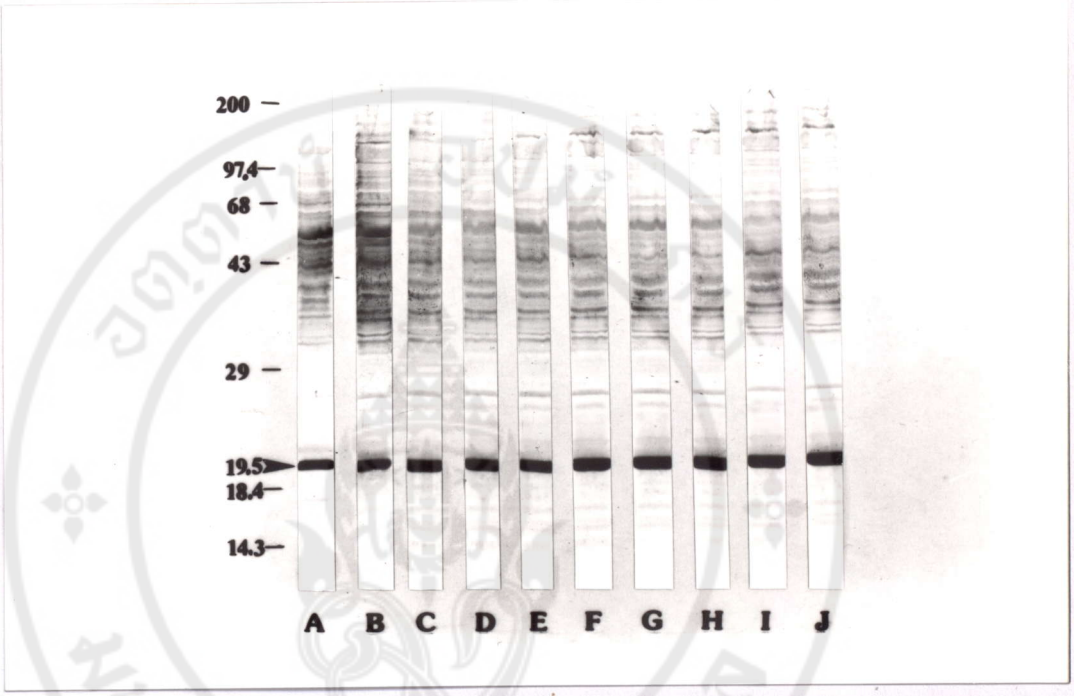

Figure 6. Representative immunoblotting patterns of the unfractionated whole cell antigens from ten strains of *P. pseudomallei* probed with pooled melioidosis serum. All strains exhibited a very strong immunoreactive component at a 19.5-kDa position (arrow head). Molecular weight standard markers are shown on the left.

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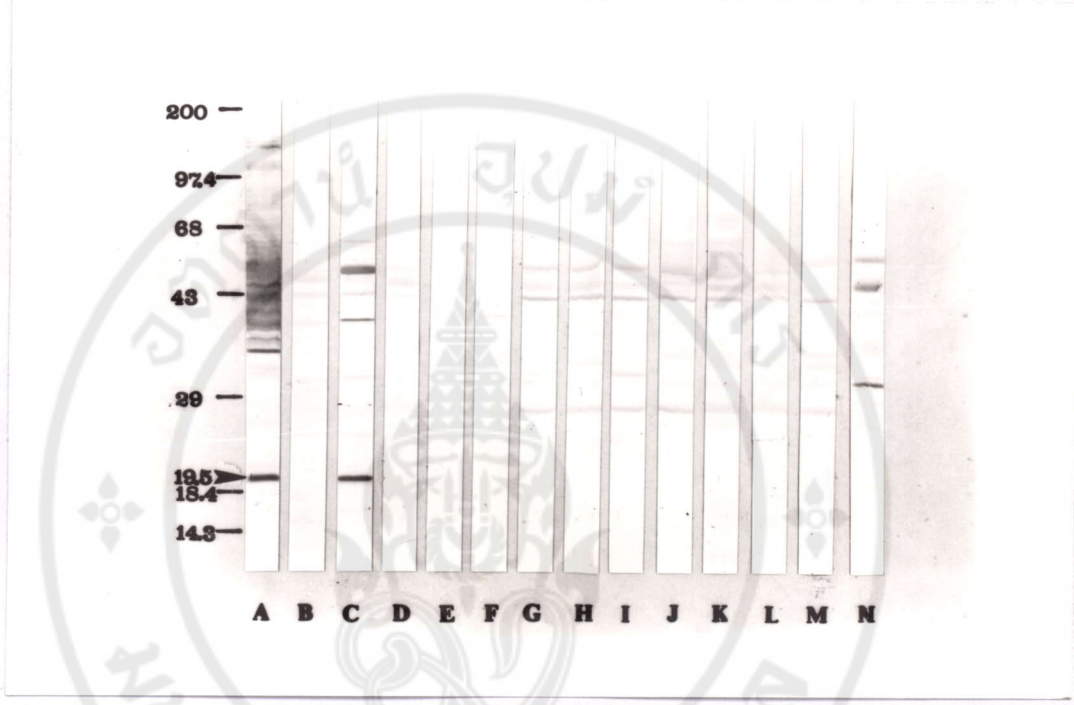

Figure 7. Immunoblotting analysis of the unfractionated whole cells antigen from PP844 (A), *P. aeruginosa* (B), *P. cepacia* (C), *P. putida* (D), *P. maltophila* (E), *P. alcaligenes* (F), *K. pneumoniae* (G), *E. cloaca* (H), *E. coli* (I), *S. typhi* (J), *S. enteritidis* (K), *S. krefeld* (L), *P. mirabilis* (M) and *S. aureus* (N), probed with pooled melioidosis serum. Only the *P. cepacia* possessed the 19.5-kDa component. Molecular weight standard markers are shown on the left.

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1.3 Purification and characterization of 19.5-kDa component

The unfractionated whole cell antigen of PP844 was loaded to a SDS-PAGE preparative gel together with prestained standard markers containing a mixture of 14.3, 18.4, 29.0, 43.0, 68.0, 97.4 and 200.0 kDa components positioned in a slot on both sides of the antigen through. After the electrophoresis was terminated, the gel was cut, one at a 18.4-kDa position and the other at a distance 1 cm above the first cut. From the reference markers, this gel strip should contain proteins with molecular weights ranging between 18,400 and 25,500. The gel strip was then put into an Electro-eluter and the protein was eluted as described in Materials and Methods. The eluents from several such preparations were pooled, dialyzed against PBS and lyophilyzed prior to removing the SDS. In this experiment, a total of 56 mg of unfractionated whole cell antigen was loaded and the yield of the fractionated antigen was 3.33 mg, representing approximately 6% of the crude antigen.

Analysis of the fractionated 19.5-kDa antigen by SDS-PAGE and stained for its protein composition by Coomasie blue showed that although the 19.5-kDa component was a predominant protein in this partially purified fraction, it nevertheless contained several other minor components, particularly those with molecular masses of 21.0, 21.6, 23.0 and 25.5 kDa (Fig. 8 and Table 3). A trace contamination by a few higher molecular weight components was also noted (Fig. 8). On the contrary, the immunoblotting pattern of the fractionated component showed that only the 19.5-kDa component reacted with the pooled melioidosis serum (Fig. 9, lane C).

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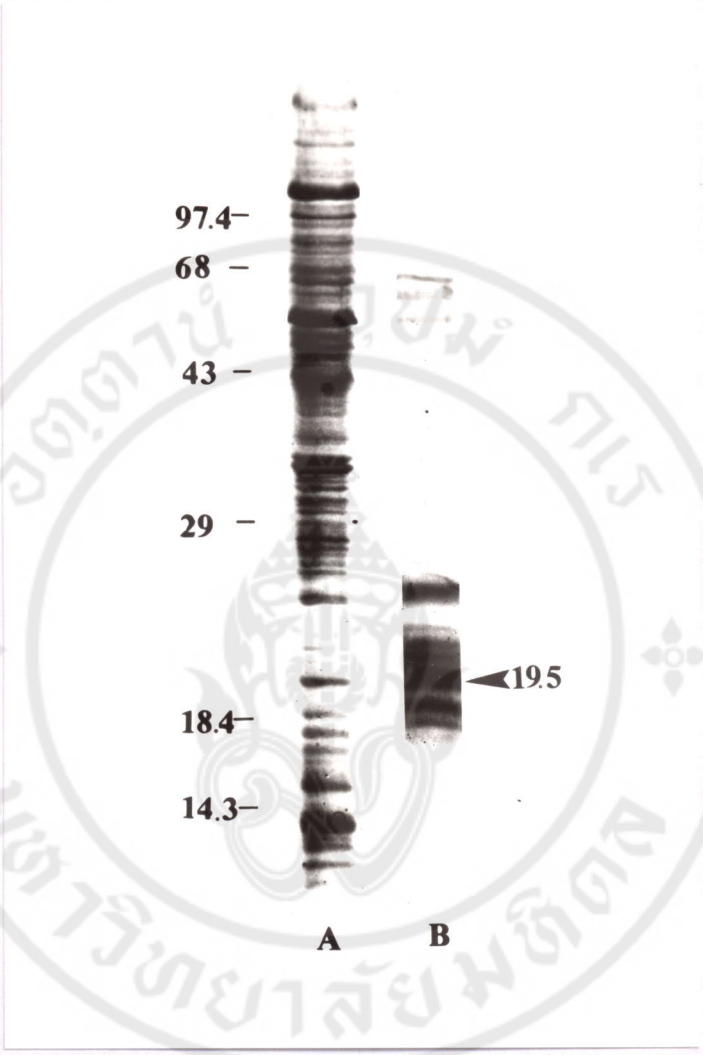

Figure 8. Coomassie blue stained SDS-PAGE profile of the fractionated antigen. Profile of the unfractionated antigen (A) was highly complicated comparing with that of fractionated one (B). The fractionated antigen was made up of a number of proteins but the predominant one was the 19.5 kDa (arrow head).

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Figure 9. Immunoblotting profiles of the fractionated 19.5-kDa antigen. Unfractionated whole cell antigen (lanes A and B) and fractionated 19.5-kDa antigen (lanes C and D) were electrophoresed, transferred and allowed to react with pooled melioidosis serum (lanes A and C) and pooled normal human serum (lanes B and D). The only immunoreactive protein in the partially purified 19.5 kDa-fraction was the 19.5-kDa component (arrow head). Molecular weight markers are shown on the left.

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1.4 Evaluation of the fractionated 19.5-kDa antigen for the diagnosis of melioidosis by indirect ELISA and immunoblotting

The fractionated 19.5-kDa antigen was evaluated for its potential in the diagnosis of acute septicemic melioidosis and the results were compared with those obtained with unfractionated whole all antigen. Both assays used the same set of clinical specimens which included 37 sera of septicemic melioidosis patients, 25 sera from septicemia associated with other bacterial infections, 25 normal human sera from Khon Kaen and 60 normal human sera from Bangkok. The optimal antigen concentrations and the appropriated serum dilution to be used in the indirect ELISA system were predetermined from checkerboard titrations (Fig. 10 for unfractionated whole cell antigen and Fig. 11 for fractionated 19.5-kDa antigen). The results obtained suggested that while the antigen concentration of 5 ug/ml could be used for both fractionated and unfractionated antigens the optimal serum dilution required for the unfractionated and fractionated antigens were 1:500 and 1:250 respectively. The ELISA results were summarized and shown in Tables 5 and 6. It is clear from the tables that a more satisfactory result (sensitivity, specificity and predictive values) was obtained with the fractionated 19.5- kDa antigen, regardless of whether a cut of limit (mean + 2SD) was established with normal sera collected from either endemic (Khon Kaen) or non-endemic (Bangkok) areas of infection. The results obtained with immunoblotting analysis showing reactivity with the 19.5-kDa protein were almost as good as those obtained with the ELISA (Table 7, Fig. 12). The immunoblot gave a sensitivity and specificity of 64.86% and 97.64% respectively.

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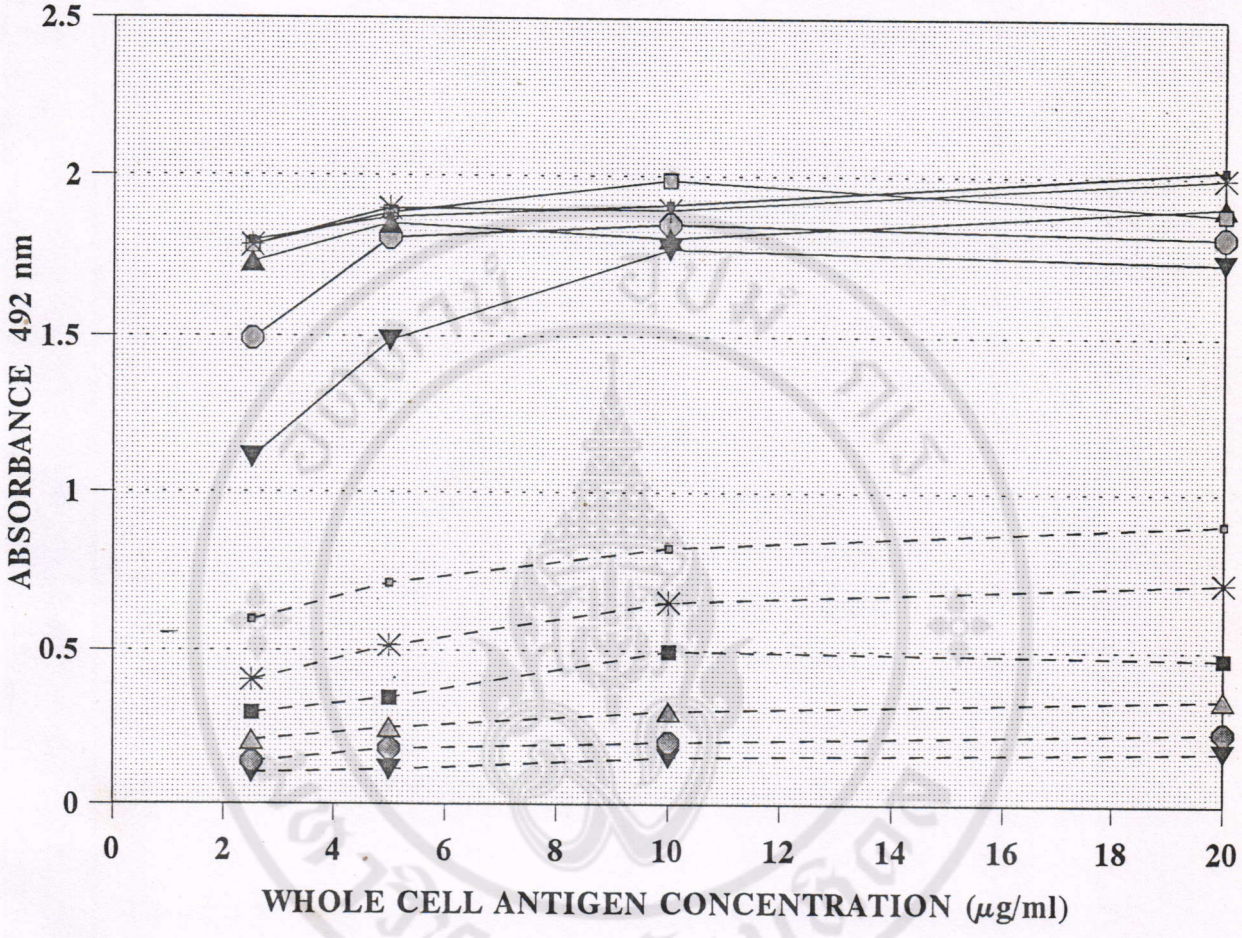

Figure 10. Checkerboard titration of the unfractionated whole cell antigen required for indirect ELISA. The positive serum used was a pooled melioidosis serum (——) and the negative serum was a pooled normal serum (----).

Serum dilutions

▪---▪ : 1:50 ✳---✳ : 1:100

■---■ : 1:200 ▲---▲ : 1:400

⬢---⬢ : 1:800 ▼---▼ : 1:1,600

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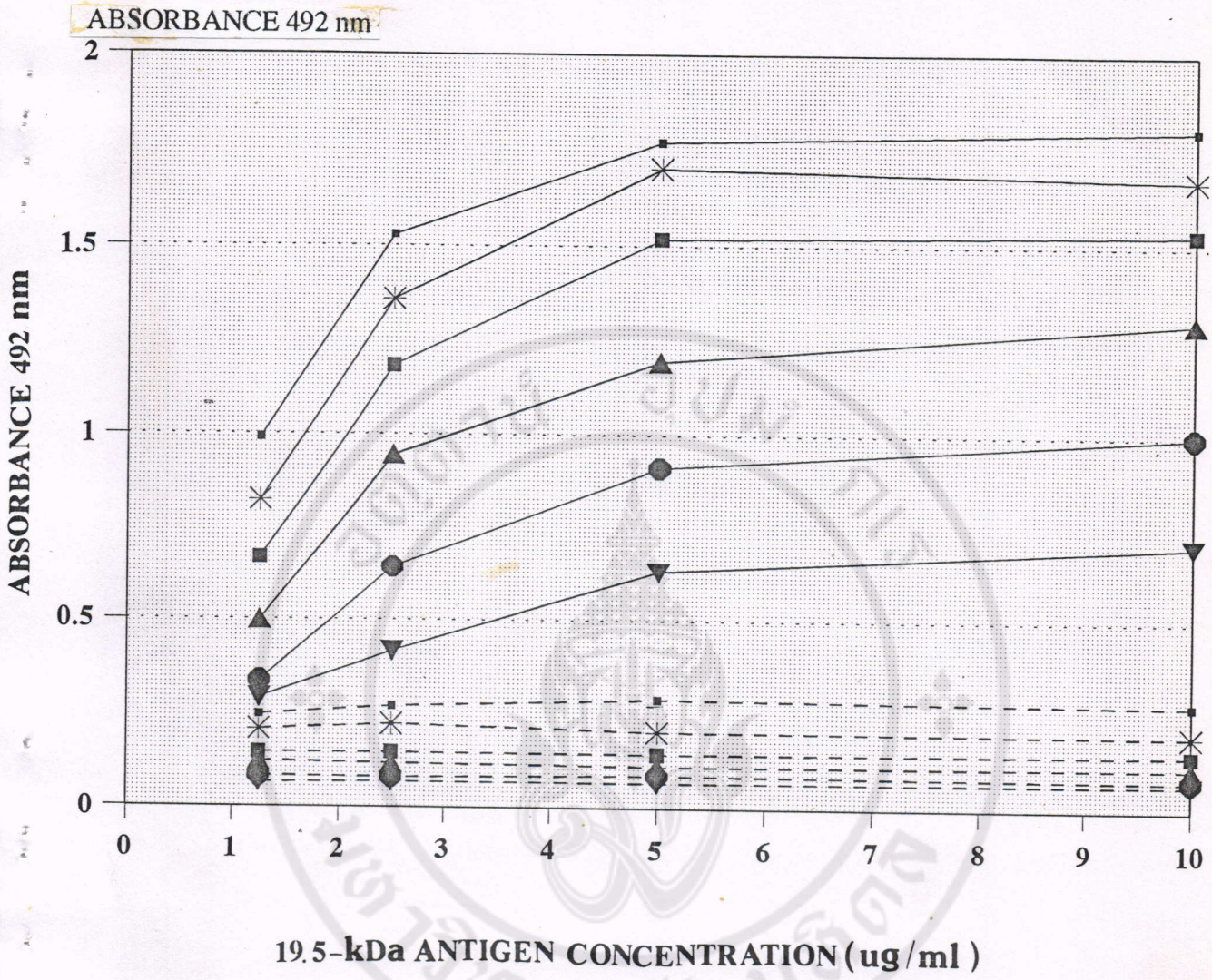

Figure 11. Checkerboard titration of the fractionated 19.5-kDa antigen required for indirect ELISA. The positive serum used was a pooled melioidosis serum (——) and the negative serum was a pooled normal human serum (----).

Serum dilutions

▪—▪ : 1:50 ✳—✳ : 1:100

■—■ : 1:200 ▲—▲ : 1:400

⬮—⬮ : 1:800 ▼—▼ : 1:1,600

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Table 5. Indirect ELISA reactivity of sera from septicemic patients with melioidosis and with other bacterial infections.

Source of specimens[a]	No. of positives / total tested			
	Unfractionated whole cell		Fractionated 19.5-kDa	
	Cut off >0.16[b]	Cut off >0.67[c]	Cut off >0.15[b]	Cut off >0.23[c]
Melioidosis	34/37 (92%)	20/37 (54%)	34/37 (92%)	30/37 (81%)
Other bacterial infections	14/25 (56%)	1/25 (4%)	6/25 (24%)	2/25 (8%)
Normal human (Khon Kaen)	19/25 (76%)	1/25 (4%)	8/25 (3.2)	0/25 (0%)
Normal human (Bangkok)	4/60 (6.6%)	0/60 (0%)	2/60 (3.3%)	1/60 (1.6%)

a : A single serum dilution of 1:500 and 1:250 were used with unfractionated and fractionated antigen, respectively.

b : A criterion used for positive cut off (mean + 2 S.D.) was based on normal values from nonendemic area (Bangkok, n = 60).

c : A criterion used for positive cut off (mean + 2 S.D.) was based on normal values from endemic area (Khon Kaen, n = 25).

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Table 6. Sensitivity, specificity and predictive values of indirect ELISA for the detection of IgG antibody in the serum of patients with septicemic melioidosis.

Antigens	% sensitivity	% specificity	% predictive value	
			Positive	Negative
Fractionated 19.5-kDa	92 (82)	91 (96)	81 (94)	96 (87)
Unfractionated whole cell	92 (54)	79 (96)	65 (91)	96 (74)

The results (see Table 5 for details) were calculated based on a cut-off limit (mean + 2 S.D.) established with normal individuals from non-endemic (Bangkok : 60) and endemic (Khon Kaen : 25; in parenthesis) areas of infection. Those infected with other agents were considered as part of the "normal" pool.

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Table 7. Immunoblotting reactivity of the unfractionated whole cell *P. pseudomallei* against sera from patients with melioidosis and other infections.

Source of Specimens*	Positive for 19.5 kDa (No. positive/No. tested)
Melioidosis	24/37 (64.86%)
Other bacterial infections	1/25 (4.00%).
Normal human (Khon Kaen)	2/25 (8.00%)
Normal human (Bangkok)	1/60 (1.60%)

* sera tested at a dilution of 1:500

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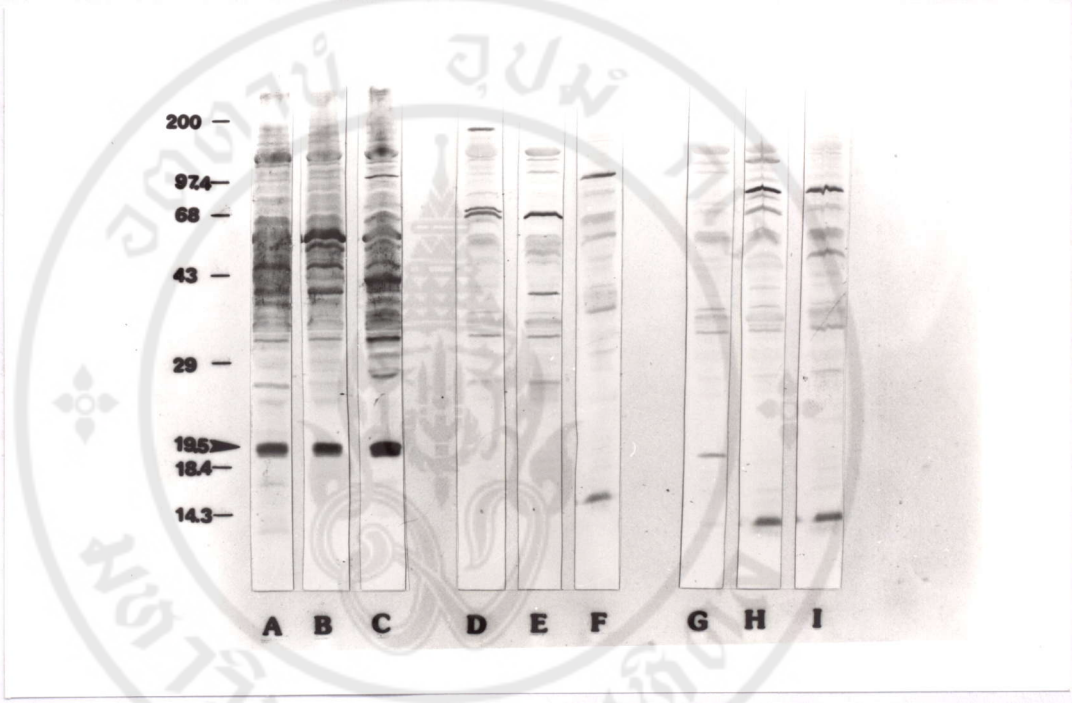

Figure 12. Representative immunoreactivity patterns of sera from 3 melioidosis patients (lanes A, B, C), 3 normal humans (lanes D, E, F) and 3 other infected patients (lanes G, H, I). Molecular weight standard markers are shown on the left. The 19.5-kDa component reacted only with serum from patients with melioidosis.

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2. Production of hybridomas

2.1 Detection of prefusion mouse sera for antibody titres and characterization of their immunoreactivity patterns.

Monoclonal antibodies (MoAbs) described in this study were obtained from 2 fusions. One fusion was from a BALB/c mouse immunized with the unfractionated whole cell antigen and another was from an animal immunized with the fractionated 19.5-kDa antigen. Antibody titers of the two prefusion sera were determined by indirect ELISA against homologous antigens and found to be 1:51,200 and 1:25,600 respectively. The immunoblotting patterns of the immune serum from these 2 animals are shown in Fig. 13. As to be expected from the antigen used for immunization, the serum from an animal immunized with the unfractionated antigen possessed antibodies reactive with a large number of higher molecular weight components (e.g. 54, 39, 36 and 32 kDa) and the one from an animal immunized with the fractionated antigen contained antibodies reactive with a fewer number of components, predominantly those with molecular masses of 25.5 kDa or lower.

2.2 Initial screening and identification of hybridomas of interest

Spleen cells from an animal immunized with unfractionated whole cell antigen were fused with the Ag 8.653 myeloma cells resulting in 32% hybridoma growing wells. Following screening by indirect ELISA using unfractionated whole cell antigen, 61 clones from the hybridoma growing wells were found to secrete antibody ($OD_{492} \geq 0.20$). The positive clones were maintained and subcultured. However, some of the positive clones were either lost or ceased to secret antibodies during the

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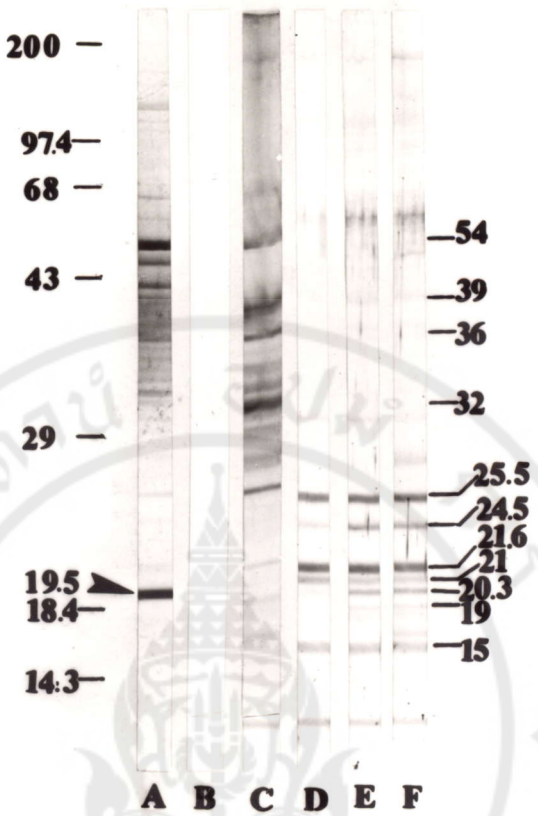

Figure 13. Immunoblotting characteristics of the immune sera from BALB/c mice immunized with either unfractionated or fractionated antigens. The immunoblotting profiles of the electrophoresed unfractionated antigen were probed with

(A) Pooled melioidosis serum (positive control)

(B) Preimmunized mouse serum (negative control)

(C) Prefusion serum from the animal immunized with unfractionated whole cell antigen

(D, E) Post-boostered serum from an animal immunized with fractionated 19.5-kDa antigen

(F) Prefusion serum from animal immunized with the fractionated 19.5-kDa antigen.

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process and only 17 clones remained and were available for specificity testing using a panel of heterologous antigens (Table A in the Appendix). Among the 17 clones, six clones (1G8, 9B10, 9C12, 9C3, 3H5 and 5F8) were found to be specific for *P. pseudomallei* and continued to secrete large quantity of antibodies (judging from the O.D. value). These 6 clones were subsequently recloned by limiting dilution method and expanded.

Spleen cells from the animal immunized with the fractionated 19.5-kDa antigen gave 57% fusion efficiency. However, only 3 clones (2A6, 2A2, 5G12) were found to be stable and secreted antibodies reactive with both the fractionated and unfractionated antigens at the same position by immunoblotting. One clone (5G12) was characterized further. It should be noted that its strongest reaction was found at a 20.3-kDa position, and not at the 19.5-kDa position as expected. A trace reactivity could also be observed at a 19.0-kDa position. It should be mentioned at this time that these figures were real as the 20.3 kDa could be readily distinguished from 19.5 kDa or 19.0 kDa bands by an experiment shown in Figure 14. Unlike the immunoblotting results, these 3 clones failed to react with both unfractionated and fractionated antigens by indirect ELISA. The reason for this is not clear but it is not due to a technical problem because both antigens could readily react with a positive control.

The isotypes of the seven selected clones are shown in Table 8.

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2.3 Specificity of monoclonal antibodies to unfractionated antigen

The six selected MoAbs (i.e., 1G8, 9B10, 9C12, 9C3, 3H5 and 5F8) from the fusion of spleen cells from the animal that was immunized

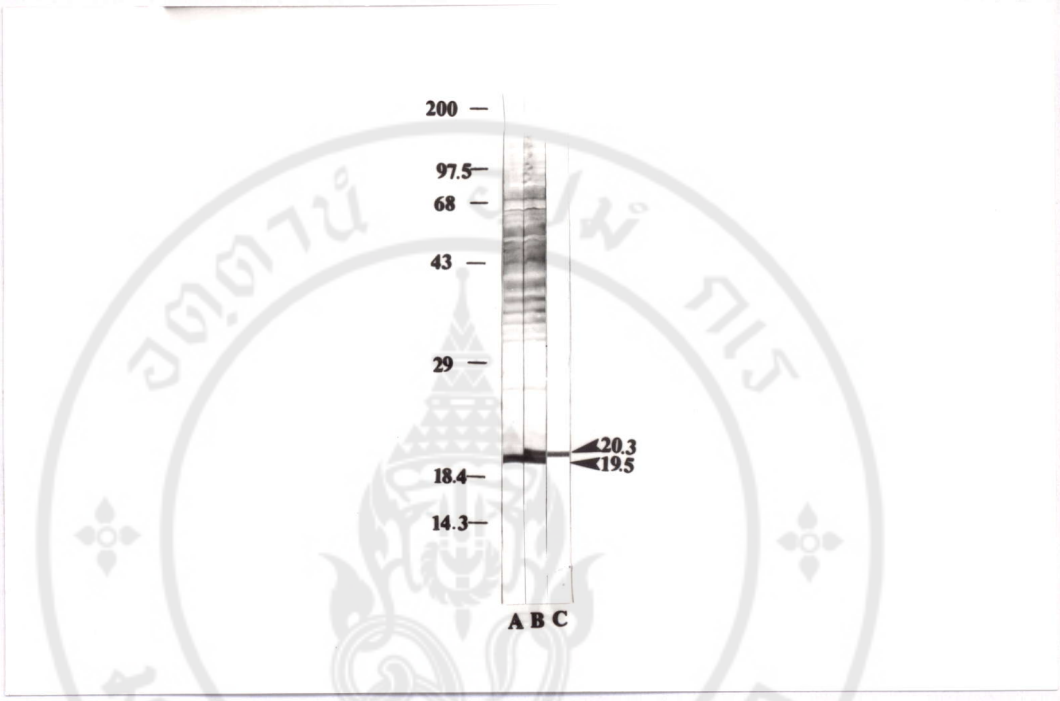

Figure 14. Experiment showing that the 20.3-kDa band developed with the 5G12 MoAb was different from the 19.5-kDa band developed with human melioidosis serum. Unfractionated whole cell antigen was electrophoresed, electrotransferred to a nitrocellulose and probed with pooled melioidosis serum (lane A), a mixture of pooled melioidosis serum and 5G12 MoAb (lane B) and 5G12 MoAb (lane C). The antibody mixture in lane B gave 2 distinct bands at 19.5 and 20.3-kDa positions (arrow heads). Molecular weight markers are shown on the left.

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Table 8. Isotypes of monoclonal antibodies selected for further characterization.

MoAb	Immunogen used	Heavy chain	Light chain
1G8	W.C.	IgG2b	k
9B10	W.C.	IgG2b	k
9C12	W.C.	IgG1	λ
9C3	W.C.	IgG1	λ
3H5	W.C.	IgG2b	k
5F8	W.C.	IgM	k
5G12	19.5-kDa	IgG1	k

W.C. : Unfractionated whole cell antigen.

19.5-kDa : Fractionated 19.5-kDa antigen.

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with unfractionated antigen were tested for specificity by indirect ELISA using a panel of unfractionated whole cell antigens from 56 strains of *P. pseudomallei*, 2 strains of *P. cepacia* and 1 strain each of *P. aeruginosa*, *P. putida*, *P. maltophila*, *P. alcaligenes*, *K. pneumoniae*, *E. cloacae*, *E. coli*, *S. thyphi*, *S. enteritidis*, *S. krefeld*, *P. mirabilis* and *S. aureus*. These antigens were used at a concentration of 5 ug/ml. The results (detailed data can be found in Table A in Appendix) were summarized as shown in Table 9. As can be seen from the Table, the 5F8 MoAb was highly specific as it reacted with all *P. pseudomallei* isolates and yet failed to react with any other bacterial antigens used for testing. These 6 selected MoAbs exhibited different immunoblotting patterns against unfractionated whole cell antigen (Fig. 15) except the 5F8 MoAb which did not give any reactive band. However, on a few occasions a very faint broad (smearing) band could be observed. 1G8, 9B10, 9C12 and 9C3 MoAbs reacted strongly at 39.0-kDa position of unfractionated antigen under both reducing and nonreducing conditions whereas 3H5 MoAb reacted with many other components but not with the 39.0-kDa component as summarized in Table 10. These MoAbs however did not give a similar immunoblotting and ELISA pattern with all *P. pseudomallei* isolates. The results presented in Figure 16 represented the examples of ELISA reactivities of 2 clones, one with high reactivity (5F8) and the other with low reactivity (1G8) against unfractionated whole cell antigen of the PPS44, testing against all *P. pseudomallei* isolates and other bacteria (Table A in Appendix). It should be noted that the reactivity against the bacterial strain 59 is rather unexpected in that the low reacting 1G8 reacted much stronger than the high reacting 5F8 clone (Fig 16).

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Table 9. A summary of immunoreactivity of seven monoclonal antibodies.

MoAb	Immunogen for immunization	Number positive / Total tested(%)			
		ELISA[a]		Immunoblotting positive[b]	
		P. pseudomallei	other bacteria	other bacteria	*P. pseudomallei*
1G8	W.C.	31/56 (55)	0/14 (0)	ND	ND
9B10	W.C.	40/56 (71)	0/14 (0)	ND	ND
9C12	W.C.	45/56 (80)	0/14 (0)	ND	ND
9C3	W.C.	47/56 (84)	0/14 (0)	ND	ND
3H5	W.C.	54/56 (96)	0/14 (0)	ND	ND
5F8	W.C.	56/56 (100)	0/14 (0)	ND	ND
5G12	19.5-kDa	ND	ND	2[c]/14 (14)	56/56 (100)

a : Positive cut-off point was ≥ 0.20

b : Immunoblotting positive at 20.3-kDa position

c : 2 strains of *P. cepacia*

ND : Not done

W.C. : Unfractionated whole cell antigen

19.5-kDa : fractionated 19.5-kDa antigen

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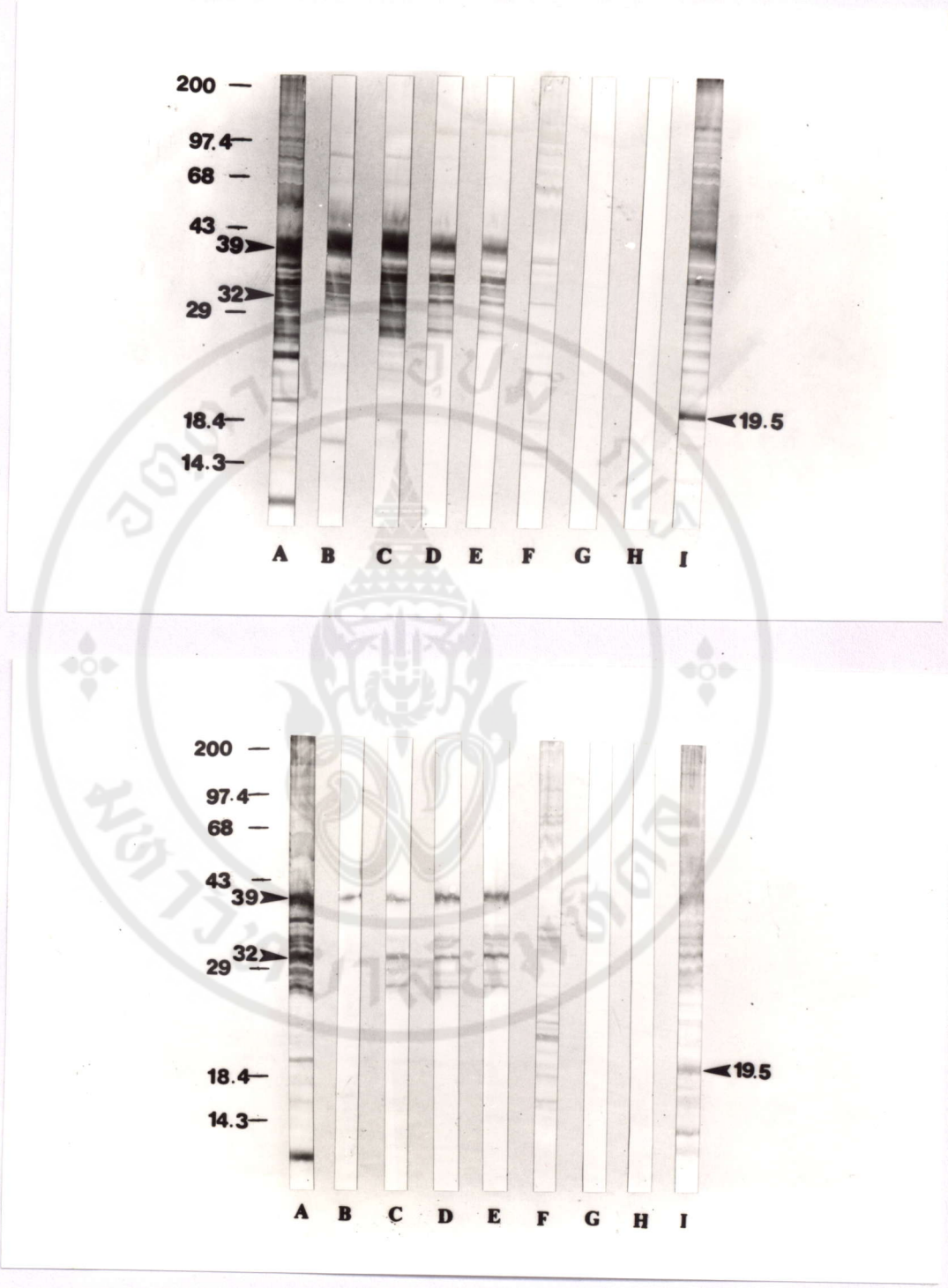

Figure 15. The immunoblotting patterns of MoAbs against unfractionated whole cell antigen under reducing condition (i) and nonreducing condition (ii). The 6 MoAbs in this experiment included, 1G8 (B), 9B10 (C), 9C12 (D), 9C3 (E), 3H5 (F), and 5F8 (G). The prefusion mouse serum (A) and melioidosis patient serum (I) were run simultaneously for comparison. Culture medium in (H) served as a negative control. The major bands are pointed (arrow heads). Molecular weight standard markers are shown on the left.

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Table 10. A summary of the immunological characteristics of selected MoAbs.

MoAbs	Immunogen	Ig Isotype	Indirect ELISA		Immunoblot profile	
			W.C.	19.5-kDa	kDa[a]	Other remarks
1G8	W.C.	IgG2b	0.68	ND	39, 33,	-
9B10	W.C.	IgG2b	1.11	ND	39, 33, 32, 27	-
9C3	W.C.	IgG1	0.74	ND	39, 33, 32 ,27	-
9C12	W.C.	IgG1	0.69	ND	39, 33, 32, 27	-
3H5	W.C.	IgG2b	0.80	ND	62, 37, 28.5, 22.5	-
5F8	W.C.	IgM	0.76	ND	-	No reaction by immunobloting.
5G12	19.5-kDa	IgG1	0.07	0.08	20.3	No reaction by indirect ELISA.

a : Major components

W.C. : unfractionated whole cell antigen

19.5-kDa : fractionated 19.5-kDa antigen

ND : not done

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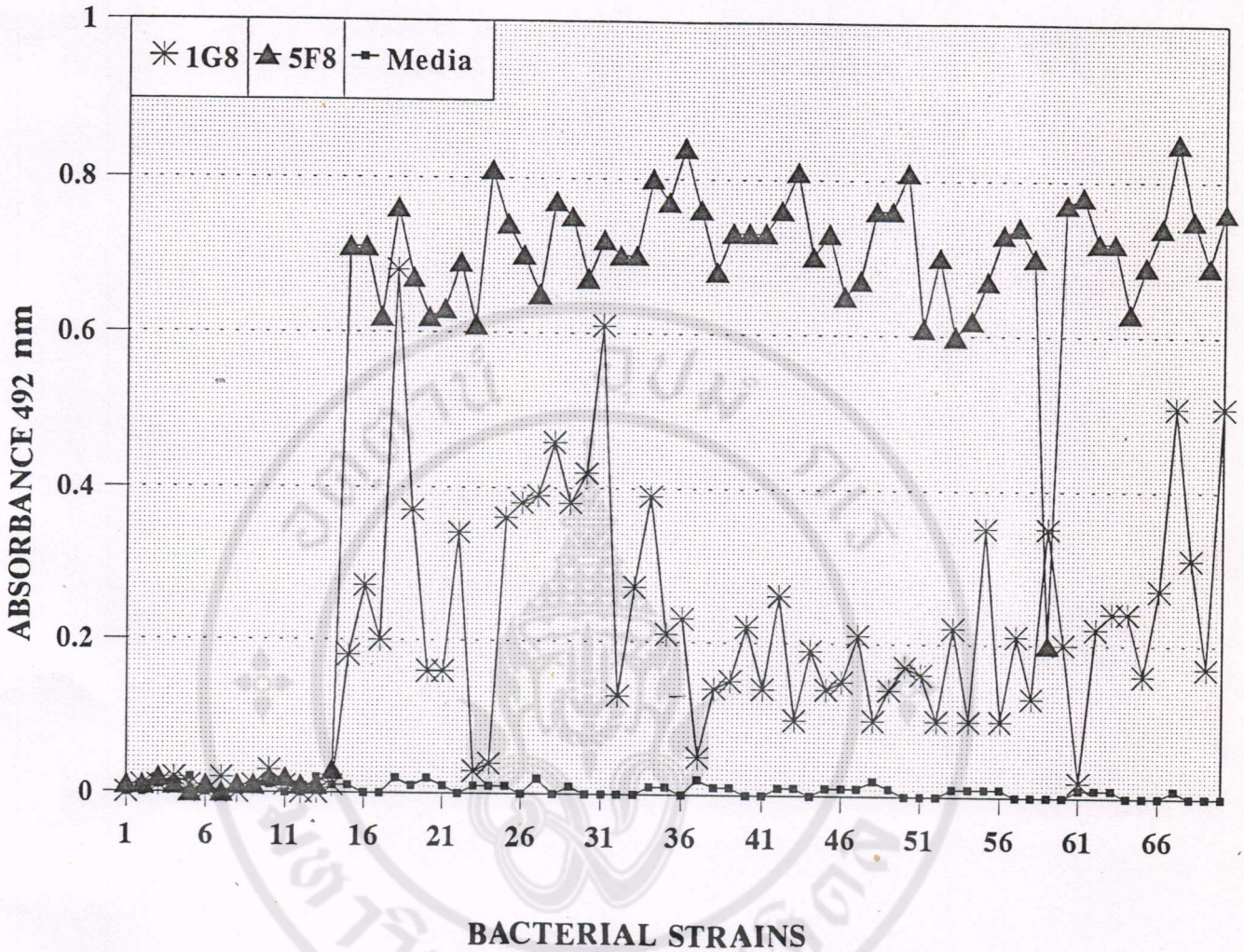

Figure 16. Immunoreactivity of the two representative MoAbs (1G8 and 5F8) against different bacterial strains. Unfractionated whole cell antigen was prepared from each bacterial isolate (see Table A in the Appendix) and were used to coat the microtiter plate at a concentration of 5 ug/ml and were allowed to react with undiluted supernatant fluid from clones 1G8 and 5F8 in indirect ELISA. The culture medium served as a negative control.

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2.4 Specificity of monoclonal antibody to fractionated antigen

The specificity of 5G12 MoAb from the mouse immunized with fractionated antigen was tested by immunoblotting. The undiluted supernatant fluid of 5G12 was used to probe against homologous and heterologous antigens shown in Figure 17. The results clearly showed no immunoreactive band with *P. aeruginosa, P. putida, P. maltophila, P. alcaligenes, K. pneumoniae, E. cloacae, E. coli, S. typhi, S. enteritidis, S. krefeld and P. mirabilis* (right strips of lanes B, D, E, F, G, H, I, J, K, L and M respectively). In contrast, this MoAb showed a prominent reactive band at a 20.3-kDa position for *P. pseudomallei* (right strip of lane A) and *P. cepacia* (right strip of lane C). With *S. aureus*, the 5G12 MoAb reacted as indistinct smearing at positions between 30 and 40 kDa (right strip of lane N). It was proved later that these positive reactions most likely represented the protein A of *S. aureus* which could bind to rabbit anti-mouse immunoglobulin that was used as a second antibody in the system. The pooled melioidosis serum from patients with melioidosis was included (left strips of every lane) as positive control. Moreover, the 5G12 was analyzed further to see if it could react with any common component of all *P. pseudomallei* isolates by performing the immunoblotting of unfractionated whole cell antigens extracted from the 56 strains of *P. pseudomallei* currently available. The results shown in Table 9 indicated that the 5G12 reacted with a component (20.3 kDa) present in all *P. pseudomallei* isolates.

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Because the 20.3-kDa and the 19.5-kDa components were less than 5% different with regard to their relative molecular sizes, the initial

impression was that this difference was not real and it was most likely an inaccuracy due to a technical error, e.g., arising as a result of inaccuracy in measuring the distances of protein migration. In order to rule out this possibility, an experiment was designed to distinguish these 2 different immunoreactive bands. This was done by running an immunoblotting experiment using unfractionated whole cell antigen probed with (i) pooled human melioidosis serum known to react with a native component at the 19.5-kDa position, (ii) 5G12 MoAb reactive with unfractionated and fractionated component at the 20.3-kDa position and (iii) a mixture of (i) and (ii). The results (Fig. 14) showed that the specimen (iii) in lane B gave 2 very closely immunoreactive bands in this region but both bands could be readily distinguished in this experiment, suggesting that these 2 components were different from one another. It was not clear why the patients did not respond to the 20.3-kDa component and at the same token why a mouse did not respond to the 19.5-kDa component.

3. Detailed characterization of monoclonal antibody 5F8

3.1 The ELISA reactivity against the culture-filtrated antigen

The indirect ELISA was performed for testing the immunoreactivity of 5F8 against the Rice, Kohst & Duthie (RKD) culture-filtrated antigen (kindly provided by Dr. Surasakdi Wongratanacheewin of Faculty of medicine, Khon Kaen University). This antigen contained biological products (protein and carbohydrate) of the pathogen that could be secreted into the culture medium. The culture-filtrated antigen at the concentration of 5 ug/ml was used for coating the microtitreplate, then allowed to react with undiluted

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Figure 17. Immunoblotting patterns of 5G12. Unfractionated whole cell antigen from PP844 (A), *P. aeruginosa* (B), *P. cepacia* (C), *P. putida* (D), *P. maltophila* (E), *P. alcaligenes* (F), *K. pneumoniae* (G), *E. cloaca* (H), *E. coli* (I), *S. typhi* (J), *S. enteritidis* (K), *S. krefeld* (L), *P. mirabilis* (M) and *S. aureus* (N) were electrophoresed in duplicate, electrotransferred and probed with either 5G12 (right hand strips) or pooled melioidosis serum (left hand strips). Positive reaction at the 20.3-kDa position exhibited by the 5G12 is noted only in lanes A and C.It should be noted that the immunoreactive bands with either *P. pseudomallei* or *P. cepacia* (20.3-kDa) developed with the 5G12 were different from those developed with positive human serum (19.5-kDa)

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supernatant from clone 5F8. The other MoAbs together with pooled melioidosis serum and pooled normal human serum were run simultaneously for comparison and for as positive and negative controls respectively. The results shown in Table 11 clearly indicated that the 5F8 could react very well with the culture-filtrated antigen. An equally strong interaction was also noted with the 3H5 MoAb.

3.2 Immunoprecipitation and autoradiography

The 5F8 MoAb was selected for use in this technic particularly because it appeared not to react with the antigens in the immunoblotting but gave strong O.D. reacting by indirect ELISA. This suggested that it might be directed at the conformational epitope. The MoAbs 5G12 and 1G8 were also used for comparison. Although the results presented in Figure 18 showed that there was still a considerable amount of non-specific interaction between the ^{125}I-labelled unfractionated whole cell antigen with either mouse Ig or protein A, a specific interaction with the 3 MoAbs could still be observed. This was particularly obvious with the 5F8 which gave specific bands at the 130, 31 and faintly at the 23 kDa (lane H). 5G12 and 1G8 also reacted specifically at the 23 kDa but like the 5F8 they exhibited rather low activity (lanes F and G).

3.3 Agglutinating activity

Slide agglutination test was performed by using the 5F8 MoAb mixed with the suspension of growing colonies of *P. pseudomallei* (3 x 10^9 bacterial suspension/ml). The hyperimmune sera and normal sera from human, rabbits and mice were used as positive and negative controls respectively. The results clearly showed that 5F8 but not other MoAbs

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Table 11. The ELISA reactivity of 5F8 MoAb against the culture-filtrated *P. pseudomallei* antigen.

antibodies	O.D. 492
5F8	1.20
1G8	0.11
9C3	0.19
9B10	0.23
9C12	0.07
3H5	1.34
+HS	1.46
NHS	0.22
medium	0.10

+HS : Pooled melioidosis serum (diluted 1:500)

NHS : Pooled normal human serum (diluted 1:500)

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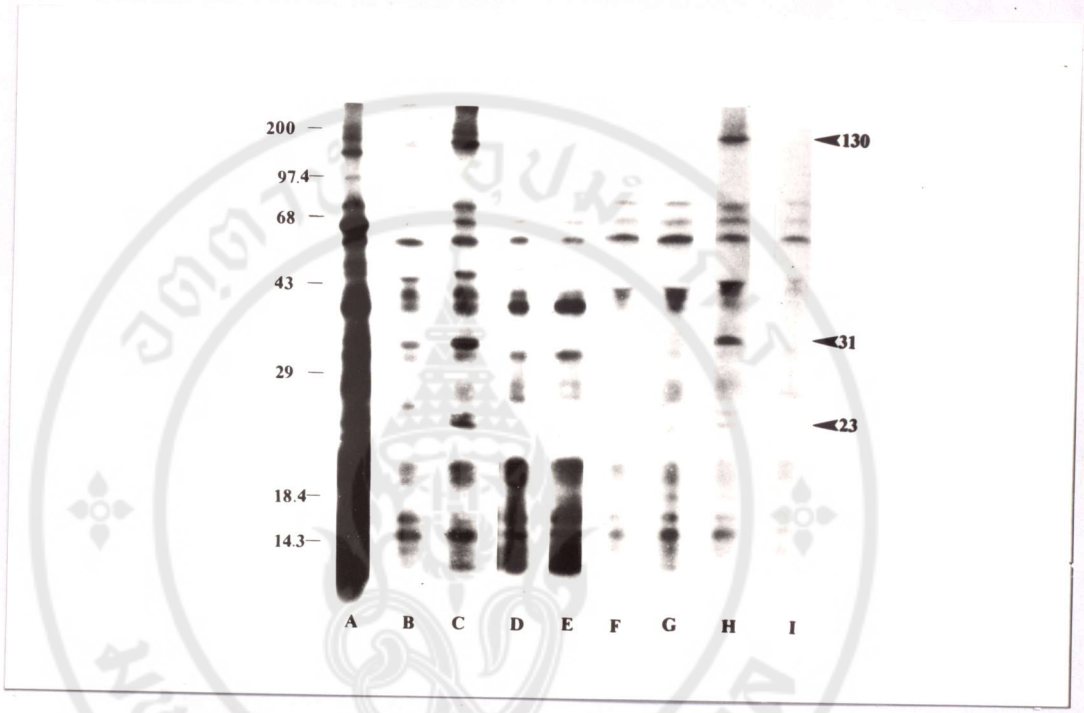

Figure 18. Autoradiographic patterns of ^{125}I-labelled unfractionated whole cell antigen before (A) and after immunoprecipitated with pooled melioidosis serum (B), prefusion mouse serum (C), pre-immunized mouse serum (D), culture medium (E), 5G12 (F), 1G8 (H) and unrelated MoAb (I). Molecular weight markers are shown on the left.

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had the capability of agglutination with homologous bacteria (Table 12).

Further, 5F8 as well as hyperimmune rabbit serum was mixed individually with the cell suspensions from 10 other strains of *P. pseudomallei* and others gram negative bacteria for agglutination. The results showed that the 5F8 could agglutinate specifically with all 10 *P. pseudomallei* isolated. As expected from other immunological tests, it did not agglutinate other bacteria. In contrast, the hyperimmune rabbit serum exhibited cross-agglutination with a number of other bacteria (Table 13).

3.4 Immunofluorescence staining

A summary of the immunofluorescent results is shown in Table 14. The 10X concentrated 5F8 MoAb was the only MoAb that gave positive fluorescence. The intensity of staining with 5F8 was as high as that stained with the hyperimmune rabbit serum (Fig. 19). It appeared from the figure that the binding site of 5F8 was associated with the surface envelopes.

4. Detection of *P. pseudomallei* antigen using 5F8 MoAb-based B-SA ELISA method

The B-SA ELISA was developed for antigen detection as described in details in Materials and Methods (section 10). The 5F8 MoAb was selected based on its specificity and reactivity against native unfractionated antigen. An appropriate amount of the antibody was captured on the ELISA-plate precoated with polyclonal rabbit anti-mouse Ig, then appropriate quantity of antigen in the form of

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Table 12. Slide agglutination test of selected MoAbs and polyclonal antibodies against *P. pseudomallei* cells.

Antibodies	Agglutination with *P. pseudomallei*
Melioidosis serum	++*
Normal human serum	+
Hyperimmune rabbit serum	++++
Normal rabbit serum	++
Hyperimmune mouse serum	+++
Normal mouse serum	0
5F8	+++
3H5	0
9C12	0
9C3	0
9B10	0
1G8	0
5G12	0

* : The degrees of agglutination were graded from 0 to ++++

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Table 13. Detailed data on agglutinating activity of 5F8 MoAb against *P. pseudomallei* and other bacterial cells.

Agglutination with	Antibodies	
	5F8 MoAb	Hyperimmune rabbit serum
P. pseudomallei (10 strains)	+++* (all 10 strains)	++++ (all 10 strains)
P. putida	0	0
P. maltophilla	0	0
P. stutzeri	0	0
P. cepacia	0	0
P. aeruginosa	0	0
E. cloaca	0	0
E. coli	0	+
S. enteritidis	0	0
S. krefeld	0	0
S. typhi	0	0
P. mirabilis	0	++
S. aureus	0	0
K. pneumoniae	0	++

* : The degrees of agglutination were graded from 0 to ++++.

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Table 14. Immunofluorescence staining of *P. pseudomallei*, *P. cepacia* and *P. aeruginosa* cells using selected MoAbs.

Antibodies	Antigens		
	P. pseudomallei	*P. cepacia*	*P. aeruginosa*
Hyperimmune rabbit serum*	+	-	-
5F8	+	-	-
3H5	-	-	-
1G8	-	-	-
9C3	-	-	-
9B10	-	-	-
9C12	-	-	-
5G12	-	-	-

* : Hyperimmune rabbit serum was used as positive control

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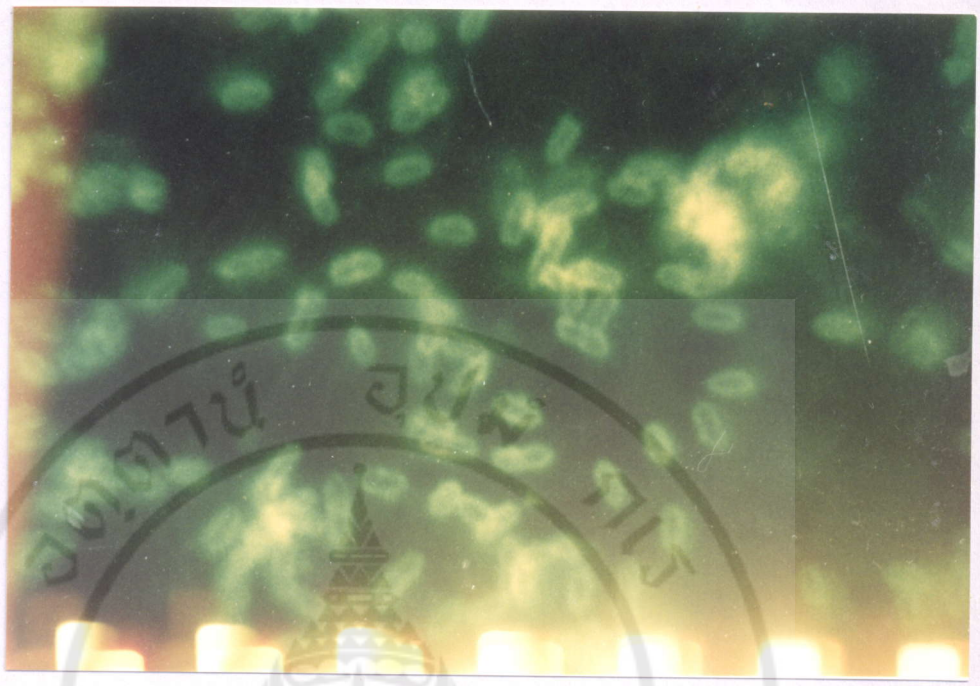
A



B

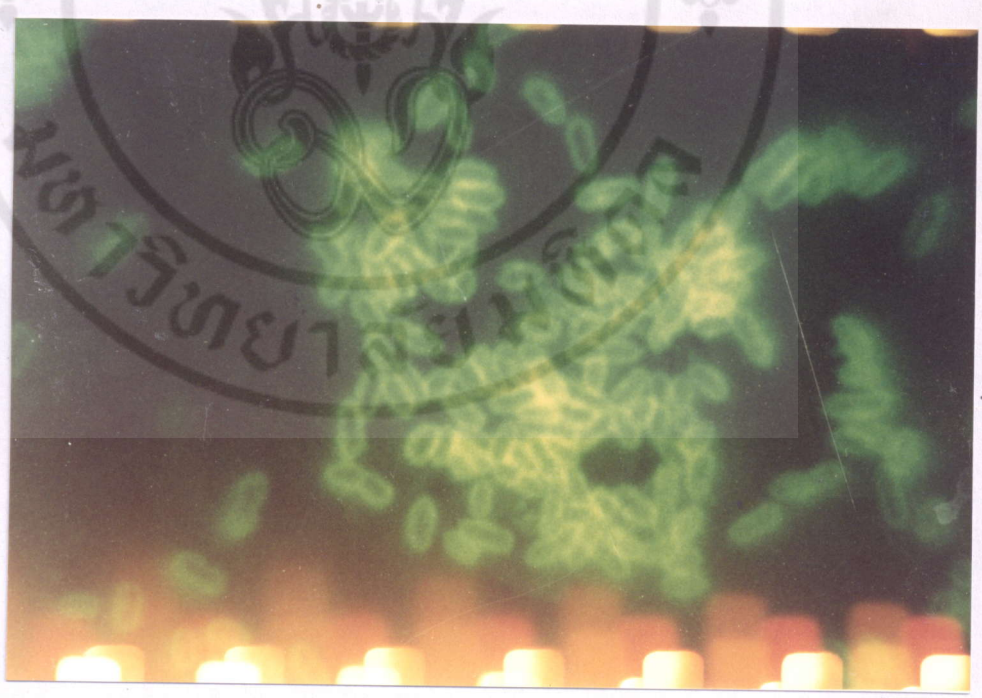


Figure 19. Immunofluorescence staining of *P. pseudomallei* cells using 5F8 MoAb (A). Hyperimmune rabbit serum (B) was used as a positive control. The positive cells showed uniform bright green fluorescence staining of the cell envelope.

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unfractionated whole cell extract or clinical specimens was added. Subsequently, biotinylated rabbit anti-unfractionated whole cell antigen, streptavidin-conjugated peroxidase and o-phenylenediamine were added in that order. The color developed was then converted to the antigen protein based on a standard curve constructed with known concentrations of unfractionated whole cell extract.

4.1 Determination of optimal concentrations of reagents used

The appropriate concentrations of all reagents used in this assay system were predetermined by checkerboard titrations. This step was accomplished by varying one parameter at a time while keeping the other reagents at an arbitrarily constant value (Figs. 20-22). With this protocol, the optimal concentrations or dilutions of the reagents used in this system were found to be 10 ug/ml for rabbit anti-mouse Ig, 1:200 dilution for biotinylated rabbit IgG antibody against unfractionated antigen, 20 ug/ml for 5F8 MoAb and 1:200 dilution for streptavidin.

4.2 Sensitivity of B-SA ELISA method

The sensitivity of the assay system was determined from a standard curve established with the unfractionated whole cell antigen at concentrations varied between 3.12 and 1,000 ng/ml. The criterion for a positive result would be equivalent to the antigen concentration giving O.D. value twice as high as that of the background. In a typical set up using optimal reagent concentrations mentioned in the previous section, the sensitivity of the method would be 10 ng/ml (Fig. 23). The assay system developed herein was highly reproducible as shown by the standard curves set up on different occasions and the sensitivities

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calculated from these curves (Fig. 24) were not noticeably different from one another (12.5 and 15 ng/ml). The mean value calculated from 3 standard curves was 12.5 ng/ml.

The standard curves set up using other MoAbs were compared with the one established with the 5F8 showed that only the 3H5 was just as sensitive as the 5F8 (Fig. 25). The sensitivities for the antigen detection by this assay system, using the various MoAbs as well as the mixture of 5F8 and 3H5, are shown in Table 15.

4.3 Preliminary testing of the antigen detection system for the antigen that may be present in urine

The feasibility of using the B-SA ELISA for antigen detection in the urine from patients with acute melioidosis was evaluated. This was done by determining a possibility of interference of urine in this assay system, using an arbitrary mixtures of known concentrations of antigen with urine from 2 different healthy volunteers. The standard curves run in the presence of urine were very similar to that of the normal standard curve run in parallel in its absence, particularly with regard to the limit of sensitivity of the system (Fig. 26). The O.D. values obtained at low antigen concentrations (12.5 to 50 ng/ml) were not different from one another. However, as the curves deviated from a straight line as found, for example, at higher antigen concentrations, the difference was more obvious.

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4.4 Preliminary evaluation of the assay system for antigen detection in serum

The potential of the assay system in detecting the antigen

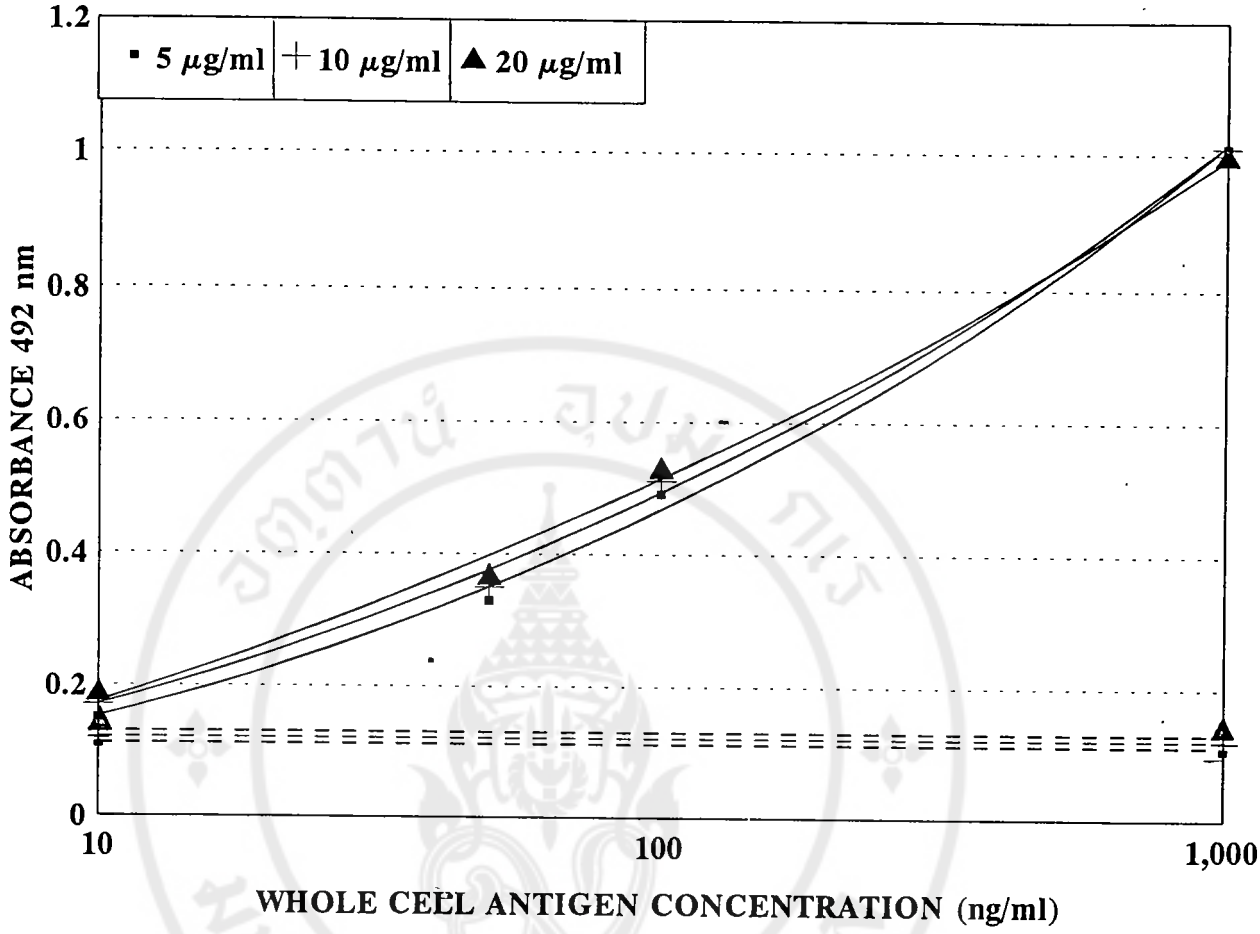

Figure 20. Checkerboard titration of rabbit anti-mouse Ig to be used for capturing MoAb on the microtitre plate for the detection of *P. pseudomallei* antigen via B-SA ELISA. 5F8 and biotinylated rabbit antiserum against unfractionated whole cell antigen used were arbitrarily fixed at 20 ug/ml and at a dilution of 1:100 respectively. The working concentration of rabbit anti-mouse Ig to be used in subsequent studies was 10 ug/ml.

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(——) B-SA ELISA with antigen.

(----) B-SA ELISA without antigen.

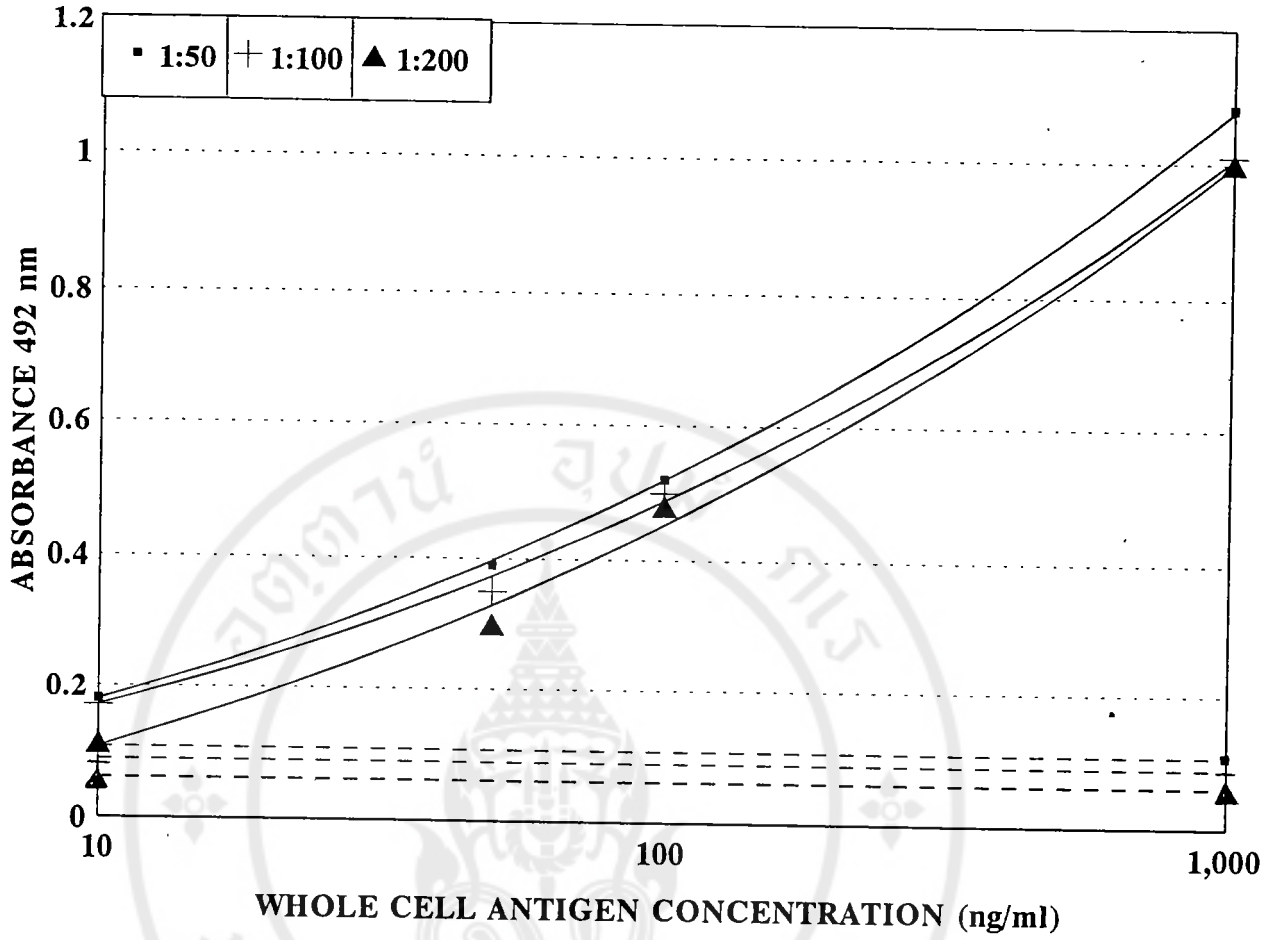

Figure 21. Optimization of the biotinylated rabbit IgG antibody against unfractionated whole cell antigen. B-SA ELISA was performed by coating the plate with 10 ug/ml rabbit anti-mouse Ig and to be used for the capturing of 5F8 (20 ug/ml). Then various concentrations of antigen (——) or the diluent (----) were added and the reaction mixtures were allowed to react with 3 dilutions of biotinylated rabbit antiserum against unfractionated whole cell antigen (1:50, 1:100, 1:200). The optimal dilution of biotinylated antibody was at 1:200.

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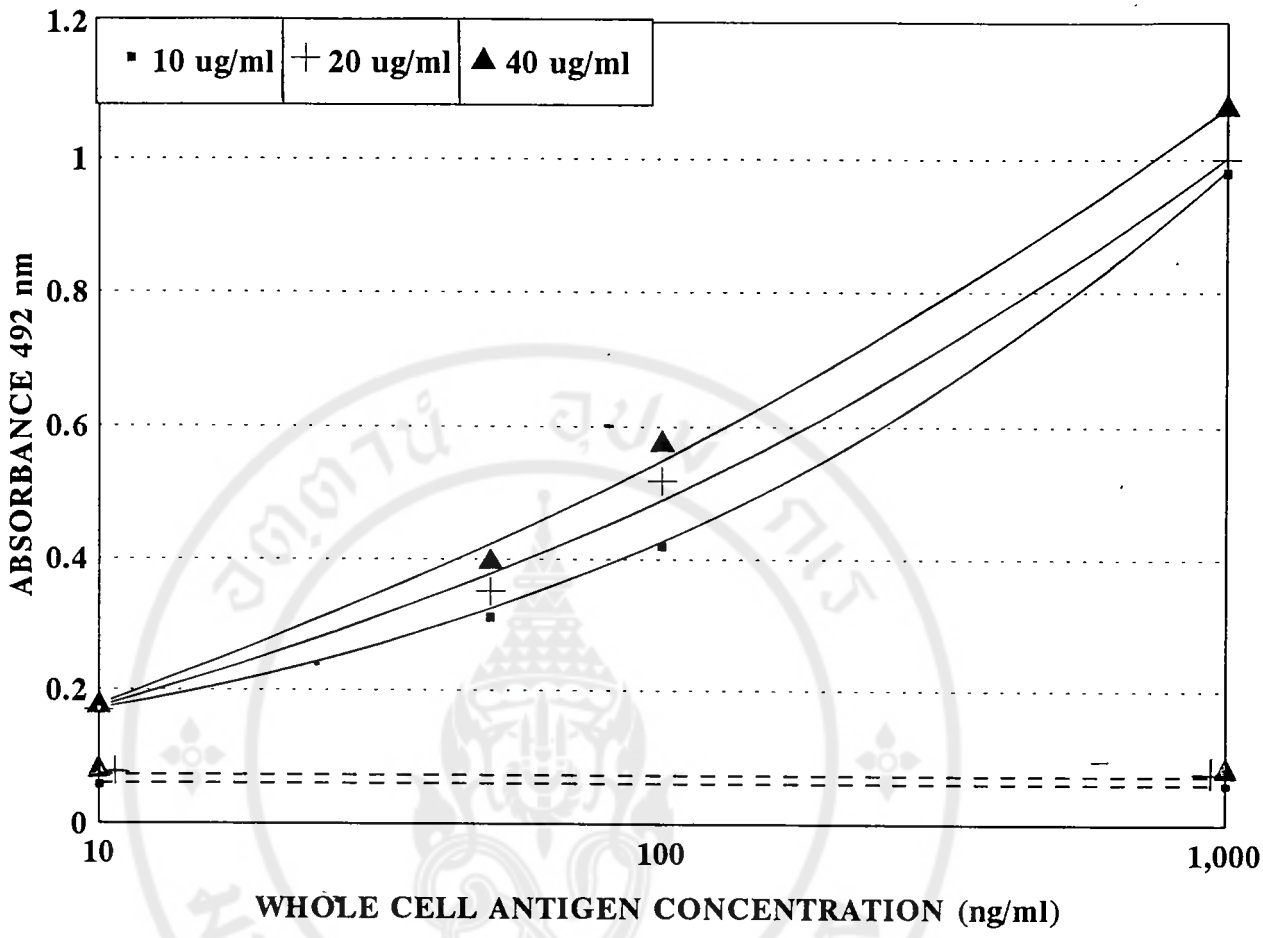

Figure 22. Optimization of 5F8 concentration for B-SA ELISA. The plate previously coated with 10 ug/ml rabbit anti-mouse Ig were allowed to react with 3 concentrations of 5F8 (10, 20, 40 ug/ml) in the presence of various concentrations of antigen (———) or in the absence of antigen (----). The biotinylated rabbit IgG antibody against unfractionated whole cell antigen and streptavidin-conjugated peroxidase were arbitrarily fixed at 1:100 and 1:2,000 respectively. The concentration of 5F8 chosen for all subsequent studies was 20 ug/ml.

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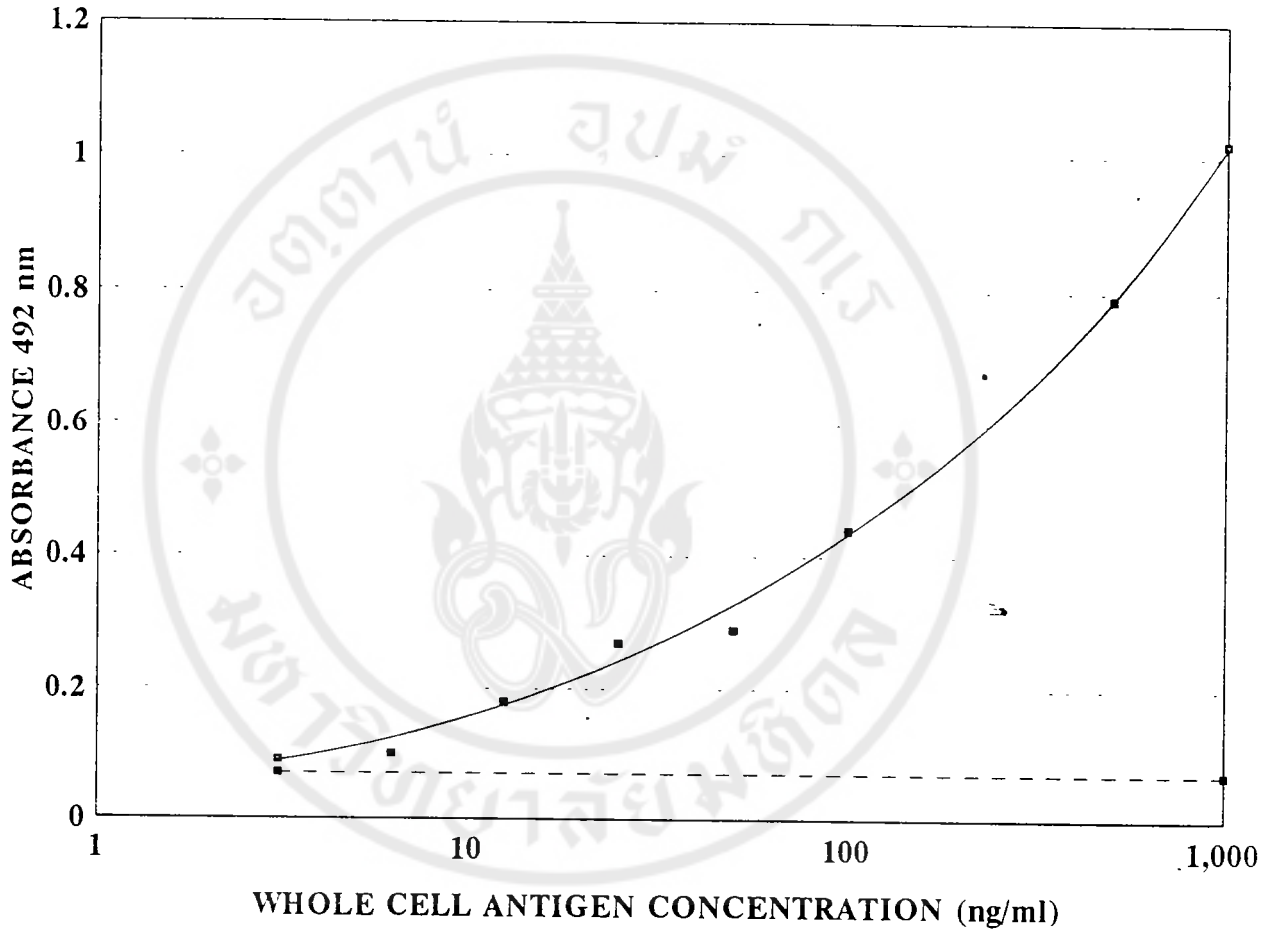

Figure 23. A typical standard curve for the detection of *P. pseudomallei* antigen by B-SA ELISA using optimal conditions standardized as described in Figures 20-22. A sensitivity of the method found from this curve was 10 ug/ml.

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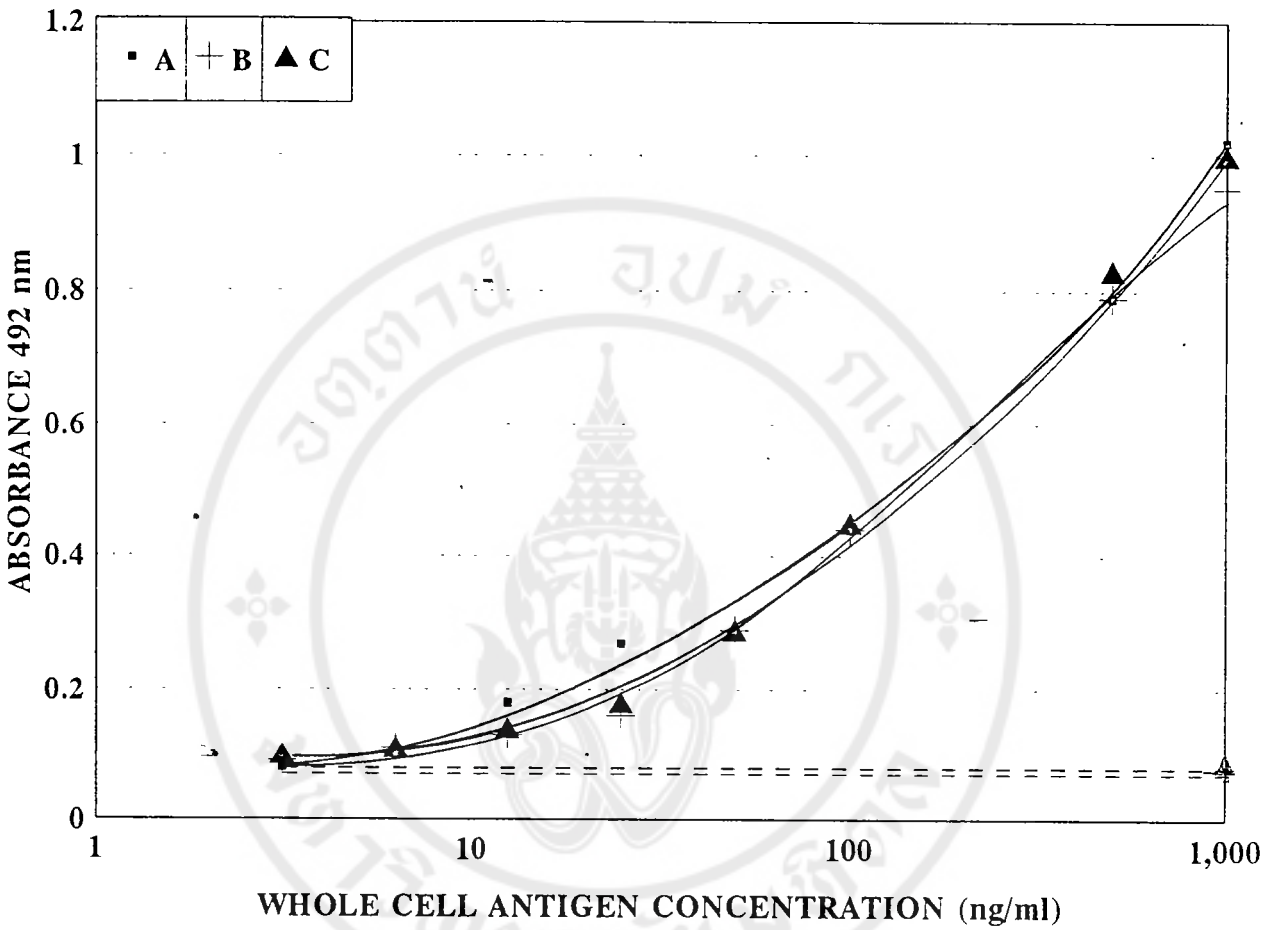

Figure 24. Reproducibility of B-SA ELISA. Standard curves run in tripicate were performed on different days and the limits of detection calculated from the standard curves A, B, and C were 10, 12.5 and 15 ng/ml respectively, thus the mean sensitivity for the three working days was 12.5 ng/ml.

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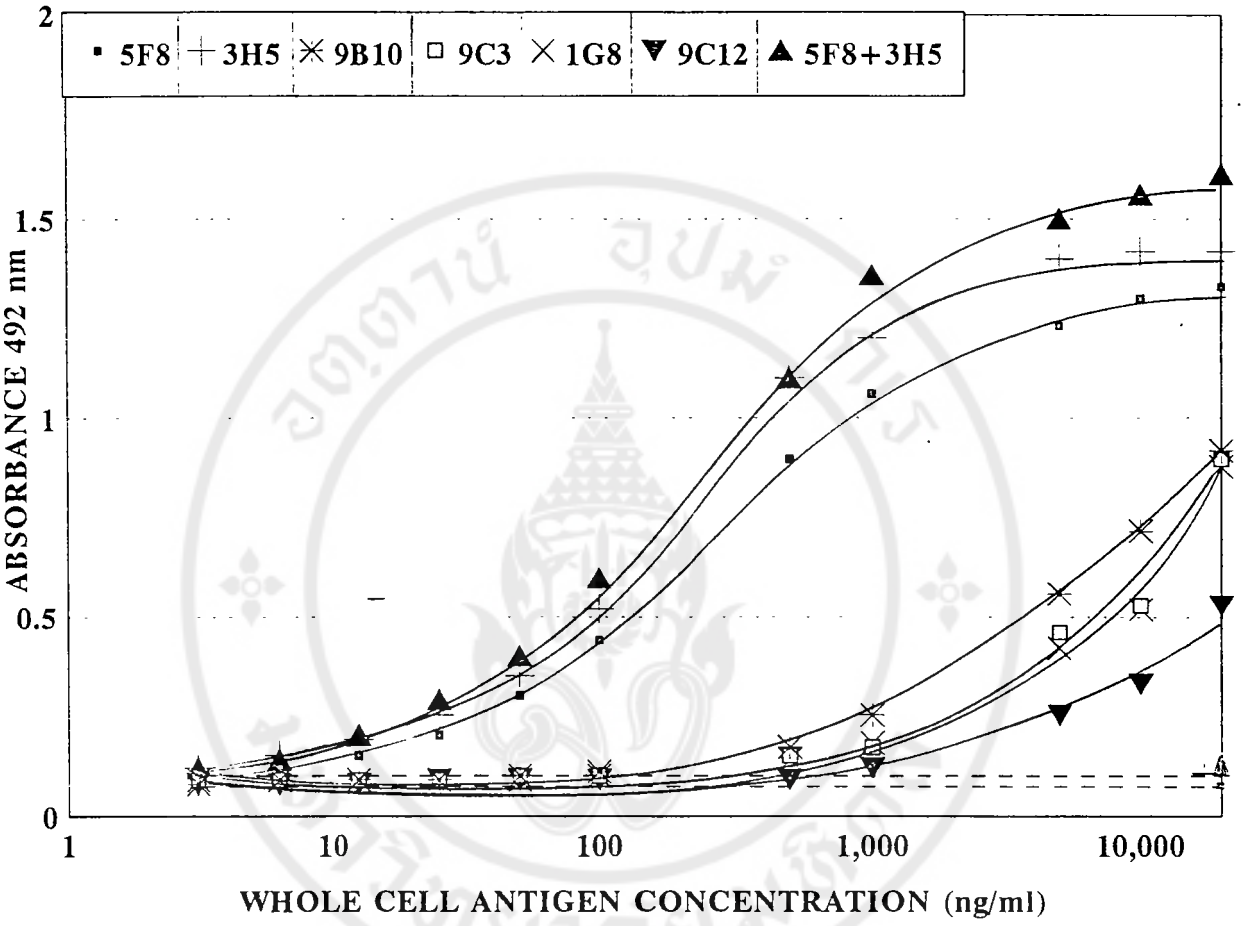

Figure 25. The efficacy of selected MoAbs used for detecting antigen based on B-SA ELISA. The concentration of 20 ug/ml of MoAbs (3H5, 5F8, 9B10, 9C3, 1G8, 9C12 or the 5F8+3H5 mixture) were used in the presence (———) or absence of antigen (-----). The other conditions used were the same as mentioned earlier.

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Table 15. A summary of the sensitivities for antigen detection by B-SA ELISA using different monoclonal antibodies.

MoAbs	Sensitivities (ng/ml)
5F8	12.5
3H5	12.5
9B10	500.0
9C3	1,000.0
1G8	1,000.0
9C12	2,100.0
5F8+3H5	12.5

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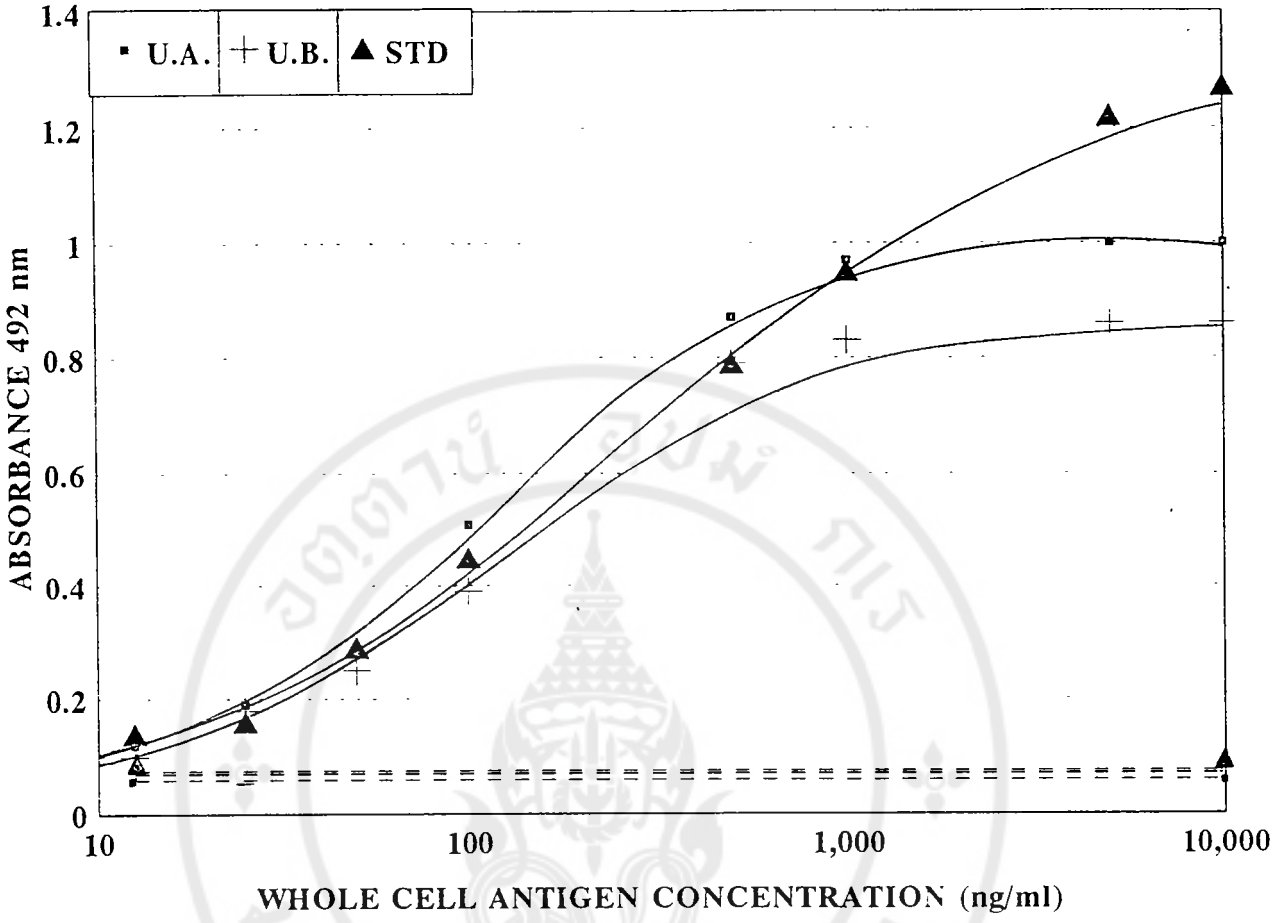

Figure 26. Preliminary testing of antigen detection system in urine specimens. Various concentrations of antigens (———) diluted in 2 normal urine specimens (U.A. and U.B.) were used for the construction of standard curves via B-SA ELISA. The standard curve established with antigen in 1% BSA-PBST (STD) was done as positive control and the samples without antigen (----) were used as negative controls. The sensitivity of antigen detection in the presence of urine were 19 and 16 ng/ml respectively, slightly less sensitive comparing with a value of 12.5 ng/ml obtained from a normal standard curve.

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that might be present in the serum of patients with acute melioidosis was evaluated in a similar manner as in the urine described in the previous section. The result presented in Figure 27 showed that the presence of normal human serum or normal rabbit serum considerably interfere with the antigen detection. The limit of sensitivity decreased from 12.5 ng/ml (for standard condition) to 100 and 71 ng/ml respectively. When the test was carried out by mixing the free antigen with serum from hyperimmune rabbit serum, the result got worse with the sensitivity decreased further to 390 ng/ml (Fig. 27). The presence of hyperimmune rabbit serum therefore drastically interfere with the quantitation by this assay system such that it was not at all possible to get any reliable reading from this artificial mixture.

The poor results obtained in the presence of hyperimmune serum might have been caused by the presence of immune complexes, either preformed *in vivo* or formed as a result of adding free antigen into the artificial system. In order to look into the mechanism that might be responsible for this phenomenon, an experiment was performed by comparing the data obtained as such with those obtained after dissociation of the immune complexs (Table 16). In this experiment, different methods to dissociate the immune complexes were used including polyethylene glycol (PEG) (87), ethylenediamine tetraacetic acid (EDTA) (88) and acid (0.5 N HCl) (89). A known concentration of unfractionated whole cell antigen was added to normal human serum, normal rabbit serum, hyperimmune rabbit serum or to 1% BSA-PBST as a control. The mixtures were allowed to stand at room temperature for 20 minutes prior to treatment with all 3 methods as previously described. These treated samples were subsequently used in the assay system and

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the results were compared with those obtained before treatment. The results showed that these methods could not restore the original readings (Table 16). Regardless of this difficulty, the B-SA ELISA was tested using sera from 3 melioidosis cases, 3 other bacterial infections sera and 5 normal human sera. None of these sera gave positive results.

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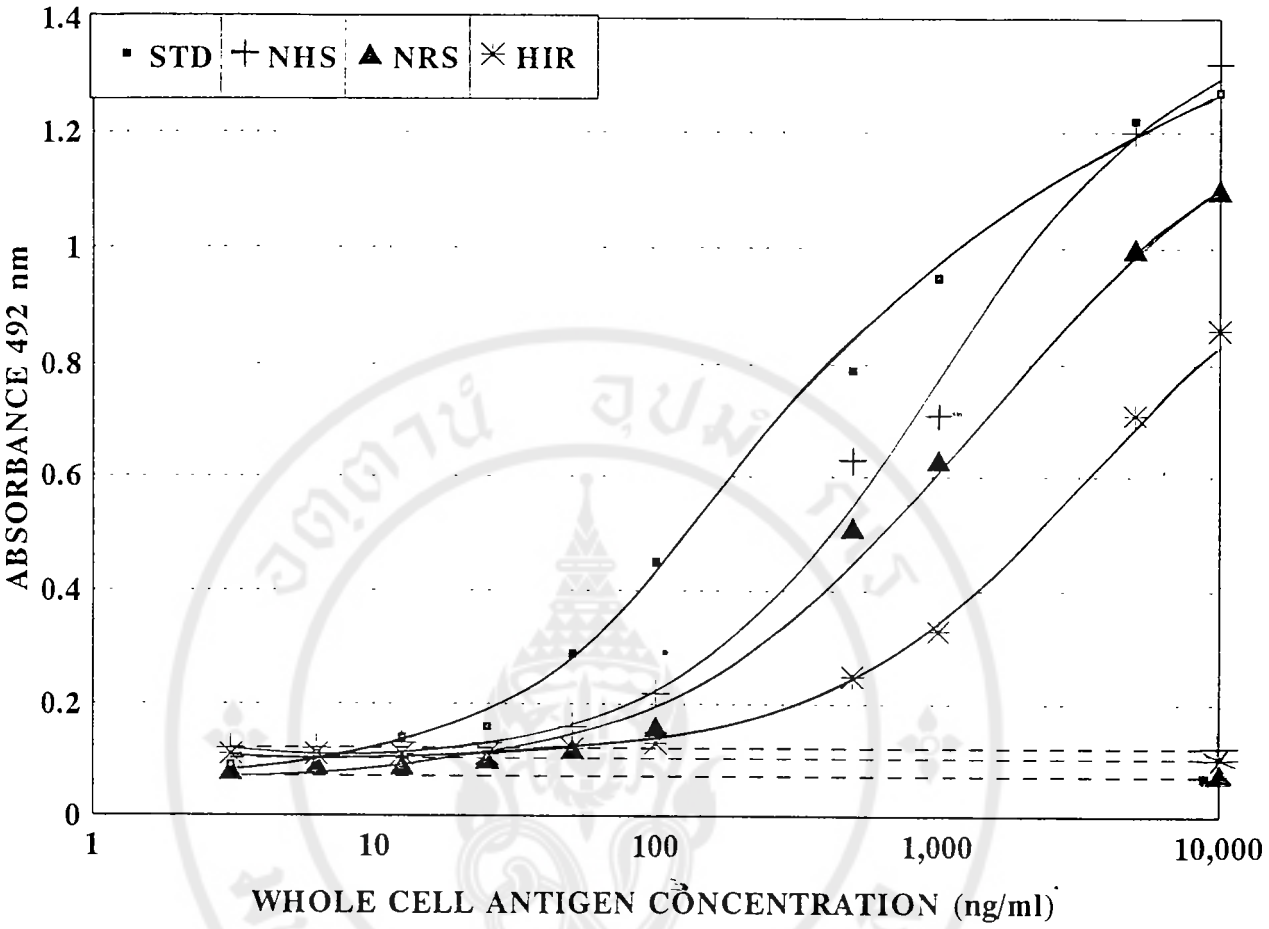

Figure 27. Preliminary testing of antigen detection system in serum specimens. Various concentrations of antigen mixed with pooled normal serum (NHS), normal rabbit serum (NRS), hyperimmune rabbit serum (HIR) and 1% BSA-PBST (STD) were used for the construction of standard curves via B-SA ELISA. The calculated sensitivities of antigen detection in the presence of NHS, NRS and HIR were found to be 100, 71 and 360 ng/ml respectively. The sensitivity of standard curve (STD) was 12.5 ng/ml.

(———) Samples with antigen

(----) Samples without antigen

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Table 16. Attempt to overcome the interfere of antigen detection by dissociation of immune complexes

Antigen* added to	B-SA ELISA (OD_{492})				
	untreated	PEG (1 min)	PEG (5 min)	EDTA	Acid
1% BSA-PBST	1.06	0.11	0.13	0.12	0.22
Normal human serum	0.68	0.45	0.44	0.22	0.27
Normal rabbit serum	0.60	0.50	0.29	0.09	0.23
Hyperimmune rabbit serum	0.11	0.13 (18)**	0.12 (9)	0.12 (9)	0.09 (<0)
Hyperimmune rabbit serum diluted 1:10	0.41	0.38 (<0)	0.37 (<0)	0.11 (<0)	0.19 (<0)

* : 1 ug of unfractionated whole cell antigen was added into 1 ml of diluent (1% BSA-PBST) or sera prior to dissociating the immune complexes.

** : The numbers in parenthesis represent % recovery of antigen in the artificial mixture $\frac{\text{O.D. After - O.D. before}}{\text{O.D. before}} \times 100$

Abbreviations

PEG : Polyethylene glycol

EDTA : Ethylenediamine tetraacetic acid

Acid : 0.5 N HCl

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CHAPTER V

DISCUSSION

Melioidosis is difficult to diagnose because it displays a variety of clinical manifestations which can mimic a number of other diseases, thus commonly known as "the great imitator". More importantly, the acute septicemic form of the disease can result in a very high mortality rate. Therefore, a sensitive, specific, rapid, and relatively simple diagnostic method is required for proper management. The most reliable method currently available is a bacteriological method but it takes time and often the results are not available at the time when a decision of antibiotic treatment has to be made. Thus, the physicians have to give an antibiotic regimen that would cover melioidosis. This is an economically poor decision as ones must use a relatively expensive antibiotic for a long period. Therefore, an availability of a reliable rapid diagnostic method would be most welcome.

In recent years, a number of serological methods for the detection of antibody have been developed to circumvent this problem. IHA had been developed by Nigg (90) and Ileri (51) for detecting antibody against polysaccharide or lipopolysaccharide of *P. pseudomallei* antigen in the sera of suspected individuals. This method was highly sensitive, moderately specific and simple to perform. Although the IHA is currently the most commonly used serological test for the diagnosis of melioidosis, it still has a number of problems because it was found that healthy individuals in endemic area of infection often exhibited high background IHA antibody titers (90).

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Consequently, a number of other serological tests such as CF (46), IgM-IFA (91), IgM-ELISA (53) and IgM antibody capture ELISA (MAC-ELISA) (92) have been developed. However, these methods are still confronted with the problem of a relatively high background antibody level in people resided in the area where the infection is endemic. The later interferes with the interpretation of the results. Such a problem may be associated with the use of crude antigen, thus resulting in poor specificity. Therefore, attempt was made in the present study to identify specific component(s) of *P. pseudomallei* for developing a more reliable antibody detection system.

Using immunoblot analysis, Lertmemongkolchai et al (5) identified in the Barber protein of *P. pseudomallei*, a 45-kDa component that appeared to be present in all 62 strains and it was recognized by all 3 patients' sera used for testing. They therefore concluded that this component might be useful for the diagnosis of acute melioidosis. More recently, Wongratanacheewin et al (58) used a similar method to characterize antigens in the culture filtrate of *P. pseudomallei*. These investigators showed that a 40-kDa component secreted in the culture filtrate was specific for *P. pseudomallei*. In the present study, unfractionated whole cell antigen was used as a starting material for the identification of specific antigen because it was felt that with the BP previously used by others investigators (5, 55) some valuable antigens might have been lost or denatured during the preparation.To circumvent this problem, the unfractionated whole cell extract used as a starting material was first electrophoresed and then relevant component(s) was identified. The gel strip possessing these antigens were cut and the protein was eluted for further analysis. The results

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showed clearly that the 19.5-kDa component eluted as such was highly specific for *P. pseudomallei* (Tables 5 & 6). It could be found in all 56 strains of *P. pseudomallei* available for testing, but was absent in 4 other *Pseudomonas* species and 8 other gram-negative bacteria. However, this component was present in 2 strains of *P. cepacia* (one from Bangkok and the other from Khon Kaen) However, this was not entirely unexpected because both species of *Pseudomonas* are biochemically classified in the same group based on nucleic acid homology (Fig.1). Further studies showed that 65% of the sera from melioidosis patients reacted with the 19.5-kDa component via immunoblotting (Table 7). It is possible that 13 of the 37 patients whose sera failed to react with the 19.5-kDa position by immunoblotting may be due to the sensitivity of the assay system because 10 of these 13 non-reactive sera were subsequently found to be positive by indirect ELISA using the fractionated 19.5-kDa as antigen. The remaining 3 sera were negative by both assay systems (Table 5 & 7). It could not be determined at this time whether the negative ELISA was really due to the absence of antibody or due to interference by other factors, e.g., immune complexes. In order to clarify this point, one should look for the presence of circulating antigen in these specimens. Altogether the data currently available suggested that the 19.5-kDa component is a specific antigen common to all *P. pseudomallei* isolates and has potential for the diagnosis of acute melioidosis.

The protein elution method used as described herein gave a relatively pure 19.5-kDa fraction which contained a 19.5-kDa protein as a predominant component (Fig.8). The method was relatively efficient with a yield of 6%. However this method has one significant drawback.

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That is, with the protocol used, the purified protein might have been denatured because the sample had to be heated, treated with SDS, and reduced by 2-ME (to get better separation) prior to being electrophoresed. Therefore, following electrophoresis, protein elution and SDS removal by ion-retardation resin, a relatively labile protein component(s) may be irreversibly denatured and conformational epitopes might have been destroyed. Therefore, the remaining epitopes available for antibody binding would be sequential epitopes. Following a natural infection, serum antibodies should have been formed against both conformational and sequential epitopes of these antigens. The results from indirect ELISA (Tables 5 & 6) using this form of fractionated antigen were positive. Therefore, there must be a sufficient amount of antibodies with diverse specificities formed against both conformational and sequential epitopes in these patients.

In this study, it was clearly demonstrated that although the fractionated 19.5-kDa antigen might have been denatured to some degrees during the purification process, it is still a valuable diagnostic reagent for the diagnosis of acute septicemic melioidosis via indirect ELISA (Table 6). It is worthy to note that the present assay system using either unfractionated or fractionated antigens was highly reliable in detecting IgG antibody in the acute phase sera. It was rather surprising to note further that of this antibody isotype could be found in the sera taken from the patients even on the day admission (Table 1). The detection of IgM antibody using fractionated 19.5-kDa antigen had been tried but the results were not as promising as recommended by other investigators (53, 91, 92). The results presented herein differed considerably from those presented earlier reporting

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more satisfactory results with the IgM antibody detection methods (53,91,92). It is possible that in our series the patients who resided in the endemic area may have been exposed to previous subclinical *P. pseudomallei* infection and that the current episode mostly represented a recrudescence of latent infection and not a primary infection. Since it had been observed in animal models by many investigators (93-95) that *P. pseudomallei* could form microcolonies in the tissues and could survive or even multiply in phagocytes without presenting any sign or symptom of infection. However, during this latent period, whenever the host well being is disturbed, the disease could flare up. This situation may be the case of a recrudescence in the patients who may have subclinical infection. Following a flare-up with acute septicemic phase, a high titre of IgG instead of IgM antibody would be produced. The results presented in this study are consistent with those of Petkanjanapong et al (64) using the endotoxin antigen from *P.pseudomallei* for detecting IgG antibody in acute septicemic melioidosis patients via indirect ELISA. Interestingly enough, the use of fractionated 19.5-kDa antigen appeared to be as sensitive as that of the unfractionated whole cell antigen and was more specific in this assay (Table 6). This result differed from those of Ashdown et al (53) who previously reported that the detection of IgM antibody using a crude sonicated antigen was quite sensitive and specific. Such a discrepancy could be explained based on the fact that the majority of non-melioidosis specimens used by these investigators were from patients with unrelated disorder and resided outside the endemic area of infection, thus these patients had lower background antibody titres than those of non-melioidosis patients available in the present study in which a large portion of septicemic patients were associated with

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other gram-negative bacterial infections and resided in endemic areas of melioidosis infection (Table 2).

As an alternative to the antibody detection methods, the detection of antigen in those with really acute infection prior to the time of antibody formation should be another reliable approach. This approach requires a highly specific reagent. The use of MoAb to detect the antigen either in the circulation or urine of patients with acute infection represents such an approach. This idea was tested by immunizing mice with the fractionated 19.5-kDa antigen which subsequently produced specific MoAb. The later would be theoretically useful for detecting the 19.5-kDa antigen in clinical specimens. Unfortunately, these animals failed to produce any antibody that would recognize 19.5-kDa component in its native form (Fig.13). Some of the antibodies produced, however, recognized other *P. pseudomallei* specific components; for instance, those with molecular masses of 19.0, 20.3, 21.0, and 21.6 kDa which were not what we wanted. These results implied that the fractionated 19.5-kDa antigen was denatured during the purification process and that the animals recognized and preferentially responded to these denatured rather than native epitopes. Another possible explanation is that the immunized animals recognize epitopes different from those recognized by humans who are exposed to the antigen by a natural route. Nonetheless, the 5G12 MoAb obtained from the animal immunized with the fractionated antigen reacted with a 20.3-kDa component present in both fractionated and unfractionated antigens from all 56 strains of pathogen via immunoblotting but not via indirect ELISA (Table 10).These results may be explained based on the fact that this MoAb is specific for the

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sequential epitopes and that this should be the case since the result from immunoprecipitation of labelled unfractionated whole cell antigen incubated with 5G12 did not give a 20.3-kDa band (Fig 18). This conclusion is based on the assumption that the antigen was not denatured during the labelling procedure and immunoprecipretation.

Because of the problem discussed above, the unfractionated native whole cell antigen was used as immunogen for the production of MoAbs that would hopefully recognize the native 19.5-kDa antigen. Unfortunately, none of the 5 immunized animals recognized and produced antibodies against the 19.5-kDa component. Instead, their immune sera recognized the antigens migrated to positions between 29 and 43 kDa (Fig.13). One possible explanation is that the animals recognize unfractionated antigen differently from the humans. Therefore, in order to imitate the human immune response against *P. pseudomallei* antigen, an experimental animal model artificially infected with the bacteria should be used. However, in order to have a successful infection, a special route of infection has to be used (94) and it is not possible to conduct such an experiment at this time as the animal facilities are not geared for this kind of study.

There was a number interesting characteristics regarding the immune response in mice stimulated with unfractionated whole cell antigen. For example, the clone 5F8 that reacted strongly with all 56 strains of *P. pseudomallei* failed to react with its close relative, *P. cepacia* (Table 9) and is therefore highly specific for the *P. pseudomallei* antigen. Another interesting point that should be noted is that even with the immunization protocol used with a number of booster injections, the 5F8 was of IgM isotype. Continuous production of IgM

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antibody is not unusual particularly when it is produced against T-independent antigens, i.e., macromolecules with repeating antigenic determinants. The immunogen used for this immunization contained, in addition to protein, some amount of polysaccharide [The crude cell extract of this pathogen contained about 6% carbohydrate (96)] which is known to be a potent IgM inducer. In fact, *P. pseudomallei* possessed a glycocalyx which is exopolysaccharide in nature (39). This component could theoretically induce the mouse to produce IgM antibody continuously. There are two relevant evidence in this study to support this hypothesis. Firstly, the 5F8 could react strongly with homologous antigen via indirect ELISA but failed to give a detectable reaction in the immunoblotting, so it is possible that the 5F8 recognizes non-protein components. Secondly, the 5F8 showed positive results in agglutination test and immunofluorescence staining of *P. pseudomallei* showed positive reaction on the surface envelope (Tables 12 & 13). These results suggested that the 5F8 bind at the surface of bacterial cells and that the binding sites may be related to the glycocalyx or lipopolysaccharide on the cell envelope and IgM antibody is known to be an excellent agglutinating antibody.

Although the 5F8 failed to react or reacted poorly with the unfractionated antigen by immunoblotting, it could bind specifically to the labelled unfractionated whole cell antigen, particularly with the one migrated to a 130-kDa position by radioimmunoprecipitation (Fig. 18). These results, together with those of the immunoblotting suggested that 5F8 recognize only the conformational epitopes.

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Because of the elevated background antibody titres in some individuals in the endemic area, the antibody detection approach has

its own drawback. The more ideal approach is to detect antigen, both in acute or chronic phase, as the presence of antigen would more truly represent active infection. However, there could be some exception, theoretically at least. That is if there is a circulating polysaccharide component, it may theoretically remain in the circulation for a long period due to inefficiency in eliminating such a component from the circulation. The earlier experiment for detecting circulating antigen was done by Ismail et al (6). They used a competitive ELISA, based on the use of monoclonal antitoxin, for the detection of *P. pseudomallei* exotoxin in the animal model. The assay could detect the presence of exotoxin at a level of 16 ng/ml. However, the presence of exotoxin in quantity sufficient for its detection in the patients remains to be investigated. More recently, Wongratanacheewin et al (55) developed the B-SA ELISA using rabbit polyclonal antibody to Barber protein for detecting *P. pseudomallei* antigen and found that this assay worked very nicely since it could detect the antigen at a concentration as low as 3.9 ng/ml. However, this method have not yet been tested on clinical specimens. In the present study, the antigen detection assay based on the use of MoAb in B-SA ELISA was developed with the hope that specific MoAb may make this approach feasible. The results showed that this system could detect the antigen at a concentration as low as 12.5 ng/ml. An attempt to increase the sensitivity of the method further by the use of a MoAb mixture in the test system was not successfully (Fig. 25). However, the use of the MoAb mixture may theoretically help increasing the chance to detecting the antigen, i.e., by increasing the number of reactive epitopes.

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The method also worked quite well when the antigen was arbitrarily mixed with urine samples (Fig. 26) thus making it possible to detect any antigen that might be excreted in the urine. However, a similar approach to detect the antigen in serum were not satisfactory probably due to interference by immune complexes (Fig. 27) or excess circulating antibodies in serum of the patients. The latter would theoretically bind to the antigen added and therefore preventing the binding to MoAb in B-SA ELISA system. Such an interference could occur eventhough the two antibodies are directed against different epitopes, e.g. by stearic hindrance. However, it was thought that this problem might be solvable, if one could completely dissociate the immune complexes prior to detecting the antigen. Therefore, different dissociation methods including PEG (87), 0.1 M EDTA (88) and acid treatments were evaluated (89). Unfortunately, the experiment did not work. This difficulty might be due to the fact that these methods could not dissociate unfractionated whole cell antigen from specific antibodies contained in the hyperimmune rabbit serum used for testing (Table 16).It is suggested that one should look for a more suitable technique for breaking up the immune complexes. Apart from the problem associated with the immune complexes, the failure of antigen detection in the sera may result from a prolonged storage of sera, a process which could denature or destroy the antigen.In conclusion, although the antigen detection method seems to have potential for detecting antigen in the urine specimens, data currently available are not too promising regarding the use of serum specimens.The real application must await the availability of these specimens which should become available during the next rainy season.

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CHAPTER VI

CONCLUSION

P. pseudomallei antigen with a molecular mass of 19.5 kDa was found to be highly specific for *P. pseudomallei*. It was purified from the unfractionated whole cell antigen that was previously separated by SDS-PAGE. A strip of gel containing components with molecular weights ranging between 18.4 and 25.5 kDa was cut from the SDS-PAGE of unfractionated *P. pseudomallei* antigen and the protein was electroeluted by using an Electro-elutor (Bio-rad, Laboratories). The yield of the fractionated antigen obtained from this method was about 6 %. Although the proteins eluted as such contained more than just the 19.5-kDa component, the latter was a predominant component in the eluent and it was the only component that reacted with pooled melioidosis serum. The immunoreactivity of this component was determined by indirect ELISA and immunoblotting analyses. Its diagnostic potential was evaluated using sera from 37 acute septicemic melioidosis patients, 25 sera from patients with other bacterial infections, 25 sera from healthy volunteers from an endemic (Khon Kaen) and 60 sera from blood doners from a nonendemic (Bangkok) area of infection. These sera were used at a dilution of 1:250 and the antigen was used at a concentration of 5 ug/ml. By indirect ELISA, the results were high satisfactory with 92% sensitivity, 91% specificity, 81% positive predictive value and 96% negative predictive value based on the background obtained with sera from the area where the infection is nonendemic. These values were 82% sensitivity, 96% specificity, 94% positive predictive value and 87% negative predictive value based on the background obtained with sera from the area where infection

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is endemic.

Another possible approach for the diagnosis of acute melioidosis was to use the antigen detection method. One such attempt was to produce monoclonal antibody (MoAb) specific for *P. pseudomallei* antigen and to use it for antigen detection. The attempt to produce MoAb by using the fractionated 19.5-kDa protein as immunogen was not successful because the MoAb obtained did not react with the native 19.5-kDa component. However, several MoAbs reactive with native antigens were produced by immunizing BALB/c animals with the unfractionated whole cell antigen. Among these specific MoAbs, one clone (5F8) appeared to be useful for antigen detection. It could be produced in large quantity by *in vitro* culture. This MoAb reacted with all 56 strains of *P. pseudomallei* by indirect ELISA and could readily agglutinate *P. pseudomallei* by slide agglutination method. The latter might be related to the fact that it was of an IgM isotype and reacted with bacterial envelope, judging from immunofluorescence test. When this MoAb was captured on the microtiter plates and used for the detection of antigen by biotin-streptavidin (B-SA) ELISA, it was found to be highly sensitive, capable of detecting native antigen at a concentration as low as 12.5 ng/ml. It was slightly less sensitive in detection the antigen artificially mixed with the urine from normal volunteers (16-20 ng/ml).

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APPENDIX

Protein determination

a. Stock solution :

2% K or Na tartrate,
1% $CuSO_4$ $5H_2O$,
2% Na_2CO_3 in 0.1 N NaOH
and 2 N Phenol

b. Working solution A :

2% K or Na tartrate –	0.5 ml
1% $CuSO_4$ $5H_2O$	0.5 ml
2% Na_2CO_3 in 0.1 N NaOH	49.0 ml

c. Working solution B :

2 N Phenol	1 vol
Distilled water	1 vol

SDS - PAGE

a. Stock acrylamide solution (30%) :

Acrylamide (Sigma)	30.0 gm
N,N-bis-methylene acrylamide (Sigma)	0.8 gm
Distilled water to	100.0 ml

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b. Working acrylamide solution (12% and 3%) :

	12 %	3%	
Stock acrylamide	8.0	1.0	ml
1.875 M Tris-HCl pH 8.8	4.0	-	ml
1.875 M Tris-HCl pH 6.8	-	0.66	ml
10% SDS	0.2	0.10	ml
100% TEMED	10	6	µl
Distilled water	7.7	8.2	ml
10% Ammonium persulfate (Sigma)	67	25	µl

c. Sample buffer 5X pH 6.8 :

Trizma base (Sigma)	0.38	ml
SDS	0.5	ml
Glycerol	5.0	ml
2-mercaptoethanol	2.5	ml
Bromphenol blue	5.0	µl
Adjust pH to 6.8 with 1 N HCl		
Distilled water to	10.0	ml

d. Electrophoresis buffer pH 8.3 :

Trizma base (Sigma)	3.0	gm
Glycine	14.4	gm
SDS	1.0	gm
Distilled water to	1.0	liter

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Coomassie blue staining

a. Staining solution :

Coomassie brilliant blue R	0.2 gm
Glacial acetic acid	7.0 ml
Methanol	46.5 ml
Distilled water	46.5 ml

b. Destaining solution :

Methanol	50.0 ml
Glacial acetic acid	70.0 ml
Distilled water to	1.0 liter

Western blotting and Immunoenzyme staining

a. Towbin buffer :

Trizma base	3.03 gm
Glycine	14.41 gm
Methanol	200.00 ml
Distilled water to	1.00 liter

b. 0.15 M phosphate buffered saline pH 7.2 (PBS) :

NaCl	8.50 gm
Na_2HPO_4	1.07 gm
NaH_2PO_4 $2H_2O$	0.39 gm
Distilled water to	1.00 liter

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c. Substrate solution :

Diaminobenzidine (Sigma)	6	mg
50 mM Tris-HCl pH 7.4	20	ml
35% H_2O_2	30	µl

Indirect ELISA

a. 0.05 M Carbonate buffer pH 9.6 :

Na_2CO_3	1.59 gm
Na_2HCO_3	2.93 gm
Adjust pH to 9.6	
Distilled water to	1.00 liter

b. Incubation buffer :

NaCl	8.90 gm
Na_2HPO_4	1.28 gm
Na_2HPO_4 2 H_2O	0.15 gm
Tween 20	0.50 ml
Distilled water to	1.00 liter

c. Washing buffer :

NaCl	45.0 gm
Tween 20	2.5 ml
Distilled water to	1.0 liter

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d. Substrate buffer (Phosphate-citrate buffer, pH 5.0) :

Na_2HPO_4	7.19 gm
Citric acid	5.19 gm

Distilled water to	1.00 liter

e. Substrate stock :

Orthophenylene diamine(Sigma)	10.0 mg
Methanol	1.0 ml

f. Substrate working solution :

Substrate buffer	19.8 ml
Substrate stock	200.0 µl
3% H_2O_2	20.0 µl

Fusion medium for P3-X63-Ag8-U1

a. Aminopterin (100x) :

Aminopterine (Lyophilized) (GIBCO)	1 Bottle
Deionized distilled water (D.D.W.)	10 ml

Filter through 0.2 µm millipore membrane

b. Hypoxanthine and Thymidine (HT)(100x) :

Hypoxanthine (Sigma)	136 mg
Thymidine (Sigma)	38 mg
D.D.W. to	100 ml

Heat at 70^0c until soluble

Filter through 0.2 µm millipore membrane

c. Polyethylene glycol (PEG)(33%):

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Sterile PEG (Sigma)	0.74 ml
Complete medium	1.26 ml

d. Oxaloacetate :

Oxaloacetic acid (Sigma)	0.5	gm
D.D.W.	38.5	ml
Filter through 0.2 µm millipore membrane		

e. 0.5 mM Na-Pyruvate :

Pyruvic acid Sodium salt (Fluka)	5.5	mg
D.D.W.	100.0	ml
Filter through 0.2 µm millipore membrane		

f. Stock RPMI :

RPMI 1640 (Flow Laboratory)	1	pack
$NaHCO_3$	2.00	gm
HEPES (Sigma)	3.57	gm
D.D.W. to	1.00	liter
Filter through 0.2 µm millipore membrane		

g. Complete medium :

Stock RPMI	100	ml
NCTC135 (GIBCO)	10	ml
Fetal calf serum (Flow Laboratory)	15	ml
Humulin R (100 µg/ml)	8	µl
Stock 0.5 mM Na-Pyruvate	1	µl
2 mM L-glutamine (GIBCO)	1	ml
Oxaloacetate	1	ml
Gentamycin sulfate (40 ng/ml)	0.15	ml

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h. Incomplete medium :

Stock RPMI	500	ml
Gentamycin sulfate	0.75	ml

i. HAT medium :

Complete medium	92	ml
2 mM L-glutamine	1	ml
Fetal Calf serum	5	ml
100x Hypoxanthine & Thymidine	1	ml
100x Aminopterin	1	ml

j. HT medium :

Complete medium	93	ml
2 mM L-glutamine	1	ml
Fetal Calf serum	5	ml
100x Hypoxanthine & Thymidine	1	ml

k. Freezing medium :

Dimethy sulfoxide (GIBCO)	0.5	ml
Complete medium	2.5	ml
Fetal Calf serum	2.0	ml

Fusion medium for X63-Ag8.653

a. Complete medium :

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Stock RPMI	75	ml
Fetal calf serum (Flow Laboratory)	25	ml
200 mM L-glutamine (GIBCO)	1	ml

2-Mercaptoethanol (0.01M)	0.5	ml

b. HAT medium :

Complete medium	100	ml
100x Hypoxanthine & Thymidine	1	ml
100x Aminopterin	1	ml

c. HT medium :

Complete medium	100	ml
100x Hypoxanthine & Thymidine	1	ml

d. 41.3% PEG :-

Autoclaved PEG4000 (Accurate Chemical & Scientific Corp)	20	gm
15% DMSO in PBS	28	ml

DEAE-Cellulose buffer

a. 0.02 M K_2HPO_4 pH 8.0 :

K_2HPO_4	3.48	gm
D.W.	500.00	ml

Adjust pH to 8.0 with 0.02 M KH_2PO_4

b. 0.02 M KH_2PO_4 :

KH_2PO_4	2.72	gm
D.W.	1.00	liter

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Radioimmunoprecipitation

a. 1 M Sodium phosphate buffer (20x) :

Solution A :	Na_2HPO_4	141.96 gm
	Distilled water to	1000.00 ml
Solution B :	NaH_2PO_4	137.99 gm
	Distilled water to	1000.00 ml
To use :	Solution A	810.00 ml
	Solution B	190.00 ml
	Adjust pH to 7.4	

b. 5 mg/ml Choramine-T :

Choramine-T (Sigma)	0.005 gm
Distilled water	1.000 ml

c. 1 mg/ml Sodium metabisulfite :

Sodium metabisulfite (Sigma)	0.005 gm
Distilled water	5.000 ml

d. 50 mg/ml Sodium iodide :

Sodium iodide (Sigma)	0.050 gm
Distilled water	1.000 ml

e. 0.5% BSA :

Bovine serum albumin (Sigma)	0.005 gm
Distilled water	1.000 ml

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f. 200 mM Tris-HCl pH 8.1 :

Trizma-base (Sigma)	24.22 gm
Distilled water to	1000.00 ml
Adjust pH to 8.1	

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Table A Reactivity of selected monoclonal antibodies against unfractionated whole cell antigen from different bacterial strains

No	Strains	Source of specimens	ELISA (OD_{492})							I.M.
			1G8	9B10	9C12	9C3	3H5	5F8	media	5G12
1	*P. aruginosa*	unknown	.00	.04	.00	.03	.02	.01	.00	-
2	*P. cepacia* ‡	"	.01	.04	.00	.03	.03	.01	.00	+
3	*P. cepacia* ‡‡	"	.01	.05	.00	.04	.03	.02	.01	+
4	*P. putida*	"	.03	.05	.01	.04	.03	.01	.01	-
5	*P. maltophila*	"	.01	.05	.01	.03	.02	.00	.02	-
6	*P. alcalogenes*	"	.00	.02	.01	.02	.00	.01	.00	-
7	*K. pneumoniae*	"	.02	.03	.00	.02	.01	.00	.00	-
8	*E. cloacae*	"	.00	.03	.00	.02	.02	.01	.01	-
9	*E. coli*	"	.01	.03	.00	.02	.01	.01	.01	-
10	*S. typhi*	"	.03	.04	.00	.02	.01	.02	.01	-
11	*S. enteritidis*	"	.01	.03	.00	.02	.02	.02	.00	-
12	*S. krefeld*	"	.00	.02	.00	.02	.00	.01	.00	-
13	*P. mirabilis*	"	.00	.04	.01	.03	.02	.01	.02	-
14	*S. aurous*	"	.01	.08	.01	.03	.03	.01	.01	-
15	PP 276	blood	.18	.23	.36	.35	.72	.71	.01	+
16	PP 453	"	.27	.76	.38	.39	.59	.71	.00	+
17	PP 247	"	.20	.88	.36	.38	.72	.62	.00	+
18	PP 844	"	.68	1.11	.69	.74	.80	.76	.02	+
19	PP 84	"	.37	.93	.45	.41	.78	.67	.01	+
20	PP 1278	"	.16	.59	.16	.17	.77	.62	.02	+
21	PP 1104	"	.16	.18	.35	.36	.71	.63	.01	+
22	PP 439	"	.34	.88	.45	.43	.74	.69	.00	+
23	PP 309	"	.03	.48	.07	.10	.12	.61	.01	+
24	PP RAMA	"	.04	.05	.03	.06	.57	.81	.01	+
25	PP 1140	"	.36	.32	.62	.58	.78	.74	.01	+
26	PP 183	"	.38	.94	.46	.45	.70	.70	.00	+
27	PP 1322	"	.39	1.02	.58	.55	.78	.65	.02	+
28	PP 951-3	"	.46	1.00	.63	.61	.82	.77	.00	+
29	PP 951	"	.38	.94	.45	.49	.71	.75	.01	+
30	PP 539	"	.42	.19	.37	.38	.60	.67	.00	+
31	PP 130	"	.61	1.06	.66	.65	.97	.72	.00	+
32	PP 639	"	.13	.45	.13	.14	.37	.70	.00	+
33	PP 727	"	.27	.15	.30	.33	.59	.70	.00	+
34	PP 704	"	.39	1.03	.56	.56	.86	.80	.01	+
35	PP 667	"	.21	.16	.31	.31	.92	.77	.01	+
36	PP 745	"	.23	.28	.38	.47	.19	.84	.00	+
37	PP 206	"	.05	.11	.11	.14	.90	.76	.02	+
38	PP 474	"	.14	.13	.21	.26	.75	.68	.01	+
39	PP 342	"	.15	.96	.42	.39	.74	.73	.01	+
40	PP 675	"	.22	.84	.36	.37	.77	.73	.00	+

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Table A (continue)

No	Strains	Source of specimens	ELISA (OD_{492})							I.M.
			1G8	9B10	9C12	9C3	3H5	5F8	media	5G12
41	PP 762	blood	.14	.20	.25	.30	.85	.73	.00	+
42	PP 711	"	.26	.80	.34	.32	.79	.76	.01	+
43	PP Bruncha	"	.10	.10	.17	.21	.86	.81	.01	+
44	PP Siriporn	"	.19	.11	.25	.22	.73	.70	.00	+
45	PP Piboon	"	.14	.61	.16	.19	.74	.73	.01	+
46	PP Pongsan	"	.15	.10	.20	.24	.58	.65	.01	+
47	PP 200	pus	.21	.82	.32	.35	.75	.67	.01	+
48	PP 421	"	.10	.15	.16	.20	.78	.76	.02	+
49	PP 508	"	.14	.13	.29	.31	.77	.76	.01	+
50	PP 215	"	.17	.90	.31	.33	.98	.81	.00	+
51	PP 190	"	.16	.14	.28	.26	.50	.61	.00	+
52	PP 2160	"	.10	.13	.20	.23	.72	.70	.00	+
53	PP 342	"	.22	.95	.42	.39	.74	.60	.01	+
54	PP 22	"	.10	.78	.22	.18	.70	.62	.01	+
55	PP 799	"	.35	.91	.46	.40	.69	.67	.01	+
56	PP 472	"	.10	.65	.17	.20	.58	.73	.01	+
57	PP 80	"	.21	.17	.23	.28	.82	.74	.00	+
58	PP 880	"	.13	.68	.16	.22	.97	.70	.00	+
59	PP 821	"	.35	.80	.38	.41	.89	.20	.00	+
60	PP 1702	"	.20	.81	.33	.37	.86	.77	.00	+
61	PP 1293	"	.02	.12	.06	.14	.89	.78	.01	+
62	PP 2050	"	.22	.14	.27	.29	.86	.72	.01	+
63	PP 1399	"	.24	.73	.22	.24	.76	.72	.01	+
64	PP 310	sputum	.24	.20	.20	.10	.34	.63	.00	+
65	PP 2305	"	.16	.84	.33	.32	.68	.69	.00	+
66	PP 844	"	.27	.83	.40	.45	.87	.74	.00	+
67	PP 2271	"	.51	1.00	.66	.64	.89	.85	.01	+
68	PP 505	fluid	.31	.19	.40	.43	.72	.75	.00	+
69	PP 001	"	.17	.66	.20	.21	.70	.69	.00	+
70	PP 258	"	.51	1.16	.70	.74	1.01	.76	.00	+

IM : Immunoreactivity of 5G12 at 20.3 kDa band of homologous or heterologous bacteria

* : Strain from Ramathibodi hospital

** : Strain from Khon Kaen

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